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Research Article

The 3' terminal sequence of the inosine monophosphate dehydrogenase gene encodes an active domain in the yeast *Schizosaccharomyces pombe*

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Abstract

The *gua1* gene encoding inosine monophosphate dehydrogenase (IMPDH), which catalyses the first step in *de novo* biosynthesis of guanosine monophosphate (GMP), was cloned in the yeast *Schizosaccharomyces pombe* by functional complementation of a *gua1ura4*-D18 mutant strain from a *S. pombe* DNA genomic library. Complementation analysis revealed a 1.2 kb fragment which segregation analysis confirmed did not code for a suppressor gene. Only 446 nucleotides of the *gua1* gene encoding the IMPDH C-terminal residues were found within this 1.2 kb sequence (GenBank, AJ293460). The comparison of this wild-type fragment with the same fragment from the *gua1ura4*-D18 mutant revealed that there was a point mutation at position 1261 (guanine → adenine) from the 5' end, corresponding to the amino acid residue 421 (glycine → serine) of the enzyme. Dot and Northern analyses showed that the *gua1* gene was expressed in transformants as well as in the wild-type and the *gua1ura4*-D18 mutant, but enzyme activity was only detected in wild-type and transformant cells. It seems likely that a 446 bp fragment from the 3' end of the *gua1* gene abolished the point mutation in the mutant strain, suggesting that this fragment participates in the sequences encoding the active domain of IMPDH in *S. pombe*.

Key words: Schizosaccharomyces pombe, inosine monophosphate dehydrogenase, *gua1* gene, purine nucleotide pathway.

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Introduction

The *de novo* biosynthesis of purine nucleotides is essentially the same in all groups of organisms studied so far (Henderson and Paterson, 1973; Michal, 1999) and inosine monophosphate dehydrogenase (IMPDH; E.C.1.1.1.205) is one of the key enzymes for the regulation of this pathway. This enzyme catalyses the NAD-dependent conversion of inosine monophosphate (IMP), which serves as a branch point between the adenine and guanine specific branches, to xanthosine monophosphate (XMP) which is the rate-limiting step in *de novo* guanine nucleotide biosynthesis (Hedstrom, 1999). Inhibition of IMPDH causes a reduction in the guanine nucleotide pool with subsequent interruption of DNA and RNA synthesis which results in cytotoxicity. The reduction in guanine nucleotides also compromises the ability of G-proteins to function as transducers of intracellular signals (Manzoli *et al*., 1995). Increased IMPDH activity and consequently GMP synthesis has been shown in a variety of cancer cell lines and it appears that IMPDH may be a target for cancer chemotherapy and the development of immunosuppressive drugs (Weber, 1983). The structural and functional properties of IMPDHs from different organisms and its mode of action and inhibition have been well documented (Hedstrom, 1999) and IMPDH sequences from at least 163 organisms from bacteria to plants have been reported to GenBank (http://www.ncbi.nlm.nih.gov/sutils/blink.cgi?pid=39959).

The yeast *Schizosaccharomyces pombe* is an attractive model system for eukaryotic cell and molecular biology studies. This yeast is known to have 12 loci *(ade1* to *ade10* plus *gua1* and *gua2*) involved in the *de novo* biosynthesis of purine nucleotides (Heslot, 1972). The chromosomal location of all these genes have been determined and the *ade1*, *ade2*, *ade4*, *ade6* and *ade10* genes have been cloned and sequenced (McKenzie *et al*., 1987; Szankasi *et al*., 1988; Speiser *et al*., 1992; Ludin *et al*., 1994; Liedtke *et al*., 1998). Pourquié (1974) conducted the first study of the genes belonging to the *S. pombe* guanine nucleotide biosynthesis pathway and identified two types of genetically

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unlinked auxotrophic mutants designated *gua1* and *gua2*, the *gua1* mutant having no IMPDH activity. Haploidization and tetrad analyses showed that the *gua1* gene was located at the centromeric region of chromosome II (Oraler *et al*., 1990).

