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An evolutionary perspective on glutathione transferases inferred from class-Theta glutathione transferase cDNA sequences

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We report the cDNA sequence for rat glutathione transferase (GST) subunit 5, which is one of at least three class Theta subunits in this species. This sequence, when compared with that of subunit 12 recently published by Ogura, Nishiyama, Okada, Kajita, Narihata, Watabe, Hiratsuka & Watabe [(1991) Biochem. Biophys. Res. Commun. **181**, 1294–1300] proves that Theta is a separate multigene class of GST with little amino acid sequence identity with Mu-, Alpha- or Pi-class enzymes. The amino acid sequence identity of class-Theta subunits is highly conserved in rat, the fruitfly *Drosophila*, maize (*Zea mays*) and *Methylobacterium*, which suggests that this family is representative of the ancient progenitor GST gene and originates from the endosymbioses of a purple bacterium leading to the mitochondrion. The high conservation of class Theta brings into prominence that Alpha-, Mu- and Pi-class enzymes, which are not present in plants, derive from a Theta-class gene duplication before the divergence of fungi and animals and, given the binding properties of the Alpha-, Mu- and Pi-classes, suggests a role for these in the evolution of fungi and animals.

INTRODUCTION

Soluble glutathione transferases (GST; EC 2.5.1.18) are a supergene family of proteins which not only catalyse the conjugation of glutathione to a variety of electrophiles, but also bind steroids, bilirubin, carcinogens and numerous organic anions [3–5]. The proteins are dimers assembled from subunits which have been put into four classes, namely Alpha, Mu, Pi [5] and Theta [2,4], based on percentage *N*-terminal amino acid identity and immunocross-reactivity with subunits of human GSTs. Most GSTs are abundant and have K_m values for GSH between 0.01 and 0.2 mM, but the Theta family, and in particular rat GST 5-5, is exceptional, since GST 5-5 is of low abundance, labile and possesses a high K_m for GSH [2–6]. Of particular interest is the dehalogenase activity of GST 5-5 with dichloromethane (DCM), because this activity may be responsible for the *in vitro* genotoxicity of DCM and for the carcinogenicity of DCM in mice, since the reaction proceeds via *S*-chloromethylglutathione, which then yields formaldehyde as final product [2]. We report the cloning of rat GST 5-5 and show that the GST Theta family is highly conserved throughout evolution, implying an as yet unrecognized basal function for this family.

EXPERIMENTAL

Materials

Rat liver and hepatoma cDNA libraries in λgt10 were prepared as described by Pemble *et al.* [1]. Restriction and modifying enzymes used for cloning procedures were obtained from Gibco–BRL, Pharmacia and New England Biolabs. Nylon membranes (Hybond N) used in Northern and Southern blotting were from Amersham. NA45 paper was supplied by Schleicher and Schuell. All other chemicals were from Sigma or BDH. Qiagen columns used in the purification of plasmid DNA were supplied by Hybaid (Teddington, Middx., U.K.). PCR reactions were carried out with Amplitaq kits from Cetus Corporation using oligonucleotides obtained from Oswel DNA service, University of Edinburgh, Edinburgh, Scotland, U.K.

Methods

Preparation of cDNA fragments using PCR. Rat liver and hepatoma first-strand cDNAs were prepared as described in Pemble *et al.* [1] and used without further purification in subsequent PCR reactions. Rat hepatoma λgt10 cDNA library (10^{11} plaque-forming units/ml) was diluted 10-fold in sterile distilled water, heated at 70 °C for 10 min and then centrifuged for 2 min in a Microfuge. A 1 μl portion of the supernatant was used for each PCR reaction, which was carried out in a 50 μl volume of 10 mM-Tris/HCl, pH 8.3, containing 50 mM-KCl, 1 mM-2-mercaptoethanol, 1% (w/v) gelatin, 1 mM-$MgCl_2$, 0.2 mM-(all four) deoxynucleotide triphosphates/Amplitaq (2.5 units) and either 1.5 μl of first-strand cDNA or 1 μl of 10-fold diluted hepatoma λgt10 lysate. Primers were used at concentrations over the range 0.2–0.5 μM.

Analysis of PCR products. PCR products for subcloning were blunt-ended using Klenow fragment and polynucleotide kinase and then separated on a 2% (w/v) agarose gel. Products were isolated by electroelution on to NA45 paper, eluted at 70 °C in 1.5 M-NaCl and were subsequently subjected to ethanol precipitation. After centrifugation, DNA fragments were ligated to *Hin*cII-restricted pUC18 and transfected into *Escherichia coli* strain JM83. Recombinant plasmid DNA was prepared for sequencing and further subcloning using Qiagen columns as described in the manufacturer's protocol. All sequencing was carried out by using Sequenase and the protocol for double-stranded sequencing described by the manufacturer.

