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CLONING AND SEQUENCING OF THE GENE ENCODING THE LARGE SUBUNIT OF GLUTATHIONE SYNTHETASE OF SCHIZOSACCHAROMYCES POMBE

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SUMMARY: The gene for the large subunit of glutathione synthetase (EC 6.3.2.3) of Schizosaccharomyces pombe was cloned from a S. pombe genomic DNA library by complementation of cadmium hypersensitivity of a glutathione synthetase deficient mutant of S. pombe. A long open reading frame was found in the cloned DNA sequence. Amino acid sequence predicted from the long open reading frame coincided with amino acid sequences of peptides obtained by V8 protease digestion of the large subunit of the purified glutathione synthetase. The glutathione synthetase deficient mutant which harbored plasmids containing the glutathione synthetase large subunit gene exhibited glutathione synthetase activity higher than the activity in the wild type strain, though the plasmid did not contain the gene for the small subunit of the enzyme.

Glutathione is usually the most abundant intracellular thiol compound. It functions in the reduction of the disulfide bridge of proteins and other molecules, in the synthesis of the deoxyribonucleotide, in the protection of cells against the effects of radiation, oxydative damage and toxic compounds (1-3). In addition to the functions described above, some fungi and plants use enzymes involved in glutathione biosynthesis to synthesize heavy metal chelating peptides called cadystins (4) or phytochelatins (5). Mutants of S. pombe defective in glutathione biosynthesis is hypersensitive to cadmium salts due to the inability to synthesize cadystins (4).

Glutathione is synthesized from its constituent amino acids by two consecutive reactions involving ATP (1). In the inital reaction glutamic acid and cysteine are converted to γ-glutamylcysteine by the enzyme γ-glutamylcysteine synthetase (EC 6.3.2.2). Addition of glycine to

Abbreviation: SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

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the γ-glutamylcysteine to yield glutathione is catalyzed by glutathione synthetase.

In order to clone genes involved in glutathione biosynthesis we transformed S. pombe mutants deficient in glutathione biosynthesis with a S. pombe genomic DNA library and selected cadmium resistant clones. Here we describe cloning and sequencing of a gene which complement cadmium hypersensitivity of a glutathione synthetase deficient mutant. Amino acid sequence predicted from the cloned DNA agreed with partial amino acid sequences of the large subunit of glutathione synthetase of S. pombe.

Materials and Methods

Strains, plasmid and growth conditions - S. pombe strains L972h$^-$, HM123 (h$^-$, leu-1), and MN101 (glutathione synthetase deficient mutant of HM123) were descrived previously (4, 6). Escherichia coli DH5 (F$^-$, endA1, hsdR17, supE44, thi-1, recA1, gyrA96, relA1, λ$^-$) was used as a host for plasmids. JM101 (F'traD36, proAB, lacI^{q}ΔM15; Δlacpro, thi, supE) was used to propagate recombinant M13 phages for DNA sequencing. E. coli - S. pombe shuttle vector pDB248'(7) was a gift from Dr. M. Yamamoto of Uiversity of Tokyo. M13 mp18 and mp19 RF DNA were purchased from Nippon Gene Co., LTD. S. pombe strains were grown in YPD medium (2% polypeptone, 1% yeast extract, 2% glucose) or YNB medium (0.67% yeast nitrogen base without amino acids, 2% glucose, 50 μg/ml required amino acid) at 30°C with vigorous shaking. E. coli strains were grown in L broth (1% Bacto trypton, 0.5% yeast extract, 0.5% NaCl) at 37°C. Solid media for both S. pombe and E. coli were supplemented with 1.5% agar.

Assay for glutathione synthetase activity - Cells grown in 200 ml of YNB medium to mid logarithmic phase were harvested and washed twice with 50 mM Tris-HCl pH 8.5, 80 mM KCl, 40 mM $MgCl_2$ (TKM buffer). About 0.3 g of cell pellet was ground with 0.6 g of quartz sand for 10 min with a mortar and a pestle and extracted with 0.6 ml of TKM buffer. Quartz sand and cell debris were removed by centrifugation at 10,000 x g for 10 min and then particulate fraction was removed by centrifugation at 100,000 x g for 60 min. Supernatant of the centrifugation was used to assay glutathione synthetase activity as described previously (4) except that 20 nmole of cold glycine was added to the reaction mixture and the pH was adjusted to 8.5. Protein concentration of cell extract was determined by the method of Lowry et al. (8) using bovine serum albumin as a standard.

