Differential tissue expression of the glutathione transferase multigene family

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The content of GSH transferase mRNAs in poly(A)-containing RNA isolated from eight rat tissues was examined by immunoprecipitation of cell-free translation products and by Northern blotting. Considerable tissue-specific distribution and heterogeneity of immunoprecipitable GSH transferase subunits 1 and 2 synthesized *in vitro* was observed. These results were confirmed by Northern blotting using a ³²P-labelled subunit 1 cDNA probe. The same probe, used in a Southern blot analysis of genomic DNA, provided confirmation that GSH transferase subunits 1 and 2 comprise a multigenic family in the rat. The results show that the selection of cDNA clones coding for chosen subunits can be made easier by making use of qualitative and quantitative tissue differences in GSH transferase mRNAs.

INTRODUCTION

Soluble glutathione (GSH) transferases are a family of dimeric enzymes with a diversity of properties including the catalysis of the conjugation of GSH with electrophiles, the reduction of free fatty hydroperoxides and the isomerization of certain double bonds. In addition they have affinity for many lipophilic non-substrate ligands. Not surprisingly they have a number of roles in physiology and toxicology including leukotriene C biosynthesis, the detoxication of metabolites of drugs and carcinogens, the prevention of the damaging effects of lipid peroxidation and the hepatic uptake of bilirubin. The regulation of the expression of these enzymes is of interest since each tissue has a characteristic distribution of isoenzymes which may change during development and be modulated by endogenous compounds such as hormones and interferon, or exogenous compounds including carcinogens and inducers of drug-metabolizing enzymes (Ketterer *et al.*, 1986; Mannervik, 1985; Wolf *et al.*, 1986).

The rat GSH transferases have been studied most. They have a new nomenclature which is based on the subunit composition, each subunit having been given a number in the chronological order of its characterization (Jakoby *et al.*, 1984). So far seven subunits have been named, but a number of others are known to exist. Of the seven well-characterized subunits, five fall into two families. These are the subunit 1/2 family and the subunit 3/4/6 family, within which the proteins share homology. In addition there are several reports of heterogeneity within particular subunits (Beale *et al.*, 1982, 1983; Hayes, 1983; Tu & Reddy, 1985) and this has been substantiated for subunit 1 (formerly called Ya) by nucleotide sequence analysis of cDNA recombinants derived from mRNA isolated from rat liver (Lai *et al.*, 1984; Pickett *et al.*, 1984). Here we demonstrate the occurrence of a number of mRNA species which encode GSH transferase subunits and study their distribution in

various tissues. We also show by Southern blot analysis of genomic DNA that there are likely to be a number of genes complementary to subunit 1 and 2 mRNA. Taken *in toto* the evidence suggests that the GSH transferases are encoded by an unexpectedly complex family of genes whose expression is regulated in a tissue-specific manner.

MATERIALS AND METHODS

Materials

Guanidinium chloride was obtained from Sigma Chemical Co. Oligo(dT)-cellulose was obtained from Bethesda Research Laboratories through Gibco Life Technologies, Paisley, Scotland, U.K. All other materials, enzymes, chemicals and solvents were obtained from sources described previously (see Allison *et al.*, 1981; Craig *et al.*, 1981; Taylor *et al.*, 1984; Edbrooke *et al.*, 1985).

Animals

All rat tissues were obtained from male Wistar rats weighing 180–200 g inbred at the Courtauld Institute of Biochemistry.

Buffers and medium

Buffers used during the isolation of total RNA from rat tissues were essentially as described by Deeley *et al.* (1977) and were as follows: solution A comprised 100 mM-sodium acetate, pH 5.0, containing 6.65 M-guanidinium chloride and 1 mM-dithiothreitol; solution B comprised 100 mM-sodium acetate, pH 7.0, containing 6.65 M-guanidinium chloride and 25 mM-EDTA; and solution C comprised 10 mM-sodium acetate, pH 7.5, containing 100 mM-NaCl, 1 mM-EDTA and 0.1% SDS.

Other buffers and media were as described by Taylor *et al.* (1984) and references therein.

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Antibodies

A polyclonal antibody to the basic GSH transferase subunits 1, 2 and 3, and its immunoreactivity towards cell-free translation products has been previously described in Taylor *et al.* (1984).