Cloning of rat subunit 5. Discovery PCR primers both contained a 64-degenerate mixture. Primer 497G was a 24-mer (CGCC ATG GGN CTC/G GAA/G CTC/G TAC/T CT) deduced from the identical *N*-terminal heptapeptide sequences of rat subunit 12 and human GST θ [5] (MGLELYL which assumes an initiator methionine codon, ATG) with an additional four nucleotides (CGCC) to create an *Nco*I site. A reduced codon

Abbreviations used: GST, glutathione *S*-transferase; DCM, dichloromethane; 1 × SSC, 0.15 M-NaCl/0.015 M-sodium citrate.

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X67654.

degeneracy based on codon frequency usage was used for the leucine residues (CTC/G). Primer 91/329 was an antisense 17-mer (TG NGG G/ATA G/ACA G/ATG G/ATC) deduced from a hexapeptide sequence DHCYPQ present in a CNBr-cleaved peptide of rat subunit 5 [2]. This peptide included the sequence SVAIL, which is characteristic of a highly conserved region of GSTs centred on residues 70–77 of known GSTs. Hence the anti-sense primer should correspond to 15 amino acid residues downstream of this region, and therefore this primer combination would yield a PCR product between 255 and 276 [i.e. $(70+15)\times 3$ and $(77+15)\times 3$] bp in length. With rat liver first-strand cDNA as a template, these primers yielded four PCR products when revealed on an agarose gel, of which one of approx. 280 bp was cloned in pUC18. Sequencing revealed that it was a partial clone encoding 88 amino acid residues of subunit 5. From this sequence, a sense-strand primer 91/414 (ATCCCG-TTCCAGATGCATACTG; underlined in Fig. 1 below) and an anti-sense primer 91/415 (CTTCATGGGGTTCACCTGGGC, underlined in Fig. 1 below) were synthesized. 91/414 and a non-specific primer 89/535 [TTTTTTTTTTTTTTTGCGGCCGC; designed to hybridize to the poly(A) tail of all cDNAs] yielded a 900 bp PCR product from first-strand rat liver cDNA which overlapped perfectly with the partial clone. The sequence was subsequently identified in direct screening of rat hepatoma and liver libraries using the PCR clones as probes.

Northern and Southern blotting. Cloned fragments for use as probes were released from recombinants using the *Sph*I and *Eco*RI sites of the flanking multicloning linker (neither site appears in the full-length cDNA) and purified by two cycles of gel separation and electroelution. Probes were radiolabelled by incorporation of ^{32}P-labelled dCTP using random priming. The preparation of Northern and Southern Blots, and the hybridization and washing conditions for Northern blots have been described previously [1]. Southern blots were hybridized at 65 °C overnight in 1 M-NaCl/1 % SDS, containing heat-denatured herring testis DNA (100 μg/ml), poly(A) (10 μg/ml) and $(1–2)\times 10^6$ c.p.m. of probe/ml [$(1–3)\times 10^9$ c.p.m./μg]. Blots were washed twice for 15 min in 2 × SSC (1 × SSC is 0.15 M-NaCl/0.015 M-sodium citrate)/1 % SDS at room temperature, followed by 2 × SSC/0.1 % SDS at 65 °C and autoradiography. High-stringency conditions included a final wash at 0.1 × SSC at 65 °C.

RESULTS AND DISCUSSION

GST class Theta has recently been defined by limited peptide sequence data which include two *N*-terminal sequences for rat subunits 5 and 12 [2,6], an *N*-terminal sequence of a subunit of human GST θ [2] and a cDNA clone encoding rat subunit 12 [7]. We have cloned a full-length cDNA encoding rat subunit 5 (Fig. 1) and several peptides unique to subunit 5 [2] are contained in the derived sequence (underlined in Fig. 1). Southern-blot analysis using the full-length subunit-5 probe under high stringency shows either two or three cross-hybridizing bands in rat DNA restricted with *Eco*RI, *Hind*III or *Sst*I and, in particular, two bands in DNA digested with *Pst*I (Fig. 2*a*). Since the cDNA has an internal *Pst*I site, these results are consistent with a single gene encoding sequences of high nucleotide identity with subunit 5. This result is not surprising, since GST subunit 12 has only 80 % nucleotide sequence identity with subunit 5, with the scattered nucleotide changes presumably preventing cross-hybridization. Peptide sequence data from Meyer *et al.* [2] also suggested that these proteins differ considerably, except at their *N*-termini. We have also cloned a partial cDNA which codes for a protein (GLELYLDLMSQPCRAVYIFAKKNGI; nucleotide sequence not shown) with high *N*-terminal amino-acid identity with both subunits 5 and 12. This gene also cannot hybridize to the subunit 5 probe under stringent conditions. With the subunit 5 probe, cross-hybridization with human DNA was seen, which was also consistent with a single subunit-5-like gene in humans (results not shown).

