

Molecular cloning of *Drosophila* γ -glutamylcysteine synthetase by functional complementation of a yeast mutant

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Abstract γ -Glutamylcysteine synthetase (GCS) catalyses a critical, rate-limiting step in glutathione synthesis. In this study we describe the isolation and characterisation of a GCS cDNA (pDmGCS4.3.3) from *Drosophila melanogaster* by functional complementation of a *Saccharomyces cerevisiae* *gsh1* mutant. Expression of pDmGCS4.3.3 in the yeast mutant partially restored glutathione levels and conferred resistance to methylglyoxal. The pDmGCS4.3.3 cDNA was found to be approx. 4.6 kb in length, containing a 2 kb fragment encoding an open reading frame with a high degree of deduced amino acid sequence identity with previously reported GCS sequences. In situ hybridisation revealed that the *Drosophila* GCS gene maps to 7D6–9 on the X chromosome.

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Key words: Glutathione; γ -Glutamylcysteine synthetase; Functional complementation; *Drosophila melanogaster*

1. Introduction

Glutathione is a principal component of cellular antioxidant and detoxification systems [1] and it (or a similar derivative) is present in the vast majority of eukaryotes, as well as in many prokaryotes [2,3]. Glutathione is synthesised in the cell cytosol by γ -glutamylcysteine synthetase (GCS) and glutathione synthetase [2]. GCS is proposed to catalyse the rate-limiting step in glutathione synthesis as it is subject to feedback inhibition by glutathione [4]. Factors that regulate the expression and activity of GCS are therefore of considerable interest, as GCS appears have a principal role in modulating glutathione homeostasis and consequently the capacity of the cell to withstand the deleterious effects of oxidative stress.

In mammals, GCS is a heterodimer comprising a catalytic heavy subunit (73 kDa, GCS_h) and a regulatory light subunit (30 kDa, GCS_l) [1]. cDNA clones encoding GCS have been isolated from several different eukaryotic and prokaryotic sources. Whilst there seems to be a remarkable degree of deduced amino acid sequence similarity between the majority of eukaryotic GCS cDNAs, the amino acid sequence of *Escherichia coli* GCS [5] is strikingly dissimilar to all of the eukaryotic GCS sequences described to date. Interestingly, the deduced amino acid sequence of *E. coli* GCS was found to be more similar to rat kidney GCS_l than GCS_h, although

the possible functional significance of this is unclear [6]. Despite the relatively high degree of conservation in primary structure of GCS in most eukaryotes, *Arabidopsis thaliana* appears to contain a GCS protein that is structurally distinct. The deduced amino acid sequence from *A. thaliana* was found to be less than 20% identical to any GCS described so far [7,8]. Thus, it appears that there has not been a strict requirement for the conservation of GCS structure throughout evolution.

To date no GCS sequence from any insect has been described. We wished to clone the *Drosophila melanogaster* GCS gene: the potential role of GCS and glutathione in resistance to insecticides makes GCS in insects an attractive target for study. Furthermore, the potential for genetic manipulation in *Drosophila* is well established and highly sophisticated. The isolation of a *Drosophila* GCS cDNA will facilitate future genetic experiments which will allow fundamental questions about the functions of glutathione to be addressed. In this study we have isolated a cDNA clone encoding GCS from *Drosophila* by functional complementation of a yeast strain mutant for GCS, and show that its deduced amino acid sequence has a high degree of similarity to the majority of eukaryotic GCS sequences.

2. Materials and methods

2.1. Functional complementation

All yeast media were prepared as described in [9].

Saccharomyces cerevisiae strains YPH499 and YPH499/5 were a gift from Dr Pascaline Ullmann (University of Strasbourg). The *D. melanogaster* S2 cell line cDNA library [10] was obtained from Prof. T. Orr-Weaver (MIT), and is constructed in the yeast expression vector pBL15 in which expression is driven by an *ADHI* promoter. This vector uses *ARG4* as its selection marker and plasmid sequences derived from pUC18, including the β -lactamase gene.

An *arg4* deletion mutant was induced in the YPH499/5 strain by recombination with the PCR product described below. Two 70 base oligonucleotides were synthesised, each contained 50 bases of homology to *ARG4*, and 20 bases of homology to the sequences flanking *TRP1*, contained within the plasmid pRS314 [11]. pRS314 was a gift from Dr D. Stirling (University of Dundee). The oligonucleotide sequences were as follows: oligonucleotide 10910: CAATTGAAGAGCTCAAAAGCAGGTAACATATATAACAAGACTAAGGCAACAGATTGTACTGAGAGTGCAC; oligonucleotide 10911: GATGAAGTCTAGAAAGTACCAGACCTGATGAAATCTTGCAGATAACGTCCTGTGCGGTATTTACACCG.