During the study described in this paper we used complementation techniques to clone a partial *gua1* gene from a *S. pombe* genomic library and, interestingly, found that the transformant containing only a 446 bp long fragment from the 3' end of the gene was able to produce an active enzyme.

Material and Methods

Strains, plasmids and growth conditions

The *Schizosaccharomyces pombe* wild-type strains $972h^-$ and $975h^+$ plus the IMPDH-negative *gua1* mutant and the *ura4*-D18 mutant containing a full deletion of the *ura4* gene (Grimm *et al*., 1988) were obtained from Istanbul University, Molecular Biology Laboratory collection (Address Above). All the *S. pombe* strains were grown using minimal media (MM) or enriched media (EM) broth or agar and sporulated in synthetic sporulation agar (SPA) as previously described by Gutz *et al*. (1974). The media were supplemented with guanine and uracil (50 mg/L) as required.

Two types of plasmid were used, the pUR19 yeast shuttle cloning vector and the pUC18 bacterial cloning vector. The *Escherichia coli* DH5 α was used for plasmid amplification. DNA manipulations, including plasmid preparation, subcloning, restriction mapping, agarose gel electrophoresis, and transformation and *E. coli* growth techniques were performed according to standard protocols (Sambrook *et al*., 1989). All enzymes for restriction mapping and subcloning were obtained from MBI Fermentas (Lithuania).

Gene isolation and subcloning

The *gua1* gene was isolated by complementation using an *S. pombe* genomic library established in pUR19 (provided by Dr. Clive Price, University of Sheffield, Department of Molecular Biology and Biotechnology). To obtain the *gua1ura4*-D18 double-mutant, strains were crossed on SPA and the double-mutant selected from tetrads according to its guanine and uracil requirements. The mating type of the double-mutant was determined as described by Leupold (1970) and was transformed using 3.5 μg of DNA for each experiment (Warshawsky and Miller, 1994). Plasmids from transformants were rescued according to the protocol of Topal *et al*. (1997). To determine the smallest fragment containing the *gua1* gene, subcloning was performed in pUR19 and plasmids were transformed into the *gua1ura4*-D18 mutant strain.

Suppressor gene analysis, sequencing and RNA isolation and analysis

Randomly selected *S. pombe* transformants carrying the insert in their genome were crossed with the wild-type $975h^+$ and genotypes of the spores were determined on selective media by tetrad analysis (McKenzie *et al*., 1987).

For sequencing, the smallest DNA fragment carrying the *gua1* gene (determined by complementation) was cloned to pUC18. Sequencing reactions of this fragment and the PCR product of the mutant allele from the *gua1* strain were performed using a Pharmacia Fluorescence Kit and a Perkin Elmer model 377 automatic DNA sequencer with a universal M13 reverse primer followed by primers, corresponding to the internal sequences of the insert. Sequence analysis of the insert was evaluated using the UWGCG (University of Wisconsin Genetic Computer Group) programs. A search of the GenBank database was made using the NCBI BLASTP 2.2.5 program (Altschul *et al*., 1997).

Total RNA was isolated as described by Burke *et al*. (2000) and dot and Northern hybridizations performed using the DNA Labeling and Detection (DIG) Kit according to the manufacturer's instructions (Boehringer Mannheim). The RNA samples (~10 μg) were blotted on a nylon membrane (Schleicher & Schuell) for dot hybridization and the RNAs separated on 1.2 % (w/v) agarose gel containing 0.66 M formaldehyde and 0.5 μg/mL ethidium bromide and transferred to the membrane using a capillary system. A DIG-11-dUTP labeled DNA fragment carrying the *gua1* gene was used as a probe for hybridizations.