Since Southern-blot analysis using the subunit-5 probe is consistent with a single cross-hybridizing gene, then Northern blot analysis to discover the tissue distribution of subunit-5 mRNA is feasible. By using full-length cDNA for subunit 5 under stringent conditions, this analysis shows a single hybridizing band of 1100 nucleotides in rat liver, epididymis and hepatoma (Fig. 2*b*) and also adrenal gland and kidney, but not in lung, testis, spleen, heart or skeletal muscle (results not shown). The size of the mRNA is similar to that reported for other GST subunits, but its abundance is considerably lower.

The deduced amino acid sequences of the subunit-5 clone is shown in Figs. 1 and 3. The mature subunit-5 protein would be 239 amino acids long with an M_r of 27340; this is a notably longer protein than those found in the other GST families (Alpha, Mu, Pi; 218–9, 220–1, 209 amino acids long respectively). Screening of the NBRF (protein sequences) database for similar proteins selected sequences of GSTs from *D. melanogaster* [8], chicken [9] and maize (*Zea mays*) [10] (Fig. 3*a*). Also shown is the recently published *N*-terminal sequence of a GST from dog liver [11] (Fig. 3*b*) and the deduced amino acid sequences of rat subunit 12 [7] and of dichloromethane (DCM) dehalogenase of *Methylobacterium* sp. strain DM4 [12]. The *Methylobacterium* and *D. melanogaster* GST subunits in particular have a high sequence identity with those of rat class Theta. It is noteworthy that rat GST subunit 5 also has DCM dehalogenase activity [2] and also that both the *Methylobacterium* and *D. melanogaster* enzymes are encoded by intronless genes [7], although this gene structure does not apply to the rat class-Theta genes (S. Pemble, unpublished work).

The high identity of sequence of the class-Theta enzymes of rat, *D. melanogaster* and *Methylobacterium* is remarkable given the evolutionary time between the three species. This conservation of sequence identity is also visible in the Plant Kingdom (maize [9] and tobacco (*Nicotiana tabacum*) [13]; Fig. 3*a*) and the implication is that class Theta is similar to the progenitor GST. This conclusion is further supported by the incidence of class-Theta *N*-terminal sequences in numerous species (Fig. 3*b*) and in a rat mitochondrial GST [14] (coded by a nuclear gene presumably arising from the bacterial endosymbioses leading to mitochondria). This maintenance of sequence identity with a GST of the mitochondrial matrix also supports the view that there is a fundamental role for this family of GSTs. This role probably relates to the evolution of GSH in purple bacteria and cyanobacteria to protect against oxygen toxicity [15] and is consistent with the genes for γ-glutamylcysteine synthetase, GSH synthetase, GSH reductase, formate dehydrogenase and the glyoxylase enzymes, and their consequent oxygen defence system, all being obtained by eukaryotes from their mitochondrial endosymbiont, since this complement of enzymes is limited to some purple bacteria and cyanobacteria [15]. It is noteworthy that the inclusion of a class-Theta GST in this enzyme contingent may have contributed not merely to oxygen-toxicity defences through a GSH peroxidase activity but may also have aided the symbiont to tolerate its new environment of host cytoplasm. Although activities of class-Theta GST are still being discovered, its conservation does allow construction of the evolutionary relationships of a number of structurally related, but functionally disparate, proteins in the following analysis, which also suggests a role for the Alpha, Mu and Pi classes of GST.

Identity in protein sequences suggests that their genes arose from a gene duplication which, in order to facilitate evolution, requires one gene to conserve its function, whereas its duplicate