Using these oligonucleotides as primers, the *TRP1* gene was amplified from this plasmid and used to transform the YPH499/5 yeast strain. *arg4* mutants were selected on minimal medium supplemented with arginine, uracil, lysine, adenine and leucine but lacking tryptophan. Sensitivity to methylglyoxal was subsequently verified. One of these strains was selected for use in this work, and was named YRS001.

The yeast strains used in this work are as described below:

The *Drosophila* cDNA library was used to transform YRS001 using

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YPH499	<i>MATa, ura3-52, lys2-801, ade2-101, trp1-Δ63, leu2-Δ1, his3-Δ200</i>
YPH499/5	<i>MATa, ura3-52, lys2-801, ade2-101, trp1-Δ63, leu2-Δ1, his3-Δ200, gsh1::HIS3</i>
YRS001	<i>MATa, ura3-52, lys2-801, ade2-101, trp1-Δ63, leu2-Δ1, his3-Δ200, gsh1::HIS3, arg4::TRP1</i>

a standard lithium acetate transformation protocol [9]. Transformed yeast cells were plated on minimal medium supplemented with 3 mM methylglyoxal [12] and amino acids required by each strain. Yeast strains YPH499/5 and YRS001 fail to grow on this medium. Colonies were picked and re-plated twice to verify rescue. Plasmid DNA (pDmGCS4.3.3) was recovered from one strain which was repeatedly observed to be viable when plated on the selective medium containing methylglyoxal by extracting total DNA from the culture and using this preparation to transform *E. coli* XL-1 Blue cells (Stratagene). Ampicillin-resistant colonies were propagated and the plasmids extracted. In order to verify that restoration of viability was due to the presence of the plasmid and not to reversion or second site mutation, the *gsh1 arg4* yeast strain was transformed with plasmid pDmGCS4.3.3. These transformants were viable when plated on medium containing methylglyoxal.

2.2. Molecular analysis

General molecular techniques were carried out as described [13]. Restriction enzymes were used as described by the manufacturer. The pDmGCS4.3.3 insert was subcloned into pBC-SK (Stratagene) in both orientations and these plasmids together with pDmGCS4.3.3 were used as templates for sequencing. Sequencing was carried out with a 'primer walking' strategy, using an ABI377 Prism automated sequencer, and terminator labeling chemistry. Sequence reads were assembled and analysed using DNASTar software (LaserGene). In situ hybridisation to *Drosophila* polytene chromosomes was carried out as described [14], using biotin-labeled DNA probes and avidin-horseradish peroxidase (ExtrAvidin-HRP, Sigma) to detect bound probe. The chromogen was diaminobenzidine (Sigma).

2.3. Glutathione assays

Yeast cultures were grown in minimal medium with supplements appropriate to their genotypes. Cells were pelleted and disrupted with glass beads as described [9]. Protein levels and glutathione concentrations (total glutathione, GSH+GSSG) were estimated as described previously [15].

3. Results and discussion

3.1. *Drosophila* cDNA clone pDmGCS4.3.3 complements the yeast mutation *gsh1*

Glutathione has been shown previously to be an important antioxidant in yeast, and *gsh1* mutants are sensitive to H₂O₂ and redox cycling agents [16]. In addition, sensitivity to the growth inhibitor methylglyoxal has been reported in yeast *gsh2* mutants, a phenotype that was utilised to isolate an *A. thaliana* glutathione synthetase cDNA by functional complementation [12]. We have followed a similar strategy to isolate GCS from *Drosophila*.

Several yeast colonies were recovered following transformation of *gsh1 arg4* cells with the *Drosophila* cDNA library and propagation on minimal medium supplemented with 3 mM methylglyoxal. On the first round of selection six colonies were chosen for further analysis. Of these, only one clone

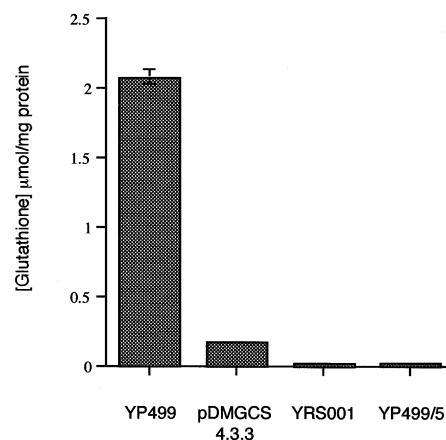


Fig. 1. Rescue of the glutathione deficiency in YPH499/5 by heterologous expression of a *Drosophila* cDNA. YPH499 is *GSH1* wild type. YPH499/5 is mutant for *gsh1*. YRS001 is mutant for *gsh1* and *arg4*. pDmGCS4.3.3 indicates YRS001 transformed with plasmid pDmGCS4.3.3. The full genotypes of these strains are listed in Section 2.