DNA sequencing - Restriction fragments from the plasmid containing a glutathione synthetase large subunit gene were isolated and cloned into M13 mp18 or mp19 phages. The resultant clones were sequenced by the dideoxynucleotide method (9) using viral plus strand DNA as templates.

Construction of a S. pombe genomic DNA library - DNA was isolated from the S. pombe strain L972h$^-$ as described by Beach et al. (7). DNA was partially digested with Sau 3AI and separated by a agarose gel electrophoresis. DNA fragments corespond to 3 to 6 kb were extracted from the gel. The extracted DNA fragments were ligated to pDB248', which was predigested with Bam HI and treated with bacterial alkaline phosphatase. The ligated DNA was used to transform E. coli DH5 to ampicillin resistance. Approximately 80,000 transformants were generated and about half of the transformants had a plasmid containing insert DNA . The average size of the inserts was 4 kb. This size of DNA library is large enough to cover entire genome of S. pombe, whose size is 1.4 Mb (9).

Yeast transformation and recovery of plasmid - Yeast transformation and recovery of plasmids from S. pombe into E. coli were performed as described by Beach et al. (7)

Peptide sequencing - Purified glutathione synthetase from S. pombe was run on SDS-PAGE to separate two subunits each other. Each subunit was extracted from the gel. The extracted polypeptide was digested with V8 protease and separated by a SDS-PAGE. Well separated bands were cut out from the gel and the peptides were extracted from the gel. Amino acid sequences of the extracted peptides were determined with a Protein Sequencer 470A (Applied Biosystems Inc).
Chemicals - Enzymes for DNA manipulation were purchased from Takara Shuzo Co., LTD. or Nippon Gene Co., LTD. V8 protease was obtained from Boehringer Mannheim. Radioactive materials were from ICN Biomedicals or NEN Research Products.

Results

S. pombe mutants deficient in glutathione synthesis were isolated as cadmium hypersensitive mutants (4). One of these mutants, strain MN101, lacks glutathione synthetase activity. MN101, which did not grow on a plate containing 0.1 mM $CdCl_2$, was transformed with a S. pombe genomic DNA library. Approximately 50,000 colonies were appeared on YNB plates containing 1.2 M sorbitol. Among these colonies 4 clones showed cadmium resistance and could grow on a YPD plate containing 0.5 mM $CdCl_2$. Plasmids were recovered from these clones by reintroduction into E. coli. Restriction analyses of the recovered plasmids showed that three of these clones had the same plasmid, which we called pYS41, and the rest had a smaller plasmid called pYS42. Inserts of these plasmids share 2.6 kb DNA sequence. Fig. 1 shows a restriction map of the insert DNA of these plasmids.

MN101 was transformed with pYS41 and pYS42 to test whether these plasmids gave cadmium resistance to MN101. The resultant transformants exhibited the same level of cadmium resistance as the parental strain HM123 (data not shown). Glutathione synthetase activities of these transformed cells were determined (Table 1). MN101 transformed with pYS41 exhibited the activity more than

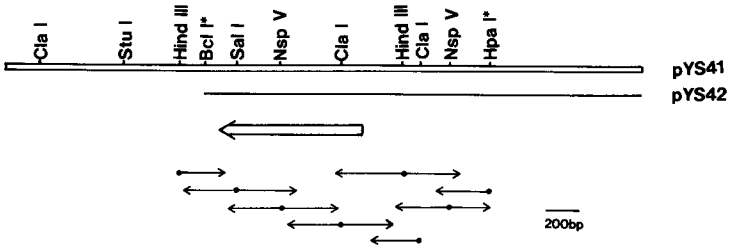

Fig. 1. Restriction map of pYS41. A restriction map of the insert DNA of the plasmid pYS41 is shown at the top. The insert DNA of pYS42 is shown below pYS41. Asterisks are the restriction sites used for subcloning experiments described in the text. Coding region for the large subunit of glutathione synthetase is shown by an open arrow. An arrow indicates the span and direction of each sequence analysis. Closed circle is an end of the restriction fragment used for sequencing.