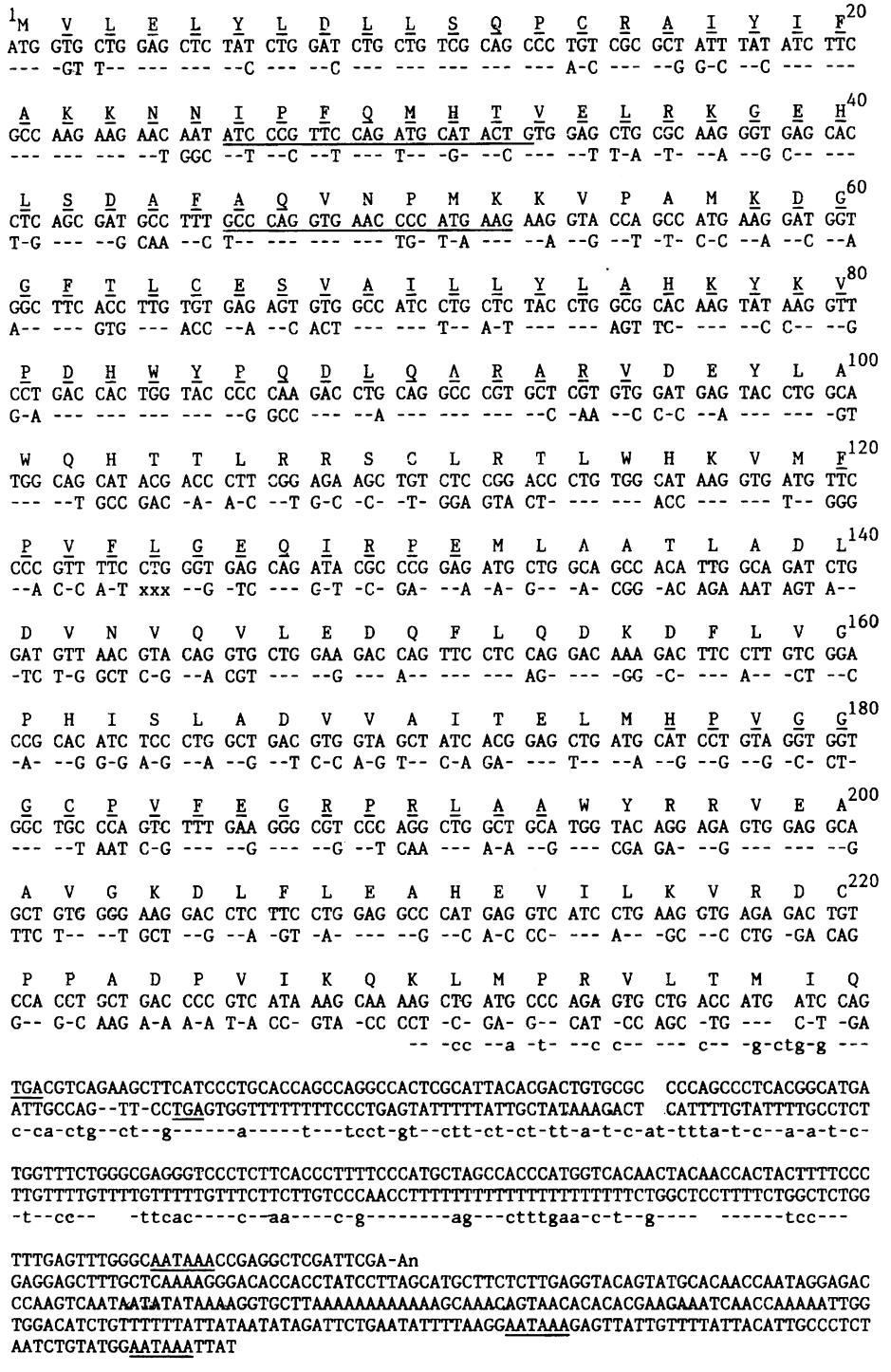

Fig. 1. Cloning of rat subunit 5

Numbers refer to the amino acid residues (single-letter code) which are deduced from the nucleotide sequence of rat subunit 5 typed immediately below. The second nucleotide sequence is that of subunit 12 [7]. Lower-case letters are from the 3′ non-coding sequences of three human class Mu cDNAs, two liver-derived cDNAs of alleles of GST M1-1 and one muscle-derived cDNA of GST M2-2 [19]. Peptides identified by Meyer *et al.* [2] are underlined. A few residues do not correspond to the published peptides, most notably that used for PCR discovery primer 91/329, which was reported as DHCYPQ (methods and [2]) and which is deduced as DHWYPQ, and also the peptide centred on residue 180 (HPVGGGCPVFEGRPRLAA), which was reported as HPVGGCP..., and which was a typographical error in Meyer *et al.* [2]. Underlined are two oligonucleotides used for identification of subunit sequences by PCR from cDNA libraries. Clones for subunit 5 were identified in both normal liver and hepatoma cDNA libraries and full sequencing on both strands showed them to be identical. A maleylated tryptic peptide sequence, XXPXADPVI (S. E. Pemble & J. B. Taylor, unpublished work; [2]) is consistent with residues numbered 219–227. Also underlined are the TGA stop codon and the AATAAA polyadenylation signal.