Isolation of poly(A)-containing RNA from rat tissues

Total RNA was prepared by a modification of the guanidinium chloride method of Deeley *et al.* (1977). Fresh tissues, frozen by immediate immersion in liquid N₂, were ground to a powder in a liquid N₂-cooled mortar and pestle, and then added to 20 vol. of ice-cold solution A and homogenized for 90 s with a Polytron. The resulting homogenate was centrifuged at 11 000 *g*_{av.} at -10 °C for 10 min in a Beckman JA-21 rotor. The supernatant was filtered through sterile muslin to remove aggregated lipid and then 0.5 vol. of pre-chilled (-20 °C) absolute ethanol was added and a precipitate was allowed to form at -20 °C for 16 h. The precipitate containing RNA was removed by centrifugation at 11 000 *g*_{av.} at -10 °C for 10 min in the Beckman rotor and resuspended in 0.1 vol. (of the original extract) of solution B. RNA was reprecipitated by the addition of 0.5 vol. of absolute ethanol at -20 °C for 4 h. This reprecipitation step was repeated a further two times in order to remove contaminating DNA. The resulting RNA pellet was dissolved in solution C and further purified by extraction with an equal volume of phenol/chloroform (1:1, v/v). After re-extraction of the organic layer with 0.5 vol. of solution C, the aqueous phases were pooled, the sodium salt concentration adjusted to 0.2 M and RNA precipitated by the addition of 2.3 vol. of absolute ethanol. Separation of poly(A)-containing RNA was then performed as previously described (Craig *et al.*, 1976).

Preparation of polyribosomes and polyribosomal poly(A)-containing RNA was as previously described (Taylor *et al.*, 1984).

Cell-free protein synthesis and immunoprecipitation

Cell-free protein synthesis was carried out in a nuclease-treated rabbit reticulocyte lysate as described by Pelham & Jackson (1976) and SDS/polyacrylamide-gel electrophoretic analysis of [³⁵S]methionine-labelled polypeptides was performed using slab gels as previously described (Craig *et al.*, 1979). Fluorography followed the procedure of Bonner & Laskey (1974) except that dimethyl sulphoxide was replaced by glacial acetic acid throughout (Burckhardt *et al.*, 1979). Immunoprecipitation procedures using antibodies raised against GSH transferase subunits 1, 2 and 3 (Fig. 1b) were as previously described (Craig *et al.*, 1976).

Preparation of high-M_r DNA from rat liver nuclei

Nuclei prepared from 5 g of rat liver by the method of Blobel & Potter (1966) were lysed at 20 °C in 10 ml of 10 mM-Tris/HCl, pH 7.5, containing 100 mM-NaCl, 10 mM-EDTA, 1% (w/v) SDS and 200 µg of proteinase K/ml. This lysate was incubated first at 45 °C for 30 min, then at 37 °C for 2 h. The resulting digest was extracted gently with 2 vol. of phenol/chloroform (1:1, v/v) and then with an equal volume of chloroform. RNA was removed from the aqueous phase by incubation with 10 µg of both DNase-free RNAase A and RNAase T₁ at 37 °C for 60 min. The solution was then made 1%

(w/v) with respect to SDS and 100 µg/ml with respect to proteinase K and incubated at 37 °C for 30 min. The solution was then extracted once with 2 vol. of phenol/chloroform (1:1, v/v), once with an equal volume of chloroform, and three times with an equal volume of water-saturated diethyl ether. Ether was removed from the solution by incubation at 37 °C for 10 min, and the DNA was recovered by ethanol precipitation, taken up in 1 mM-EDTA, pH 8, and stored at 4 °C.

Northern blotting

Poly(A)-containing RNA was analysed by agarose gel electrophoresis under denaturing conditions, then blotted onto Biotransfer membrane and probed as previously described (Taylor *et al.*, 1984).

Southern blotting

Rat liver DNA was analysed by agarose gel electrophoresis and blotted onto a GeneScreenPlus transfer membrane as previously described (Edbrooke *et al.*, 1985).

