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Disruption of the GTP-Cyclohydrolase I Gene In *Saccharomyces cerevisiae*

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GTP-cyclohydrolase I is the first enzyme in the biosynthetic pathway leading to folic acid and tetrahydrobiopterin. We determined the complete sequence of the GTP-cyclohydrolase I gene from the yeast *Saccharomyces cerevisiae*. The gene, which is located in the subtelomeric region of the right arm of chromosome VII, gives a major transcript of about 1000 nt and encodes a protein of 243 amino acids, which is highly homologous to the GTP-cyclohydrolase I from bacteria to man. We obtained by gene replacement a knock-out mutant that shows a recessive conditional lethality due to folinic acid auxotrophy, and lacks any detectable specific enzymatic activity. The gene was identified as *FOL2*, previously genetically mapped in the same region (J. Game, personal communication).

GTP-cyclohydrolase I (E.C.3.5.4.16) catalyzes the production of dihydroneopterin triphospate and formic acid from GTP. This reaction is the first step in the biosynthetic pathway leading to various pteridines. Essential biological functions depend on the two classes of pteridines conjugated and unconjugated. Conjugated pteridines are represented by folic acid derivatives which act as enzyme cofactors in one-carbon transfer reactions, involved in processes such as purine and amino acid biosynthesis. Bacteria, fungi and plants synthesize tetrahydrofolic acid while mammals depend on uptake of folates as vitamins from food. Unconjugated pteridines include tetrahydrobiopterin (BH4), molybdopterins, skin and eye pterin-pigments (1). BH4 is the most important in vertebrates as it is the essential cofactor for the hydroxylation of the aromatic amino acids, initial steps in phenylalanine degradation and neurotransmitter synthesis (2). It is also involved in the production of nitric oxide (3), in proliferation of different animal cells (4) and is an essential regulator in melanin biosynthesis (5). GTP-cyclohydrolase I has been found in every species examined. The protein sequences have been deduced from human (6), rat (7), mouse (8) and *Drosophila* (9) cDNAs and from *E. coli* (10), *Bacillus subtilis* (11), *Haemophilus influenzae* (12), *Streptococcus pneumoniae* (13) genes. Recently, the organization of the mouse and human genes has been determined (14). Partial sequences of GTP-cyclohydrolase I from other species, including *S. cerevisiae*, have been determined from cDNA fragments (15). The comparison of sequences from different organisms indicates that the enzyme has been extremely conserved throughout evolution.

Mutations in the human GTP-cyclohydrolase I gene have been detected in heterozygous and homozygous patients affected by neuronal disorders: most of them are missense mutations which cause substitution of highly conserved amino acids (14,16,17). An extensive genetic analysis of the complex *Punch* locus of *Drosophila*, encoding GTP-cyclohydrolase I, was performed by using several mutants with distinct morphological and protein phenotypes (18,19). The mammalian enzyme is regulated at the transcriptional (20,21) and activity levels (22,23). Different mRNAs have been found for GTP-cyclohydrolase I in rodents (24) and *Drosophila* (9). Little is known about the regulation of microbial GTP-cyclohydrolases I (25).

We determined the complete sequence and the chromosomal localization of the gene encoding

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the GTP-cyclohydrolase I of the yeast *S. cerevisiae*, the first gene completely sequenced in fungi. We also report on the disruption of the gene and the phenotype associated with a null mutation. This is the first report on the analysis of a knock-out mutant in the GTP-cyclohydrolase I.

MATERIALS AND METHOD

Strains, plasmids and media. S288C (*MAT αSUC2 mall mel gal2 CUP1*) was used in Southern and Northern hybridizations. The homoallelic strain W303*MATα/MATα ade2-1/ade2-1 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 ura3-1/ura3-1 trpl-1/trpl-1 can1/can1*) was used for *ORF243*disruption. The *fol2-1* strains BR414/4a, BR414/34a and BR414/39d, kindly provided by J. Game (Lawrence Berkeley Laboratory, Berkeley), and Q1/8d (a *fol2-1* segregant from the diploid strain originated by crossing BR414/34a with W303/9a, a haploid segregant from W303) were used for the complementation test. Yeast cells were grown in YEPD (1% yeast extract, 2% peptone, 2% glucose) or YNB (0,67% DIFCO yeast nitrogen base without aminoacid, 2% glucose) appropriately supplemented. When needed, folinic acid (Sigma) was added at a final concentration of 50 μg/ml. Yeast was transformed according to the lithium acetate method of Ito (26). Plasmid pLA643 was obtained by subcloning a 3485 bp *Sal*I-*Nhe*I fragment, including the entire *ORF243*, into pGEM-7Zf(+ (Promega), digested with *Xho*I and *Mlu*I.