is free to evolve a novel function advantageous to the organism. GSTs were assigned to an appropriate class by optimizing sequence identity while minimizing 'gaps' in the sequence alignments to preserve relative positions. This showed that the Alpha, Mu and Pi classes have a maximum of 7% overall sequence identity with class Theta that is due to a short, highly

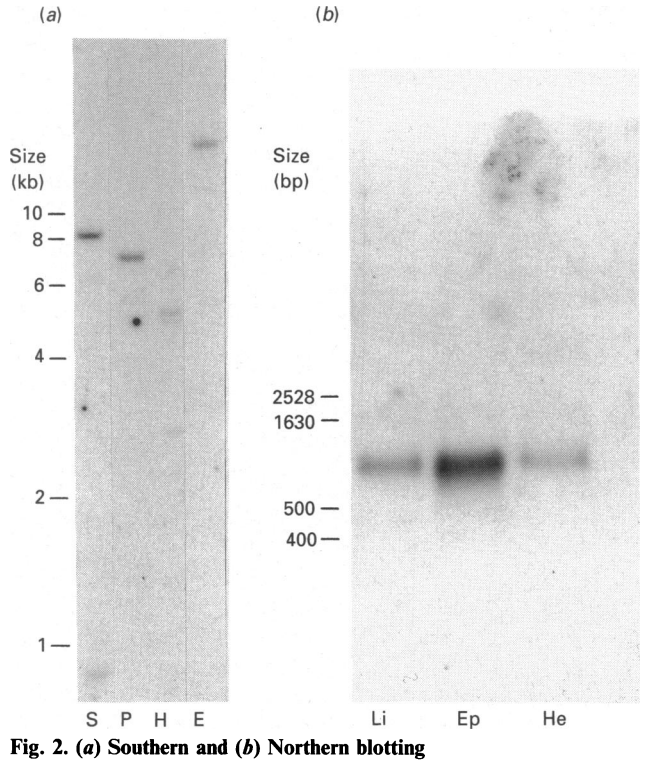

Fig. 2. (*a*) Southern and (*b*) Northern blotting

(*a*) Rat genomic DNA isolated from the liver of a single rat was digested to completion with restriction nucleases as indicated below. DNA fragments were separated by electrophoresis on a 0.6% agarose gel, transferred to Hybond N and hybridized to ^{32}P-labelled full-length rat subunit 5 cDNA and autoradiographed. The cDNA contains a site for *Pst*I. Lanes correspond to DNA digested with: S, *Sst*I; P, *Pst*,I; H, *Hin*DIII; E, *Eco*RI. Size markers (kb) correspond to a 1 kb restriction fragment ladder from Bethesda Research Laboratories. (*b*) Poly(A) RNA isolated from rat liver, epididymis and hepatoma was analysed under denaturing conditions in 1.1% (w/v) agarose gels. The RNA was transferred to Hybond N membrane, hybridized to ^{32}P-labelled full-length rat subunit 5 cDNA and autoradiographed. 10 μg of poly(A) RNA was loaded in each lane as follows: Li, liver; Ep, epididymis; He, *NN*-dimethyl-4-aminoazobenzene-induced rat hepatoma. DNA size markers (bp) were from a mixed digest of pAT153 and processed in the same way as the RNA samples.