passed two further rounds of selection, and was named pDmGCS4.3.3. It was important to verify that rescue in this strain was due to the presence of the cDNA clone, and not to a second site mutation or reversion. Total DNA was therefore extracted from this yeast strain, and used to transform *E. coli* strain XL-1 Blue. Ampicillin-resistant colonies were recovered, and plasmid was purified from one of these. Transformation of the YRS001 yeast strain with this plasmid restored viability upon plating on medium containing 3 mM methylglyoxal.

3.2. Expression of pDmGCS4.3.3 causes an increase in intracellular glutathione

We wished to correlate rescue of viability on medium supplemented with methylglyoxal with restoration of glutathione levels. Cell cultures of four yeast strains (YPH499, YPH499/5, YRS001 and YRS001 transformed with pDmGCS4.3.3) were prepared in minimal medium with appropriate supplementation. Pelleted cells were disrupted with glass beads and the glutathione levels in the soluble fraction determined (Fig. 1). The YPH499 strain was found to contain 2.050 ± 0.07 μmol glutathione per mg of soluble protein whereas the *gsh1* mutant strains had glutathione levels which bordered on the detection limit of our assay system (0.006 μmol glutathione/mg protein). Levels of glutathione in YRS001 transformed with pDmGCS4.3.3 were elevated relative to the strain of identical genotype not carrying this plasmid, but it is clear that levels were not restored to wild type levels. The glutathione levels in the complemented mutant were found to be 0.162 ± 0.002 μmol/mg protein, approximately 8% of the parental strain. It is possible that this reflects the structure of the cDNA insert within this clone, as described in Section 3.3. Alternatively, it is feasible that *Drosophila* GCS requires specific factors not

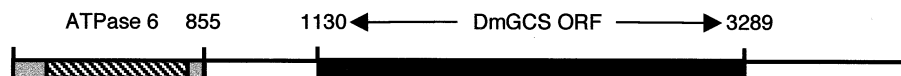


Fig. 2. Structure of clone pDmGCS4.3.3. The section of mitochondrial genome is indicated as a grey box with the hatched area representing the open reading frame. The section containing the *Drosophila* GCS cDNA (DmGCS) is indicated by the white box, with the open reading frame (ORF) shaded black.

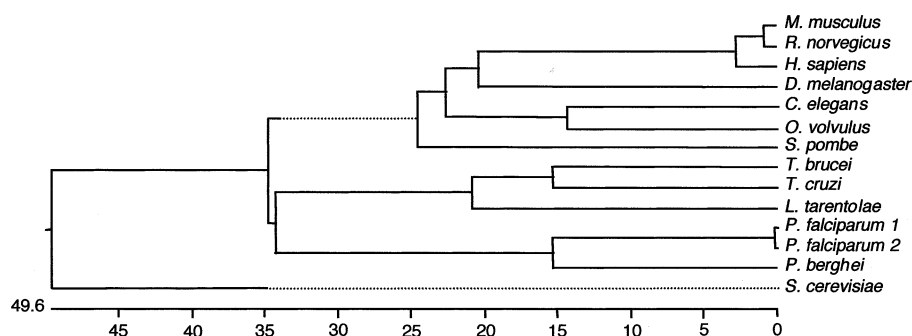


Fig. 3. A phylogenetic tree representation of sequence alignment of GCS catalytic subunit polypeptide sequences of several eukaryotes.

found in *S. cerevisiae* for full activity. For example, it is conceivable that, like mammalian GCS, the *Drosophila* enzyme requires a regulatory subunit for efficient activity. Furthermore, it was shown previously that complementation of an *S. cerevisiae* *gsh1* mutation by GCS from *A. thaliana* resulted in restoration of glutathione levels which were less than 15% of the parental yeast strain values [8] and it was proposed that cell-specific post-transcriptional mechanisms are required for full GCS activity. It will be interesting to determine whether *Drosophila* GCS enzyme activity is subject to regulation by similar mechanisms. The biochemical characterisation of *Drosophila* GCS is the subject of a future study. It is, however,

notable that even the reduced levels of glutathione synthesis restored by pDmGCS4.3.3 are sufficient to restore viability when cells are plated on medium containing 3 mM methylglyoxal.