DNA sequence analysis. A cosmid clone from strain FY1679, a direct derivative of S288C, was used for DNA sequence determination (L. Panzeri, manuscript in preparation). Sequence analysis and comparison of nucleotide and amino acid sequences and multiple sequence alignment were performed using the GCG software package (27).

Northern blot analysis. Total yeast RNA was prepared from exponentially growing cultures according with the procedure described by Sachs et al. (28). Northern blot analysis was performed as described in ref. 29. The filter carrying total yeast RNAs from α-factor-synchronized cultures (as described in ref. 30) was a kind gift from M. Ferrari (Milano).

Enzyme assay. Yeast cultures logarithmically growing in YNB complete medium, with or without folinic acid, were collected and washed three times at 4°C. About 5×10^8 cells were homogenized by disruption with glass beads (0.25 mm, Roth) in 50 mM Tris/HCl, 2.5 mM EDTA, pH 8.0. The cytosolic fraction was assayed for GTP-cyclohydrolase I activity as previously described (31). The 6-hydroxymethylpterin content of the yeast cells was measured as described (31).

RESULTS AND DISCUSSION

Complete sequence of GTP-cyclohydrolase I from S. cerevisiae. As part of the EC BIOTECH program for systematic sequencing of the yeast genome we sequenced a DNA region adjacent to the *MES1* gene, which maps on the right arm of *S. cerevisiae* chromosome VII. Search for putative ORFs in this sequence revealed the presence of a 729 bp open reading frame, named *ORF243*, encoding a putative protein of 243 amino acids (Fig. 1) whose sequence is highly homologous to known GTP-cyclohydrolases I. The identity of the yeast complete sequence with the vertebrate proteins, from human (6), rat (7) and mouse (8) is respectively 49.6%, 51.2% and 52.7%. The identity with *Drosophila* (9) is 40.5%. The global identity with eukaryots is higher than with bacteria: 37.6% with *B. subtilis* (11), 32.4% with *S. pneumoniae* (13), 32.1% with *E. coli* (10) and 31.6% with *H. influenzae* (12). Sequence comparisons failed to detect significant similarities

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  1  MHNIQLVQEI  ERHETPLNIR  PTSPYTLNPP  VERDGFSWPS  VGTRQRAEET

 51  EEEEKERIQR  ISGAIKTILT  ELGEDVNREG  LLDTPQRYAK  AMLYFTKGYQ
                              .           *  ** * *           *

101  TNIMDDVIKN  AVFEEDHDEM  VIVRDIEIYS  LCEHHLVPFF  GKVHIGYIPN
                          ..  * . **   .   **** .     *      * *

151  KKVIGLSKLA  RLAEMYARRL  QVQERLTKQI  AMALSDILKP  LGVAVVMEAS
       . ****    * .      .  * ***..              .      . *    .

201  HMCMVSRGIQ  KTGSSTVTSC  MLGGFRAHKT  REEFLTLLGR  RSI
     * *    **          * *      * *       * . .
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FIG. 1. Deduced amino acid sequence of the *S. cerevisiae* GTP-cyclohydrolase I. Amino acids identical in eukaryotic (rat, mouse, human, *Drosophila*, yeast) and prokaryotic (*E. coli, B. subtilis, H. influenzae, S. pneumoniae*) GTP cyclohydrolases I are indicated by asterisks below the sequence. Points indicate amino acids conserved in all but one the available sequences. The region corresponding to the cDNA fragment previously reported (15) is underlined.

between the 60 N-terminal amino acids and the corresponding regions of the known GTP-cyclohydrolase I sequences.