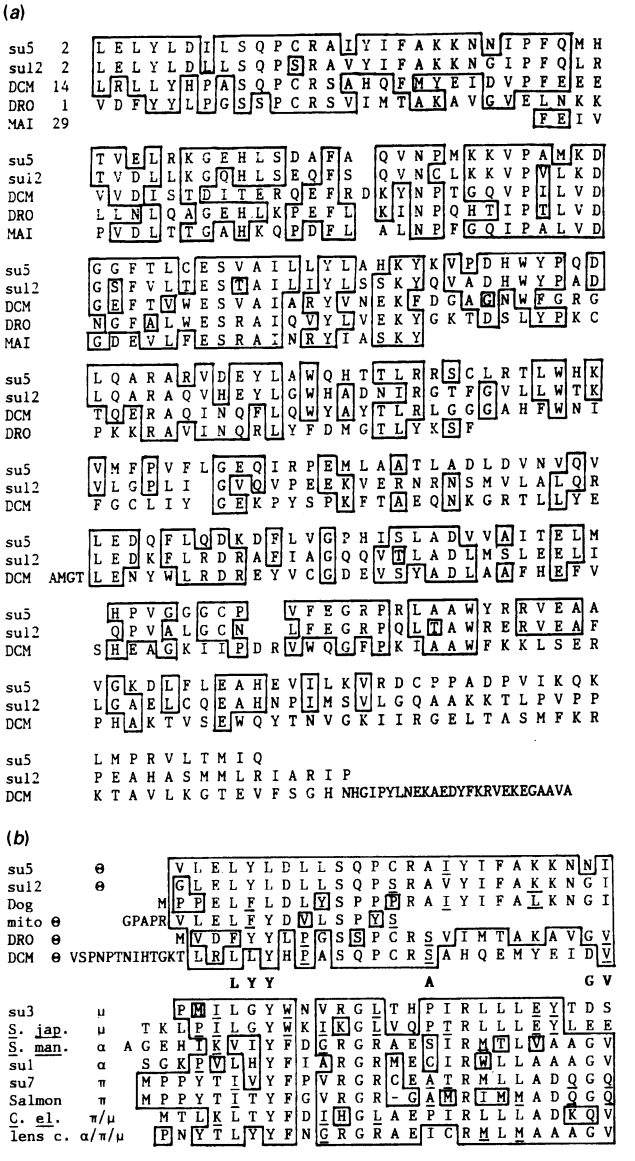

Fig. 3. Sequence comparison of GSTs

The identity comparison of full-length sequences for rat, fly, maize and *Methylobacterium* (*a*) and of *N*-terminal sequences (*b*) of some animal and bacterial GSTs. Su 5, 12, 3 and 1 are rat GST subunits (see the text); mito is the mitochondrial matrix GST; DCM is the DCM-utilizing enzyme of *Methylobacterium*; DRO is a GST of *Drosophila*; *S. jap.* and *S. man.* are from *Schistosoma japonicum* and *S. mansoni*; *C. el.* is from *Caenorhabditis elegans*; lens c. is squid lens crystallin; MAI is from maize. For convenience the class of a GST subunit is given as a greek letter (i.e. μ = class Mu etc., but note that a capital theta is used to avoid confusion with 'GST θ' in the text). In (*b*) note that a tyrosine residue near to the *N*-terminal is a feature of all GSTs, but other residues are only favoured, not conserved (in **bold**) between Theta and Alpha/Mu/Pi. The mitochondrial *N*-terminal extension is compatible with a signal sequence [14]. In (*a*) residue positions are given for the first amino acid shown (e.g. DCM:14, MAI:29) and so note that relative positions are well maintained, since few 'breaks' are inserted (a feature of GSTs [32]).

conserved sequence centred on amino acid residue 70 and seen in all GSTs, whereas two additional highly conserved regions are common to classes Alpha, Mu and Pi [16], of which the *N*-terminal region is shown compared with class Theta (Fig. 3*b*). These observations suggest that classes Alpha, Mu and Pi arose from a common progenitor, namely a duplication of a class-Theta gene (Fig. 4). This line of descent does not apply to a yeast regulatory protein, URE2 [17], which causes transcriptional down-regulation of enzymes of the glutamine-biosynthetic pathway in glutamine-supplemented media. This protein has two regions of high identity with the class-theta GSTs of maize and *Drosophila* [17] and of rat (results not shown), and not with classes Alpha, Mu and Pi. The largest conserved region, centred on residue 70 and with its core in common with all GSTs, is coincident with protein domains forming the binding site of the γ-glutamyl moiety of GSH in a porcine class-Pi GST [16,18], thus the conservation may be due to a requirement to bind a γ-glutamyl derivative, in this case glutamine. The URE2 protein therefore may have arisen either from a second duplication of a class-Theta gene or it may itself be the precursor of the GSH-binding site of GSTs (Fig. 4). This latter possibility would require the URE2 gene to be present in bacterial species which do not possess GSTs, which is unknown at present.

Sequence comparisons also suggest the parentage of proteins arising from the class-Alpha/Mu/Pi precursor. Computer-database searches did not detect any long-range similarity between the coding sequences of class-Theta genes and those of other GST classes; however, they do detect a similarity of sequence between the 3′ non-coding sequences of the subunit-5 cDNA and those of three human class-Mu cDNAs ([19]; Fig. 1). We note