RESULTS AND DISCUSSION

Tissue-specific distribution and heterogeneity of immunoprecipitable GSH transferase subunits synthesized *in vitro*

Poly(A)-containing RNA isolated from eight rat tissues was used to direct [³⁵S]methionine-labelled protein synthesis in a rabbit reticulocyte lysate system and the products were analysed by SDS/polyacrylamide-gel electrophoresis and fluorography (Fig. 1a). Each of these mRNA preparations directed the synthesis of a wide spectrum of proteins which displayed tissue-specific differences. Immunoprecipitates from these cell-free translation products, by using an antibody raised against GSH transferase subunits 1, 2 and 3 from rat liver, are shown in Fig. 1(b). The tissues show characteristic differences in the distribution of the antigens precipitated by this antibody. mRNA preparations from kidney and liver encode abundant amounts of a protein band of M_r 25000 previously shown to correspond to subunit 1 synthesized *in vitro* (Fig. 1b, lanes 3, 7 and 10, and see Taylor *et al.*, 1984) whilst mRNA from the remaining six tissues encode little or no detectable protein of this mobility. This is consistent with the distribution *in vivo* of subunit 1 which has been examined in several tissues and observed to occur in quantity only in liver and kidney (Ketterer *et al.*, 1985). Likewise, in qualitative terms the tissue distribution of subunit 2 synthesized *in vitro* (M_r 28000) is similar to that of subunit 2 *in vivo*, since it is encoded by mRNA from all the tissues under investigation with the exception of the heart (lane 9). In quantitative terms it is noteworthy that this polypeptide is the predominant product immunoprecipitated *in vitro* encoded by mRNA from the adrenal gland which reflects GSH transferase activity *in vivo* in this tissue. Such quantitative relationships between levels *in vitro* and *in vivo* are not observed in all tissues and for all subunits. For example, mRNA from testis encodes abundant cell-free translation products (see Fig. 1a, lane 4) from which the yield of immunoprecipitated proteins is unexpectedly low (see also Lai *et al.*, 1984) in view of previous reports that, *in vivo*, the testis is rich in GSH transferases (Guthenberg *et al.*, 1983; Meyer *et al.*, 1983;

Fig. 1. Autoradiographs of [^{35}S]methionine-labelled cell-free translation products of poly(A)-containing RNA from rat tissues

(a) Poly(A)-containing RNA preparations from eight rat tissues were added to a nuclease-treated rabbit reticulocyte lysate, and [^{35}S]methionine-labelled cell-free translation products were separated by SDS/polyacrylamide-gel electrophoresis and visualized by fluorography. Lane 1, reticulocyte lysate background with no added mRNA. Lanes 2–10, [^{35}S]methionine-labelled protein products directed by poly(A)-containing RNA isolated from: lane 2, adrenal gland; lane 3, kidney; lane 4, testis; lane 5, lung; lane 6, epididymis; lane 7, liver; lane 8, spleen; lane 9, heart; lane 10, free polyribosomes of liver. Arrows denote the relative mobilities of the following [^{14}C]methylated standard marker proteins of known M_r obtained from Amersham International; lysozyme (M_r 14300), carbonic anhydrase (M_r 30000), ovalbumin (M_r 46000), bovine serum albumin (M_r 69000), phosphorylase b (M_r 92500) and myosin (M_r 200000). (b) Immunoprecipitates of the cell-free translation products visualized in (a) using a polyclonal antibody recognizing subunits 1, 2 and 3 of rat liver. Each lane corresponds to tissue of origin as in (a). Arrows denote subunits 1, 2, and 3 and polypeptides of M_r 25800, M_r 24500 and M_r 23700 synthesized *in vitro*.

Ketterer *et al.*, 1985). Moreover, the reverse is true of the epididymis which has one-fifth of the levels *in vivo* of GSH transferase activities of the testis (Meyer *et al.*, 1983; D. Meyer & B. Ketterer, unpublished work) yet shows the greatest quantitative and also qualitative

tissue-specific differences in the translation products precipitated *in vitro* by this antibody (see Fig. 1b, lane 6). In addition to bands which can be related to subunits 1 and 2, protein of the mobility of subunit 3 synthesized *in vitro* (M_r 26500) was clearly present in the translation

products *in vitro* of mRNA isolated from epididymis, testis and liver (Fig. 1*b*, lanes 6, 4, 7, and see Taylor *et al.*, 1984).

Other than those immunoprecipitated proteins described above which have been related to proteins *in vivo*, three protein bands of estimated M_r 25 800, 24 500 and 23 700 are clearly visible which also show quantitative tissue-specific differences. One of these protein bands (M_r 23 700) occurs in the cell-free translation products encoded by mRNA isolated from the heart and liver (Fig. 1*b*, lanes 9 and 7; see also lane 10). This polypeptide may correspond to the fast running $Y\alpha$ polypeptide reported by Tu *et al.* (1983) (and see Lai *et al.*, 1984) to be present in liver. A polypeptide of similar size synthesized *in vitro* is also translated from mRNA selected by hybridization to a full-length subunit 2 cDNA clone which also hybridized to mRNAs encoding subunits 1 and 2 (Telakowski-Hopkins *et al.*, 1985). These mRNAs selected by hybridization to a single cDNA sequence could be expected to encode proteins which have antigenic sites in common recognized by a polyclonal antibody.