Inosine monophosphate dehydrogenase (IMPDH) assay

We prepared *S. pombe* lysates according to the method of Pourquié (1974), with a slight modification. Cells were grown in EM broth to the late log phase in a rotary shaker (30 °C, 150 rpm), harvested by centrifugation for 10 min at 0 °C and 4000 x *g*, washed twice in distilled water, re-centrifuged. The pellet was resuspended in 1.5 mL of breakage buffer (1 M Tris-HCl, pH 8.4; 0.1 M KCl) per gram of cell wet-weight and 0.45-0.50 mm ∅ glass beads were added to just below the meniscus of the suspension and the mixture homogenized in a cell Braun homogenizer chilled with CO_2 at 15 s intervals for three minutes. After cell disruption, the homogenate was clarified by ultra-centrifugation for 90 min at 4 °C and 90 000 x *g* and the resultant supernatant (crude extract) was used for the enzyme assay. Protein concentration of the crude extract was measured by the method of Lowry *et al*. (1951) and IMPDH activity was determined spectrophotometrically by monitoring the formation of NADH at 340 nm (Carr *et al*., 1993) by adding 0.9 mL of crude extract to 4.1 mL of reaction mixture (100 mM Tris-HCl, pH 8.0, 100 mM KCl, 3 mM EDTA, 200 μM inosine mono-

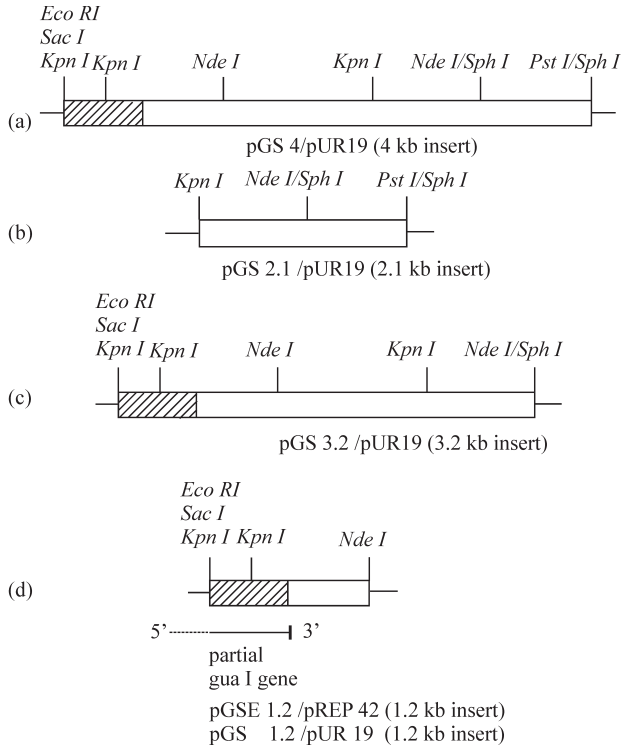

Figure 1 - The cloned 4kb *Schizosaccharomyces pombe* fragment in plasmid pUR19 containing a partial *gua1* gene (a) and three subclones (b-d). Subclone pGS1.2 contained the shortest (1.2 kb) DNA fragment that complements the *gua1* mutant. The position and orientation of the partial *gua1* gene is represented by the hatched box and arrow.

phosphate (IMP) and 400 μM NAD) and a second reaction mixture supplemented with 0.12 mM allopurinol, a potent inhibitor of IMPDH (O'Gara *et al*., 1997). After incubation at 37 °C for 15 min, the optical density was read at λ = 340 nm (OD_{340}) against a blank consisting of breakage buffer minus crude extract. Enzyme activity was expressed as OD_{340} per mg per mL of protein in the crude extract.

Results

Cloning of the gua1 *gene*

The *S. pombe gua1ura4*-D18 strain was transformed with a pUR19/*Sau*3A genomic library and cells exhibiting gua^+ura^+ phenotype were selected. Five positive colonies were obtained from approximately 3.6×10^4 transformants. The plasmids from these transformants were isolated and amplified in *E. coli,* and their sizes were determined by restriction analysis. These plasmids were named according to the size of insert as (in sequence) pGS9.3, pGS7.5, pGS4.8 (two plasmids of similar size) and pGS4. The plasmid used for further studies was pGS4. To determine the location of the *gua1* gene within the 4 kb insert, the *gua1ura4*-D18 double-mutant was transformed with several subcloned DNA fragments of variable lengths. The smallest fragment complementing the mutation was a 1.2 kb *Kpn*I/*Nde*I fragment (Figure 1). Then, this fragment was cloned into pUR19. New construct was designated as pGS1.2, and the positive *S. pombe* transformants designated as SG1.