The crystal structure of the *E. coli* GTP-cyclohydrolase I (32) showed that the region from Arg65 to Arg185 (corresponding to Arg87 and Arg207 respectively in the yeast sequence) contains the relevant residues for the active site structure. The majority of these amino acids are conserved in the *S. cerevisiae* protein: Arg65 and Lys68 (positions 87 and 90 respectively in yeast), as well as residues 110–113 and 150–153 (corresponding to 132–135 and 172–175 in yeast) are identical in the two sequences. Residues 87–89 of *E. coli*, which differ from those in the corresponding positions (111–113) in yeast, are not even conserved in other GTP-cyclohydrolases I. Residues 131–139 (153–161 in yeast) are largely conserved, with the exception of two amino acids: Leu159 and Ala160 of the yeast enzyme are different in *E. coli*, but highly conserved in other organisms. The amino acid sequence of the region spanning from Glu115 to Gly208 has been previously deduced from a cDNA fragment obtained by RT-PCR from *S. cerevisiae* (15). The highly conserved residues Leu159 and Ala160 were not found in the cDNA fragment, where Glu and Gly respectively were present. Since both the cDNA sequence and the genomic sequence were obtained from strains closely related to the wild type strain S288C, this difference might be due to the amplification procedure. A third difference (Ser206 instead of Met) was due to the primer design.

Disruption of the GTP-cyclohydrolase I gene. Southern analysis of total DNA from the strain S288C, digested with several restriction enzymes and probed with the 423 bp *Eco*RI-*Hind*III fragment, internal to *ORF243* (see Fig. 2), indicated that this sequence is unique in the yeast haploid genome (data not shown). The ORF is transcribed, as demonstrated by Northern analysis (Fig. 3, A). In order to establish whether the *ORF243* function is essential for cell viability, we performed a one-step gene replacement as described by Rothstein (33), using the deletion substitution strategy described in Fig. 2. Plasmid pLA646 was used to transform the diploid strain W303 to histidine prototrophy. Recombination of the *Vsp*I-*BamH*I fragment at the *ORF243* locus resulted in the replacement of one copy of the wild type gene with the disrupted allele *orf243Δ::HIS3 (orf243Δ)*, as confirmed by Southern analysis (data not shown). In addition, pLA646 cut with *Xho*I was used to direct the integration *HIS3* as a control. The disruption of one copy of *ORF243* does not affect the growth rate of the heterozygous transformants or their ability to sporulate. Tetrads from two transformant clones (971, 973) were dissected on both YEPD (Fig. 2, B1) and YNB plates, and showed a 2:2 lethality that was linked to the *HIS3* marker (Table I, A), since all of the viable spores were His-. Tetrads from the parental W303 strain and from a transformant carrying the plasmid integrated at the *HIS3* locus (HV5.1), dissected as control, gave rise to four colonies of normal size.

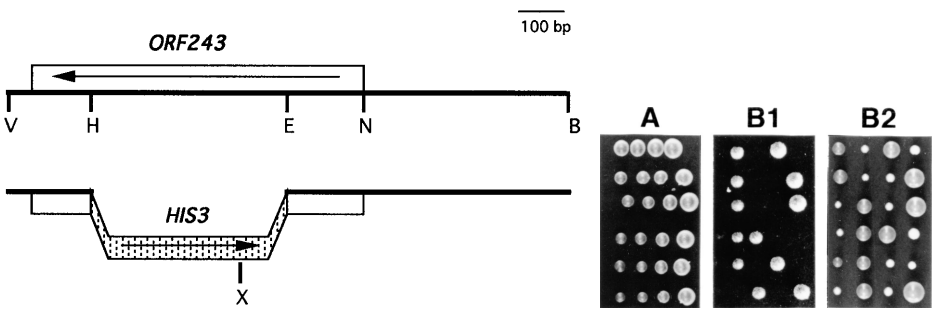

FIG. 2. Disruption of *ORF243*. Left: replacement of the 423 by *Eco*RI–*Hin*dIII fragment of plasmid pLA643, internal to the *ORF243* coding region and corresponding to residues 59–198, with a 1764 bp *Bam*HI fragment including the *HIS3* gene. The *Bam*HI–*Vsp*I fragment from the resulting plasmid pLA646 was used for a one-step gene replacement of the *ORF243* genomic resulting plasmid PLA646 was used for a one-step gene replacement of the *ORF243* genomic locus. Arrows indicate the direction of transcription. Restriction sites: B = *Bam*HI; E = *Eco*RI; H = *Hin*dIII; X = *Xho*I; N = *Nsi*I; V = *Vsp*I. Right: tetrads from strains W303 (A) and 971 (B), dissected on YEPD (A and B1) or YEPD with folinic acid (B2).