3.3. Clone pDmGCS4.3.3 contains a full length cDNA encoding the *Drosophila* homologue of GCS

Plasmid pDmGCS4.3.3 was propagated in *E. coli* strain XL-1 Blue following its extraction from YRS001. Restriction digestion with *NotI* revealed an insert of approximately 4.6 kb. This insert was subcloned into pBC-SK (Stratagene). Clones containing the insert in both orientations were recov-

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1  MGLLSEGSPLSWEETKALADHVRHGVNQFINLYHRLKDRQGDILKWGDEVEYIIIVKFDDEQKVARVALR
1  MGLLSQGSPLSWEETKRHADHVRHGIQLFLHIYHAVKDRHKDVLKWGDEVEYMLVSFDHENKKVRLVLS

71  AQDLLAQNEKELADPNGVKSILWRPEYGAYMIEGTPGKPFGLMAHFNLVEANMRYRREEVTELLAKDEC
71  GEKVLETLQEKGERTNPNHPTLWRPEYGSYMIEGTPGQPYGGTMSEFNTVEANMRKRKEATSILEENQA

141  VMSITNFPRLGAPNFTYPLAQPRPEDPLSSARSLYFPDEAIFPGHPRFKTLTRNIRKRGEKVSIKLKVF
141  LCTITSFPRLGCPGFTLPEVKPNVEG-GASKSLFFPDEAT-NKHERFSTLTRNIRHRERGEKVINVPIE

211  KDTKTKLPV-----EGAPPGEPDVLLDAMGFGMCCCLQLTFQACNITEARRLYDQLAPLCPIMLA
209  KDRNTPSPFFIETFTEDDEASRASKPDHIYMDAMGFGMCCCLQVTFQACSISEARYLYDQLATICPIVMA

273  LTAASPIYRGYLTESDCRWNVISSVDCRTEEERGLAPLDQQKFRIAKSRYDSIDSYLSPEGAKYNDVPL
279  LSAASPFYRGYVSDIDCRWGVISASVDDRTREERGLEPLKNNYRISKSRYSIDSYLSKCKEYNDIDL

343  TYDEKVYQRLVEGGIDHLLAQHVAHLFIRDITVSLFSEKVHQNEDTDHFNIEQSTNWQTMRFKPPPPNS
349  TIDKEIYEQLLQEGIDHLLAQHVAHLFIRDITLFEKIHLDANESDHFENIQSTNWQTMRFKPPPPNS

413  SIGWRVEFRPCEAQISDFENAAIVCFVLLTRVILSYQLNFLTPIISKVDENMQTAQKRDAKREKFWFRK
419  DIGWRVEFRPMEVQLTDFENSAYVVFVLLTRVILSYKLDFLIPLSKVDENMKVAQKRDAVLQGMFYFRK

483  SSKTTEQRAAKAQAAQAAQAAQAAQTNGKATLNGNGLANGNGSENGDQEEQQPLTNGSAKMGHGN
489  -----

553  GTTNGTNGSSNGSSNGTSDHTDDEENELFQLLSINEIFNGKPNVFPGLVPLIRSYLQSMVDTDTHC
489  DICKGGNAVVDGCGKAQNSTELAAEE-----YTLMSIDTIINGKEGVFPGLIPLILNSYLENMEVDVDTRC

623  TIEQYLRFIQKRAAGELITTATWMREQVLSHPDYKQDSVVSERINYLKRIQGIQEGKQVEPALLGQDY
554  SILNYLKLKKRASGELMTVARWMREFIANHPDYKQDSVITDEMNYSLILKCNQIANELCECEPELLGSAF

693  HSKTKTKDFIPPALQKQLAKNGCCEEK
624  R---KVK-YSGSKTDS-----SN

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Fig. 4. Sequence alignment of *Drosophila* and human GCS. The alignment between *Drosophila* (top) and human (bottom) GCS amino acid sequences was derived from a Clustal alignment, using the MegAlign program from the DNASTar software suite.



Fig. 5. Localisation of the *Drosophila* GCS gene to 7D6-9 on the X chromosome. In situ hybridisation of plasmid pDmGCS4.3.3 to *Drosophila* larval salivary gland polytene chromosomes was carried out as described in the text. The signal is indicated by the arrow.

ered and used along with pDmGCS4.3.3 as sequencing templates.

Sequence analysis revealed the insert to be 4638 bp in length. BLASTN and BLASTX database searches indicate this clone represents two independent segments of DNA, as illustrated in Fig. 2. Approximately 800 bp immediately adjacent to the *ADH1* promoter are derived from the *Drosophila* mitochondrial genome and contains the ATPase 6 polypeptide open reading frame in its entirety. Because this open reading frame utilises the mitochondrial genetic code, it is not expected to be expressed in *S. cerevisiae*, and indeed when translated using the nuclear genetic code, this segment of DNA contains multiple stop codons in all reading frames.