Suppressor gene analysis

Suppressor gene analysis was performed to determine whether the 1.2 kb *Kpn*I/*Nde*I fragment contained the *gua1* gene itself or an extragenic suppressor. The segregation of the *gua1* gene localized on chromosome II (Oraler *et al*., 1990) with the *ura4* gene localized on chromosome III (Gygax and Thuriaux, 1984) was investigated. To achieve this the wild-type *S. pombe* $975h^+$ strain was crossed with the stable SG1 *S. pombe* transformant, which carries the *gua1* gene integrated into its genome. We analyzed 556 spores from 139 tetrads for guanine and uracil auxotrophy and found that 43 spores were ura^- and all of them were gua^+. This indicated that the insert had integrated precisely at the *gua1* locus and that the complementation did not originate from a suppressor gene, but instead represented a cloned functional *gua1* gene.

Sequence analysis

We cloned a 1.2 kb *Kpn*I/*Nde*I DNA fragment carrying the *gua1* gene into pUC18 and the new construct (pGSC1.2) was transformed in *E. coli* DH5 α as described above. The DNA sequence of the fragment was analyzed and compared with the sequence present in cosmid c2F12 which is assumed to be carrying the putative *gua1* gene and which is available at the *S. pombe* genome project in the Sanger Center (http://srs6.ebi.ac.uk/srsbin/cgi-bin/wgetz?-id+3HhqU1NNOGG+-e+[EMBL:`SPBC2F12']+-qnum+1+-enum+12). We were surprised to find that the whole *gua1* gene did not exist within the cloned 1.2 kb fragment but only a 446 bp long region from the 3' terminus of the *gua1* gene was located in this insert, the remaining approximately 750 bp belonged to the gene encoding a kinesine-like protein. This partial sequence of the *gua1* gene has been deposited in the National Center for Biotechnology Information (NCBI) data bank under the Accession Number AJ293460. Sequence identity comparisons showed that this partial gene fragment was correlated with the C-terminal residues of the enzyme, the partial sequence revealing an open reading frame of 446 nucleotides encoding a polypeptide of 148 amino acids (Figure 2).

The size of the IMPDH gene PCR products from wild and mutant strains was also similar, leading us to determine the mutation type of the *gua1* strain. The results of the sequence analysis of the wild-type and mutant strain proved that the defect within the *gua1* gene was a point mutation at position 1261 (**g**gt → **a**gt), resulting a substitution at residue 421 (glycine → serine) in *S. pombe* IMPDH. Thus a 446 bp from the 3' terminus of the gene complemented with this mutation whereas PCR product of the remaining longer 1129 bp part did not (Figure 2).

A search of the GenBank database using NCBI BLASTP 2.2.5 program (Altschul *et al*., 1997) showed that

Figure 2 - The sequence of the *Schizosaccharomyces pombe gua1* gene showing the predicted inosine monophosphate dehydrogenase (IMPDH) amino acid residues. The cloned and complemented partial *gua1* gene discovered in this study is shadowed. The location of the point mutation is shown in bold-faced type. Altering the guanine (g) at the 1261 nucleotide position to adenine (a) caused the IMPDH 421_{Gly} residue to be substituted by a 421_{Ser} residue.

the partial IMPDH amino acid sequence had homologies with IMPDH from *Candida albicans* (64%), *Saccharomyces cerevisiae* (62%), *Drosophila melanogaster* (53%), human type I (53%), *Mus musculus* type I (52%), human type II (52%), *M. musculus* type II (52%) *Arabidopsis thaliana* (45%) and *E. coli* (40%) (Figure 3).