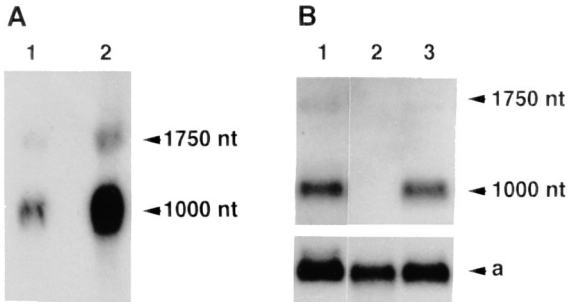

FIG. 3. Northern analysis of *ORF243*. Panel A: 5μg (lane 1) and 20 μg (lane 2) of total RNA from the standard strain S288C grown in YEPD, probed with the 423 bp *Eco*RI–*Hin*dIII fragment internal to *ORF243*.Panel B: 5 μg of total RNA from cells grown in media with folinic acid probed with the 596 bp *Nsi*I-*Hin*dIII fragment internal to *ORF243*. Lane 1: strain W303 from YNB. Lane 2: strain 971/1a (*orf243δ*) from YNB. Lane 3: strain W303 from YEPD. As control for loaded RNA the filter was hybridized with a 1.5 kb *Bam*HI–*Hin*dIII fragment from the yeast actin gene (a) (35).

In yeast defects in GTP-cyclohydrolase I are expected to impair the folate biosynthesis. Since the growth of yeast mutants deficient in the folate metabolism can be rescued by provision of exogenous folates (34), tetrads from strains 971 and 973 were dissected on YEPD supplemented with 50 μg/ml of folinic acid. In these conditions the tetrads gave four viable spores, two spores yielding colonies of normal size and two smaller colonies (Fig. 2, B2). Since W303 is a homoallelic diploid strain, segregants from the disrupted strains differ only in a *ORF243* allele. Segregation analysis showed that a 2:2 folinic acid requirement (Fol-phenotype) was linked to the *ORF243* disruption,

TABLE 1
Tetrad Analysis of Diploid W303 (*his3–11, 15/his3–11, 15*) Transformed with Plasmid pLA646

						Segregants			
A	Diploid strain	Integration site	Total number of tetrads	Viable spores per tetrad	Number of tetrads observed	His^+		His^-	
	W303	—	18	4	14	0		56	
				3	2				
				2	0				
				1	2				
	HV5.1	*HIS3*	9	4	7	14		14	
				3	1				
				2	1				
				1	0				
	971	*ORF243*	31	4	0				
				3	1				
				2	29	0		58	
				1	1				
	973	*ORF243*	19	4	0				
				3	0				
				2	16	0		32	
				1	3				
B						His^+ Fol^+	His^+ Fol^-	His^- Fol^+	His^- Fol^-
	971	*ORF243*	34	4	33	0	66	66	0
				3	1				
	973	*ORF243*	15	4	13	0	26	26	0
				3	2				

Note. Tetrads were dissected on medium without (part A) or with (part B) 50 μg/ml folinic acid. All the His^+ segregants were also "slow growing" on YEPD with folinic acid (data not shown).

since it always segregated with the His^+ and "slow growth" phenotypes (Table I, B). The deletion of *ORF243* thus causes a recessive conditional lethality.

Crosses of *orf243*Δ segregants and sporulation of diploids homozygous for the disruption demonstrated that the GTP-cyclohydrolase I gene is not required for conjugation and meiosis. After shift from YNB medium supplemented with folinic acid to not supplemented medium, haploid *orf243*Δ cells maintained the same growth rate for 2.7 cell generations and then stopped growing. Also *orf243*Δ spores from diploids heterozygous for the disruption, when germinated on medium without folinic acid, performed on average 3.4 cellular generations: the folate intracellular pool seems therefore to be sufficient for a limited number of cell divisions.

Transcriptional analysis of ORF243. Total RNA from the wild type strain S288C was analysed by Northern hybridization. The probe identified a major transcript of about 1000 nt and a much less represented transcript of about 1750 nt (Fig.3, A). Both transcripts were also detected in strain W303, used for the disruption analysis. Neither transcript was detected in the haploid segregant 971/1a carrying the disrupted allele (Fig. 3, B). The functional significance of the larger transcript will be the object of further analysis. No significant difference in the amount of the transcripts was observed (Fig. 3, B) in different growth conditions (rich YEPD medium and synthetic YNB, both supplemented with folinic acid). The steady-state level of the transcripts did not change after growth in medium without folinic acid (data not shown).