Table 1
Glutathione synthetase activity of cell extract of verious strains

Strain	Glutathione synthetase activity[a]
HM123	0.14
MN101	0.00
MN101/pYS41[b]	1.92
MN101/pYS42[c]	0.46

[a]Glutathione synthetase activity is expresed as μmole glutathione synthesized /mg protein/hour.
[b]Strain MN101 harboring the plasmid pYS41.
[c]Strain MN101 harboring the plasmid pYS42.

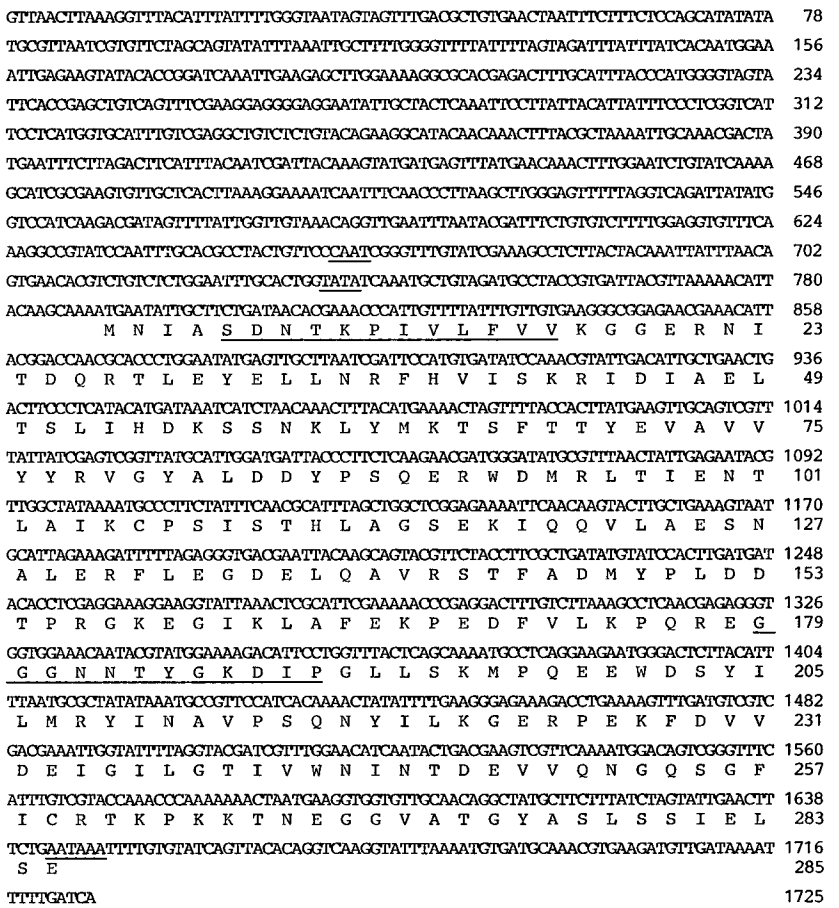

Fig. 2. Nucleotide sequence of a 1725 bp Hpa I - Bcl I fragment containing the gene for the large subunit of glutathione synthetase of S. pombe. The predicted translation product of 285 amino acid residues is indicated. Underlined amino acid sequences are those that coincided with the sequences obtained from V8 protease fragments. Putative CAAT box, TATA box and AATAAA polyadenylation signal are also underlined.

10 times higher than the activity in the parental strain HM123. Meanwhile, MN101 transformed with pYS42 exhibited activity 3 times higher than HM123.

Subcloning of the plasmid pYS42 showed that the gene which confers glutathione synthetase activity on the strain MN101 resides between the Hpa I site and the Bcl I site (data not shown). Nucleotide sequence analysis of this region revealed one long open reading frame (Fig. 2). The consensus sequence for splicing in S. pombe (11) was not found in the sequence.