Transcription analysis

To demonstrate expression of the *gua1* gene we isolated RNAs from three different types of the cells, wild-type, double-mutant (*gua1ura4*-D18) and the transformant (SG1). Dot and Northern hybridizations were carried out with a 1.2 kb fragment as a probe containing partial gene fragments of a kinesine-like protein and IMPDH. Labeling was detected with the samples tested by dot hybridization (data not shown) and two bands were observed for all samples in the Northern blot hybridizations (Figure 4). One of these bands was consistent with the size of a full-length *S. pombe* IMPDH transcript of 1575 nucleotides, indicating that expression of this gene occurred at the transcriptional level in the double mutant. The size of the other band, corresponding to the kinesine-like protein transcript was about 2 kb.

Enzyme assay

The IMPDH activities of the crude extracts from the $972h^-$ wild-type, *gua1ura4*-D18 double-mutant and the SG1 transformant were determined in the reaction mixture with or without the IMPDH inhibitor allopurinol. Significant IMPDH activity existed in wild-type and the SG1 transformant but almost none in the *gua1ura4*-D18 mutant and there was also a clear difference between the activities detected for each sample in the presence and absence of allopurinol. These results confirmed that allopurinol had an inhibitory effect on IMPDH *in vitro* (Weber, 1983) and decreased the enzyme activity (Table 1).

Discussion

We found that the *S. pombe* DNA fragment cloned in this study is part of the IMPDH gene as indicated by complementation with the *gua1ura4*-D18 double- mutant as well as comparison of the sequence of the fragment with

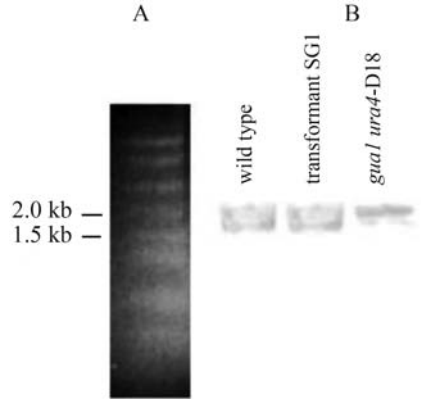

Figure 3 - Northern blot analysis of inosine monophosphate dehydrogenase (IMPDH) transcripts in the *Schizosaccharomyces pombe* wild-type, mutant and SG1 transformant. Total RNA (~10 μg) was separated using denaturing formaldehyde/agarose gel electrophoresis. RNA markers separated on agarose gel (a); Northern blot hybridization of IMPDH mRNA with a DIG-11-dUTP labeled DNA fragment carrying the *gua1* gene as a probe.

Table 1 - Inosine monophosphate dehydrogenase (IMPDH) activity (OD_{340} per mg per ml of protein) in crude extracts of *Schizosaccharomyces pombe*. Activity measurements were carried out using two different reaction mixtures, one with allopurinol (O'Gara *et al*., 1997) and the other without allopurinol (Carr *et al*., 1993). Values represent the average ± the standard deviation for three independent determinations.

	IMPDH activity	
Cell type	With allopurinol	Without allopurinol
Wild type (972h$^-$)	0.465 ± 0.057	0.759 ± 0.015
Double mutant (*gua1ura4*-D18)	0.061 ± 0.008	0.045 ± 0.003
Transformant (SG1)	0.408 ± 0.009	0.549 ± 0.014

the putative *gua1* gene cloned to chromosome II cosmid c2F12.

The BLASTP search of the GenBank database showed that the partial IMPDH amino acid sequence had 40 to 64% homology with IMPDH from, in increasing order of size, *E. coli*, *A. thaliana*, *M. musculus* type II, human type II, *Mus musculus* type I, *Drosophila melanogaster*, *S. cerevisiae* and *C. albicans* (Figure 3), indicating that this sequence seems to be conserved between unrelated species.

The observation of similar-sized IMPDH mRNAs in both the wild-type and the mutant *S. pombe* strain indicated that the defect of the *gua1* gene was due to a point mutation. Furthermore, sequence analyses showed that the point mutation was exactly at position 1261 (**g**gt → **a**gt), the first nucleotide of codon 421 which encodes a glycine residue in IMPDH (Figure 2), and that this mutation changed this residue to a serine residue.