Because of the role of tetrahydrofolate in the biosynthesis of nucleotides, GTP-cyclohydrolase I function is relevant for DNA replication. In yeast the transcript levels of several genes involved in this process fluctuate concomitantly during the cell cycle, reaching a peak at the G_1/S phase boundary (36). The regulatory motif "*Mlu*I cell cycle box", which is found upstream of most DNA-synthesis genes, is not preset in the 5′ non-coding sequence of this gene, suggesting that it is not regulated in the cell cycle. This was confirmed by Northern analysis of total RNA prepared from α-factor-synchronized cell cultures probed with *ORF243* (data not shown).

*orf243*Δ *mutants lack GTP-cyclohydrolase I activity*. The specific activity of GTP-cyclohydrolase I as well as the concentration of produced 6-hydroxymethylpterin was determined in the four segregants deriving from a tetrad of strain 971, heterozygous for the *ORF243* deletion. The results are summarized in Table II. Both the GTP-cyclohydrolase I activity and the concentration of 6-hydroxymethylpterin were below the detection limit in the two *orf243*Δ clones. No significant difference was found in the wild-type clones between cells logarithmically growing in medium with or without folinic acid. These results demonstrate that cells carrying the deletion of *ORF243* are knock-out mutants for the GTP-cyclohydrolase I function, thus confirming that in yeast this enzymatic activity is encoded by only one gene.

The gene encoding GTP-cyclohydrolase I is FOL2. Yeast mutants carrying genetic lesions in the folate metabolic pathway (*fol*) have already been described (34). Genetic analysis of the folate

TABLE 2
Specific Activity and 6-Hydroxymethylpterin Concentration in Yeast Wild-type and *orf243*Δ.

Strain	*ORF243* allele	Folinic acid in the medium	GTP-CH I specific activity pmol/(min × mg)	6-hydroxymethylpterin (pmol/10^7 cells)
971/6a	*orf243*Δ	+	—	—
971/6b	*ORF243*	−	3.27 ± 0.52	286 ± 83
		+	5.09 ± 0.76	342 ± 125
971/6c	*ORF243*	−	7.31 ± 0.49	512 ± 176
		+	4.61 ± 0.72	483 ± 65
971/6d	*orf243*Δ	+	—	—

Note. The values represent the mean ± S.D. of five individual tests. The values for *orf243*Δ clones were below the detection limit.

auxotrophs defined two genes, *FOL1* and *FOL2*. The *fol2* mutation were genetically mapped by J. Game (personal communication) in the subtelomeric region of the right arm of chromosome VII. Since *ORF243* is localized in the same region, a complementation test was performed by crossing haploid strains carrying our deleted allele with four different strains carrying the *fol2-1* mutation. The *orf243*Δ deletion and the *fol2-1* mutation, which are both recessive, fall in the same complementation group, as indicated by the Fol− phenotype of the resulting diploids. An allelism test was carried out in one of the diploid strains. The analysis of 19 tetrads revealed that all of the segregants required folinic acid, thus conferming that the two mutations are alleles of the same gene and that, therefore, *ORF243* is identical to the *FOL2* gene.

Conclusions. The GTP-cyclohydrolase I gene (*FOL2*) is present in single copy in the haploid yeast genome. Our disruption deletes a large part of the open reading frame. At the protein level the deletion eliminates all of the amino acids proposed as relevant for function in the *E. coli* enzyme, leaving only the 58 unconserved residues of the N-terminal domain. This mutation causes a recessive conditional lethality, due to folinic acid auxotrophy. We thus obtained the first knockout mutant in a GTP-cyclohydrolase I gene to have been found. To our knowledge this is also the first mutant of GTP-cyclohydrolase I reported in microorganisms.

In *S. cerevisiae* recessive lethal alleles can be maintained in heterozygous cells and the lethal phenotype can be detected in haploid segregants. The ease of genetic manipulation and analysis, combined with the possibility of maintaining viable GTP-cyclohydrolase I mutants by supplying them with exogenous folate, renders yeast a suitable system for studying various GTP-cyclohydrolase I mutations. Among them, substitutions of highly conserved residues and modifications of the variable N-terminal portion could be of particular interest.

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