The protein bands of M_r 25 800 and 24 500 are both present as abundant translation products of mRNA from the epididymis (Fig. 1*b*, lane 6). A protein of M_r 25 800 is seen in the cell-free translation products of mRNA preparations from the adrenal gland, lung and spleen (lanes 2, 5 and 8) in the last of which (spleen, lane 8) it appears to be the most abundant immunoprecipitated polypeptide. Although this protein band is of the mobility of subunit 6 (Ketterer *et al.*, 1985) it is not necessarily subunit 6 synthesized *in vitro* since another protein band, also of M_r 25 800, which is a product of mRNAs from spleen and epididymis but not adrenal

gland or lung, can be precipitated with an antibody specific for subunits 3, 4 and 6 (S. Pemble & J. Taylor, unpublished work) and may be related to the subunit 3/4/6 family. The protein band of M_r 24 500 is encoded by mRNA isolated from the adrenal gland and heart (Fig. 1*a*, lanes 2 and 9). These polypeptides were not observed in cell-free translation products isolated by *S*-hexyl-GSH-linked Sepharose affinity chromatography (Lai *et al.*, 1984) either because this affinity matrix does not bind all GSH transferases (see Meyer *et al.*, 1984) or because endogenous GSH present in the rabbit reticulocyte lysate competes with the affinity ligand for binding to GSH transferases.

Tissue distribution of GSH transferase mRNA sequences showing homology with liver subunit 1 cDNA

The clone pGSTr155 has been shown to contain a cDNA insert with an open reading frame corresponding to the first 129 amino acids of the *N*-terminus of a subunit 1 polypeptide and an additional 63 nucleotides upstream of the initiating methionine codon (Taylor *et al.*, 1984). This clone was used to prepare a ^{32}P -labelled probe for a Northern blot analysis of poly(A)-containing RNA isolated from total cellular RNA from eight rat tissues. Within poly(A)-containing RNA from liver this probe hybridizes strongly to a heterogeneous mRNA population (980 ± 75 nucleotides) believed to be subunit 1 mRNA species and less strongly to another heterogeneous mRNA species (1150 ± 100 nucleotides) which we believe to be subunit 2 mRNA (see Taylor *et al.*, 1984 and Fig. 2, lane 5). The kidney mRNA shows a pattern of hybridization similar to that of the liver, indicating the presence of a large amount of heterogeneous subunit 1 and 2 mRNAs. Subunit 1 mRNAs were also detected in

Fig. 2. Tissue distribution of mRNA species hybridizing to cDNA encoding subunit 1

Poly(A)-containing RNA isolated from eight rat tissues was analysed under denaturing conditions in 1.1% (w/v) agarose gels. The RNA was transferred to 'Biodyne A' transfer membrane, hybridized with ^{32}P -labelled pGSTr155 and autoradiographed. Sample load in each lane [μg of poly(A)-containing RNA] and tissue of origin were as follows: lane 1, 10 μg from adrenal gland; lane 2, 3 μg from kidney; lane 3, 10 μg from testis; lane 4, 3 μg from epididymis; lane 5, 3 μg from liver; lane 6, 10 μg from spleen; lane 7, 10 μg from heart; lane 8, 10 μg from lung. DNA fragments of known size were from the mixed products of a *Pst*I/*Bam*HI double digest of pAT153 (1129 base pairs) and a *Hinf*I digest of pAT153 (1641, 517, 396 and 298 base pairs), treated in the same way as the RNA (see the Materials and methods section).