Recently, glutathione synthetase of S. pombe was purified in our laboratory. It is composed of two kinds of subunits. Apparent molecular weights 33,000 and 26,000 were assigned for these subunits by SDS-PAGE (Nakagawa, C. W., et al. manuscript in preparation). The large subunit of the glutathione synthetase was digested with V8 protease. Two of the resultant peptide fragments were determined their amino acid sequences. One peptide with apparent molecular weight of 6,500 had a sequence SDNTKPIVLFVV (expressed in one letter amino acid code), which coincide with the amino acid sequence predicted from the nucleotide sequence 802 to 837 in Fig. 2. Another peptide with apparent molecular weight 8,000 had an amino acid sequence GGGNNTYGKDIP (expressed in one letter amino acid code), which is identical with the amino acid sequence predicted from the nucleotide sequence 1324 to 1359. Amino acid sequence of a peptide fragment derived from the small subunit of the enzyme was determined. The resultant amino acid sequence was not found in the amino acid sequences predicted from the nucleotide sequence described in Fig. 2 in any reading frames (data not shown). These results indicated that cloned DNA contains the gene for the large subunit of the glutathione synthetase.

Early in the open reading frame there are two methionine codons (nucleotide 544 to 546 and 790 to 792) possible to initiate translation. Molecular weight 33,000 of the gutathione synthetase large subunit is in good agreement with the predicted molecular weight 32177 of the polypeptide started at the second methionine codon (nucleotide 790 to 792) and ended at the stop codon of the nucleotide 1645 to 1647. We tentatively decided that the second methionine codon is used for a translation initiation site. On the other hand, since the peptide fragment whose N-terminal amino acid sequence is GGGNNTYGKDIP (nucleotide 1324 to 1359) has apparent molecular weight 8,000 by the determination with SDS-PAGE, the C-terminal of the large subunit of the enzyme must be close to the termination coden of this DNA sequence. At least the initiation from the first methionine codon is unlikelly on the basis of the molecular weight of the subunit and of the presence of the V8 protease peptide encoded by the nucleotide sequence nearby the termination codon. Possible TATA box and CAAT box characteristic of eukaryotic promoters (12) are found in nucleotide 785 to 788 and nucleotide 658 to 661, respectively.

The sequence AATAAA at nucleotide 1643 to 1648 is a possible poly(A) addition signal (13).

Discussion

We have cloned and sequenced the gene which encodes the large subunit of the glutathione synthetase of S. pombe. Strain MN101 harboring plasmid pYS41 or pYS42 exhibited higher glutathione synthetase activity than the parent strain. Higher enzyme activity is probably due to a gene dosage effect because pDB248' exists 5 to 10 copies in a cell (14). Strain MN101 harboring pYS41 exhibited glutathione synthetase activity higher than the activity of MN101 harboring pYS42. Since plasmid pYS42 lacks the sequence flanking 3'-terminal of the gene in comparison with pYS41, the nucleotide sequence in the 3-terminal flanking region of this gene must have some role in the expression of the gene.

The plasmids pYS41 and pYS42, which encode the gene for the large subunit of the glutathione synthetase, complement glutathione synthetase deficiency of the strain MN101. This indicated that the strain MN101 has a mutation in the gene of the large subunit of the glutathione synthetase. Although pYS41 and pYS42 contain only one gene encoding the large subunit of glutathione synthetase, S. pombe strain harboring pYS41 or pYS42 exhibited higher glutathione synthetase activity than the wild type strain. This indicated that both large and small subunits of glutathione synthetase are overproduced in a cell harboring these plasmids. How the small subunit could be synthesized in a large amount corresponding to the amount of the large subunit? One explanation is that the small subunit always exists abundantly in a cell and easily comlexed with the overproduced large subunit. Other explation is also possible, such as, large amount of the large subunit somehow induces the synthesis of the small subunit, and so on. Mechanisms of overproduction of the whole enzyme are now under study in our laboratory.

Glutathione synthetase gene have been cloned from E. coli B (15). Comparison of the nucleotide sequences of the genes from E. coli and S. pombe showed no significant homology between two genes.

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