There was significant enzyme activities in both the wild-type strain and the SG1 transformant but the enzyme activity in the mutant was so low that it could be disregarded. These results show that the SG1 transformant containing the partial *gua1* gene showed nearly as much catalytic activity as wild-type *S. pombe*. In addition, when we cloned the rest of the gene (1129 bp) into the *gua1ura4*-D18 double-mutant, no complementation was observed. Having shown that the enzyme activity obviously originated from one of the IMPDH C-terminal residues encoded by a small portion of *gua1* gene, complementing the mutation raised the interesting question of how only a partial gene sequence could give rise to a level of IMPDH expression similar to that of the wild-type *S. pombe*.

Figure 4 - Partial amino acid alignment of *Schizosaccharomyces pombe* inosine monophosphate dehydrogenase (IMPDH) (GenBank CAB97003) homologues with *Candida albicans* (O00086), *Saccharomyces cerevisiae* (P50094), *Drosophila melanogaster* (Q07152) Human type I (P20839), *Mus musculus* (P50096), Human type II (P12268), *M. musculus* type II (P24547), *Arabidopsis thaliana* (Q9SA34) and *Escherichia coli* (P06981). Abbreviations for the species are: Sp = *S. pombe*; Ca = *C. albicans*; Sc = *S. cerevisiae*; Dm = *D. melanogaster*; Hs = *Homo sapiens*; Mm = *M. musculus*; At = *A. thaliana*; Ec = *E. coli*. Identical amino acids are shown with a black background, similar amino acids with a gray background. Dashes (gaps) have been introduced for optimal alignment. The alignments revealed that the regions of putative catalytic domain amino acids (378-524) were highly conserved.

The IMPDH enzyme is a tetramer formed by monomers consisting of two domains, an α/β barrel core domain (catalytic domain) and a cystathione-β-synthase (CBS) subdomain (Carr *et al*., 1993; Huete-Perez *et al*., 1995; Zhou *et al*., 1997; Colby *et al*., 1999; Zhang *et al*., 1999). Zhang *et al*. (1999) showed that site specific mutations in the CBS subdomain of *Streptococcus pyrogenes* did not result in loss of IMPDH activity but the construction of a point mutation in the active site by changing Arg_{406} to alanine resulted in complete loss of IMPDH activity. However it had been previously reported (Zhou *et al.*, 1997) that IMPDH from *Borrelia burgdorferi* did not contain a CBS subdomain and yet maintained enzymatic activity. Nimmesgern *et al*. (1999) demonstrated the expression of the core domain and the CBS subdomain of human IMPDH separately in *E. coli* and determined that the core domain was enzymatically active while the CBS subdomain was inactive. Futer *et al*. (2002) reported that the mutations of three active site residues to alanine in the IMP binding pocket reduced IMPDH activity to less than 0.1 % of that found in human wild-type IMPDH.

All these findings suggest that the region close to the C terminus of the core domain, rather than the CBS subdomain, is responsible for IMPDH activity. Our results were also consistent with this conclusion because the SG1 transformant contained a 446 bp fragment of the gene encoding the IMPDH C-terminal residues and was capable of producing active enzyme.

However, IMPDH CBS subdomains from different species vary considerably in size and the subdomain sequences are much less conserved than the core domain sequences (Nimmesgern *et al*., 1999). Moreover, mutation studies on the gene fragment encoding the CBS domain in *Methanococcus janaschii* (Archeae) suggest that the CBS domain is responsible for the regulation of cystathione-β-synthase activity (Bateman, 1997). Thus it can be speculated that the function of the *S. pombe* IMPDH CBS domain may also be related to regulation of IMPDH expression. However, the presence of this domain in the alignment of all but one (from *B. burgdorferi*) of the 56 IMPDHs studied by Nimmesgern *et al*. (1999) still raised questions with regarding its functional role.

In our study, complementation in the SG1 transformant carrying the 446 bp part of the *gua1* gene in the plasmid pGS1.2 suggested that the insert integrated into the exact region containing the mutation in the genome by homologous recombination. Hence, it can be concluded that a 446 bp from the 3' end of *gua1* gene participates in the sequences encoding the catalytic domain of *S. pombe* IMPDH.

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