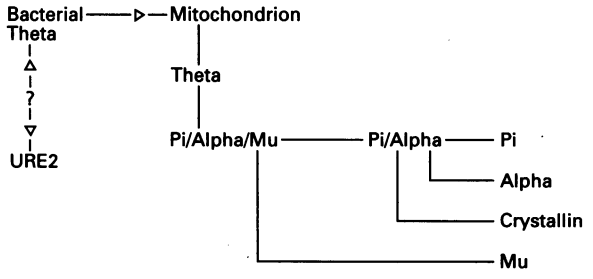

Fig. 4. Evolutionary relationships of GST classes

Animal class Alpha/Mu/Pi GSTs, together with squid lens crystallin are similar (Fig. 3*b*, lower block) and are unlike class Theta (Fig. 3*b*, upper block). Since class Theta is well conserved and appears in mitochondria, the Alpha/Mu/Pi precursor probably arose from a Theta-gene duplication. A class-Mu precursor diverged then from an Alpha/Pi/lens-crystallin precursor, and the lens crystallin subsequently diverged from the Alpha/Pi precursor (for a full-length comparison; see [22]). Part of the URE2 gene of *Saccharomyces cerevisiae* also has high identity with all GSTs (see the text), but its relationship to Theta is undetermined.

that this similarity of non-coding sequences is greater even than that between several regions of rat subunits 5 and 12 (Fig. 1). These non-coding sequences of the human cDNAs are remarkable for their 99% identity, reported to arise from gene conversion [19], and this coincidence of sequence with a class-Theta cDNA suggests that these non-coding sequences have a functional role. It also suggests that class Mu diverged from the common Alpha/Mu/Pi precursor prior to a class-Alpha/Pi divergence (Fig. 4), and further lines of evidence support this interpretation. Firstly, classes Alpha, Pi and Mu are highly similar in the incidence of their exon/intron boundaries [20], a conservation which is exemplified within the Pi class even to the single exon/intron boundary in *Caenorhabditis elegans* GST having a coincident splice site in rat/human class-Pi genes [21]. Secondly, rat class-Pi subunit 7 has best identity with rat class-Alpha subunits ([1] and references cited therein). Thirdly, squid lens crystallins are thought to have evolved from a GST subunit [22,23] and, since they have greatest similarity to mammalian classes Alpha and Pi [see 21] and also have optimal *N*-terminal identity with a squid liver class-Alpha subunit [24], then they probably derive from a progenitor of classes Alpha and Pi.