The second DNA segment contains a single open reading frame encoding a polypeptide of 719 amino acid residues with highly significant homology to GCS sequences in the sequence databases. Fig. 3 shows a phylogenetic alignment of this sequence with other GCS sequences in the EMBL and GenBank databases. Fig. 4 shows an alignment between the predicted DmGCS polypeptide sequence and human GCS. Homology between the two sequences is striking (56% similarity).

The N-terminal 15 residues are very highly conserved in eukaryotes (with the exception of protozoans), and alignment with these sequences indicates that it is likely that the pDmGCS4.3.3 open reading frame extends to include the N-terminus of the enzyme. There is an insertion of 70 residues in the DmGCS (amino acid residues 483–552) relative to mammalian GCS sequences (Fig. 4). This region contains two repetitive motifs, the QA and NG repeats. It is unclear whether these sequences represent regions of specific function.

We do not find a clearly defined polyA tail in this cDNA clone. However, there are several moderately long stretches of A residues in this section of the insert, and it is possible that one or more of these represent the polyA tail, perhaps disrupted during library construction. The open reading frame extends 3' to include the residues homologous to the C-terminus of mammalian GCS sequences. There appears to be an extended untranslated region 3' to the open reading frame, of about 800 bp.

It is possible that the low concentrations of glutathione in YRS001 complemented with pDmGCS4.3.3 result from low levels of GCS expression due to the upstream insertion of

mitochondrial DNA. This segment of DNA includes 10 methionine codons, in all three reading frames, at which translation might be expected to initiate, together with numerous stop codons in all three frames.

Plasmid pDmGCS4.3.3 was mapped to 7D6-9 on the X chromosome by in situ hybridisation (Fig. 5). In addition to determining the site of origin of this clone, this experiment confirms that the insert is indeed derived from the *Drosophila* genome. The mitochondrial segment of pDmGCS4.3.3 would not be expected to yield a site of hybridisation on polytene chromosomes. The localisation of the gene encoding GCS to 7D6-9 is of particular interest because several mutations have been mapped to this region. It is possible that one or more of these may contain a deficiency in the GCS gene, offering a powerful tool with which to investigate glutathione metabolism and function in *Drosophila*.

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References

- [1] Hayes, J.D. and McLellan, L.I. (1999) Free Radical Res. 31, 273–300.
- [2] Meister, A. and Anderson, M.E. (1983) Annu. Rev. Biochem. 52, 711–760.
- [3] Fahey, R.C. and Sundquist, A.R. (1991) Adv. Enzymol. Relat. Areas Mol. Biol. 64, 1–53.
- [4] Richman, P.G. and Meister, A. (1975) J. Biol. Chem. 250, 1422–1426.
- [5] Watanabe, K., Yamano, Y., Murata, K. and Kimura, A. (1986) Nucleic Acids Res. 14, 4393–4400.
- [6] Huang, C.-S., Anderson, M.E. and Meister, A. (1993) J. Biol. Chem. 268, 20578–20583.
- [7] May, M.J. and Leaver, C.J. (1994) Proc. Natl. Acad. Sci. USA 91, 10059–10063.
- [8] May, M.J., Vernoux, T., Sánchez-Fernández, R., Van Montagu, M. and Inzé, D. (1998) Proc. Natl. Acad. Sci. USA 95, 12049–12054.
- [9] Lundblad, V. (1997) in: Current Protocols in Molecular Biology, Vol. 2 (Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K., Eds.), Wiley, New York.
- [10] Lahue, E.E., Smith, A.V. and Orr-Weaver, T.L. (1991) Genes Dev. 5, 2166–2175.
- [11] Sikorski, R.S. and Hieter, P. (1989) Genetics 122, 19–27.
- [12] Ullmann, P., Gondet, L., Potier, S. and Bach, T.J. (1996) Eur. J. Biochem. 236, 662–669.
- [13] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- [14] Saunders, R.D.C. (1994) in: In Situ Hybridization Protocols, Vol. 33 (Choo, K.H.A., Ed.), pp. 183–191, Humana Press, Totowa, NJ.
- [15] Galloway, D.C., Blake, D.G. and McLellan, L.I. (1999) Biochim. Biophys. Acta 1446, 47–56.
- [16] Stephen, D.W.S. and Jamieson, D.J. (1996) FEMS Microbiol. Lett. 141, 207–212.