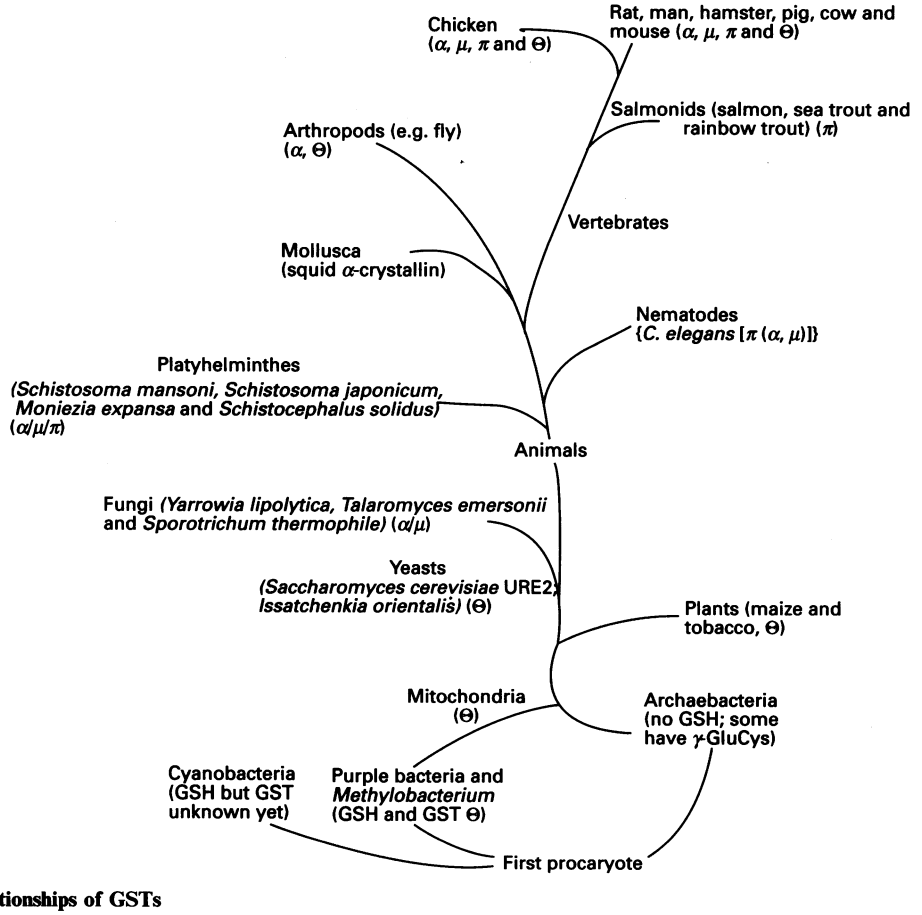

Fig. 5. Evolutionary relationships of GSTs

For convenience, greek letters indicate class of GST in the species (e.g. α = class Alpha, but note that capital theta is used to avoid confusion with 'GST θ' in the text). Source references are given in the text, in a review [5] or as follows for the latest publication with suitable references contained within: mouse [36], hamster [37], man [5], pig/cow [18], rat [5], chicken [38], salmonids [39], fly [8], squid [22,24], *C. elegans* [21], platyhelminthes (*Schistosoma mansoni*, *Schistosoma japonicum*, *Moniezia expansa*, *Schistocephalus solidus*) [30,32,33], fungi (*Yarrowia lipolytica*, *Talaromyces emersonii*, *Sporotrichum thermophile*) [26], yeast (*Saccharomyces cerevisiae*, *Issatchenkia orientalis*) [17,40], plants [10,13,41], mitochondrion [14], methylobacterium [12]. GSH and associated enzymes in cyanobacteria and purple bacteria [15]. Not included, but noteworthy, are a report of a Pi-class enzyme in freshwater shrimp (*Gammarus italicus*, crustacean, arthropod) [42], which also contains a discussion showing that class-Alpha/Mu/Pi antibodies do not cross-react with bacterial enzymes (see also [43]) and a report that GST activity was identified in 71 animals from nine Phyla [44]; however, no antibody or sequence data were included in that study.

Evolutionary analysis together with properties of a protein also permits the deduction of selection pressures which resulted in a novel gene family. For example the squid lens crystallins are thought to have been selected in response to the criteria of solubility, thermodynamic stability and optical transparency rather than GST activity [23]. However, to evolve new detoxication pathways such as those involving GSTs, a duplicate gene must not simply express a novel enzyme but also that enzyme must not be capable of yielding a product which is toxic and cannot be utilized or excreted. A simple example of this principle is the adaptive induction of the *Methylobacterium* GST, which metabolizes DCM, a man-made compound not occurring naturally [12,25]. The ultimate product of GST action is formaldehyde, which is then utilized as the sole source of carbon and energy. With this in mind, and using immunocross-reactivity data [26], in addition to amino acid sequence comparison, the incidence of GST subunits can be superimposed upon an evolutionary tree (Fig. 5) which shows that classes Alpha, Mu and Pi are restricted to animals and fungi. This suggests that the selection of the progenitor Alpha/Mu/Pi GST after gene duplication may be associated with the protective mechanisms developed as plants competed with fungi and animals for survival; plants protected themselves by biosynthesizing defensive toxins and fungi and animals countered by developing detoxication systems [27]. This temporal pattern is mimicked by the burst of cytochrome *P*-450 multigene families [27], which require pre-existing downstream conjugating and export systems to neutralize those products which are electrophilic. Since Alpha/Mu/Pi GSTs are broad-spectrum conjugating enzymes capable of sequestering lipophiles in general and, even more so, lipophile–GSH conjugates and other lipophilic anions [3,5,28–30], they offer, then, a unique potential to have contributed to the evolutionary selection of co-ordinated xenobiotic-metabolizing systems. Firstly, in order to ensure maximal binding of numerous compounds with variable affinities, binding proteins are required in high concentrations [29]. Secondly, the high solubility of the GSTs allows them to remain fully active even when induced to 10% or more of the total protein, as in the *Methylobacterium* enzyme [12,25] or tumours [31]. Thirdly, class Alpha/Mu/Pi GSTs in both higher [29] and lower [30] animals are inhibited by glutathione conjugates; that is, they bind them [29,30], and they also appear in excretions, implying crude dumping when no other export system is available; in parasites such as schistosomes, several cytoplasmic proteins, such as Alpha/Mu GSTs [32,33] and paramyosin [34], are accessible to the host immune system, and this is thought to reflect the import of potentially toxic haem (originating from their diet of host blood), and a similar phenomenon is still present in human liver, since all three binding classes of GSTs are present in the bile [35]. This non-specific dumping mechanism circumvents the need for pre-existing downstream processing enzymes for which no selective pressure had yet evolved, yet coincidentally it would exert that pressure via the necessity to maximize energy efficiency by reducing loss of protein.

In summary, GSH and GSTs are believed to have evolved in prokaryotes as part of the oxygen toxicity protection mechanism [15], and the high conservation of class Theta suggests that this may still be a direct role of that class. In fungi and animals the incidence and properties of class Alpha/Mu/Pi GSTs suggest an associated role, namely providing a sink allowing the assembly of complex waste-disposal reaction pathways such as those involving dioxygen and the mixed-function oxidases.

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