relatively small amounts in the testis. Subunit 2 mRNAs occur in large quantities in the epididymis and at much lower levels in all other tissues except the heart, in which it was not detected (note the varying amounts of poly(A)-containing RNA loaded in each lane). Overall, patterns of distribution of subunit 1 and 2 mRNAs parallel the immunoprecipitation data, but in quantitative terms anomalies are apparent. For instance the kidney and liver contain similar amounts of subunit 2 mRNAs when judged by RNA blotting (Fig. 2, lanes 2 and 5), but considerably less subunit 2 synthesized *in vitro* is immunoprecipitated after cell-free translation of the kidney mRNA compared with the liver mRNA (Fig. 1b, lanes 3 and 7). If this anomaly arises from the level of incorporated [³⁵S]methionine then it is suggestive of heterogeneity within subunit 2 (cf. subunit 1). It is noteworthy also that a low level of hybridization to mRNA from the testis is seen. This result is consistent with the low level of immunoprecipitated cell-free translation products of this mRNA (and see Lai *et al.*, 1984) but again is unexpected given the high levels of GSH transferase isoenzymes seen in soluble supernatant from homogenates of the testis (Guthenberg *et al.*, 1983; Meyer *et al.*, 1983; Ketterer *et al.*, 1985).

Southern blot analysis of genomic DNA sequences complementary to subunit 1 and subunit 2 mRNA

A nick-translated *Pst*I restriction fragment of pGSTR155, 300 base pairs in length and which hybridizes to both subunit 1 and subunit 2 mRNA (see Taylor *et al.*, 1984; Telakowski-Hopkins *et al.*, 1985) was used as a probe in a Southern blot analysis of restriction enzyme digests of rat genomic DNA (Fig. 3). At least two hybridizing fragments would be likely to result from a single gene following digestion with either *Bgl*II or *Hind*III since these enzymes have recognition sites occurring within the probe. In all lanes, however, a multiplicity of hybridizing DNA fragments of varying intensities is visible irrespective of the presence or absence, within the cDNA probe, of a cleavage site for the chosen restriction enzyme. Although it is possible to generate multiple hybridizing restriction fragments from a single genomic sequence due to intron sequences containing several recognition sites for the enzyme, such an interpretation is most unlikely in this case. Since the probe is fairly small, there is little likelihood of multiple restriction fragments arising from a single complementary genomic sequence because the intron sequences would need to contain several of each of the low frequency cleavage sites recognized by the five enzymes. The range of intensities of the bands may reflect either varying homology to the probe, for example subunit 2 and other subunit 1 genomic sequences, or non-equimolar ratios of equal length restriction fragments due to varying multiplication of highly homologous genes. The number of hybridizing restriction fragments and their varying intensities is suggestive of a number of genes (or pseudogenes) encoding subunit 1- or 2-like polypeptides. This interpretation is supported by three full-length cDNA sequences corresponding to subunit 2 and two variants of subunit 1 (Pickett *et al.*, 1984; Lai *et al.*, 1984; Telakowski-Hopkins *et al.*, 1985). Nucleotide variations between the two full length cDNA clones corresponding to subunit 1 are scattered along the coding sequences, suggesting that there are at least two genes encoding

Fig. 3. Southern blot analysis of genomic DNA sequences complementary to cDNA encoding GSH transferase subunit 1

Genomic DNA (10 μ g) isolated from the liver of a single rat was digested to completion with a restriction nuclease as indicated below. The resulting DNA fragments were separated by electrophoresis on a 0.55% agarose gel and transferred to GeneScreenPlus blot transfer membrane. The blot was probed with a ³²P-labelled *Pst*I restriction fragment of pGSTR155 which was 300 base pairs in length and which corresponds to amino acid residues 26–129 of subunit 1. This probe contains restriction sites for *Bgl*II and *Hind*III but not *Pst*I, *Eco*RI or *Pvu*II (Taylor *et al.*, 1984). Hybridizing fragments were visualised by autoradiography. Lanes correspond to genomic DNA digested with: lane 1, *Pst*I; lane 2, *Eco*RI; Lane 3, *Pvu*II; lane 4, *Bgl*II; lane 5, *Hind*III. DNA fragments of known size were from the mixed products of a *Hind*III digest of bacteriophage λ [23.7, 9.5, 6.7 and 4.25 ($\times 10^3$) base pairs], a *Pst*I/*Bam*HI double digest of pAT153 [2.53 and 1.13 ($\times 10^3$) base pairs] and a *Hinf*I digest of pAT153 (1.63 $\times 10^3$ base pairs).

subunit 1 polypeptides rather than alternative RNA splicing regimens of a single primary transcript. Likewise substantial differences occurring in the 3' untranslated region of the two clones is characteristic of multiple genes (cf. α -actins). Of particular relevance to the multigenic nature of the subunit 1/2 family has been the selection of clones of mouse genomic DNA which hybridize to a radiolabelled probe of subunit 1 cDNA (Czosnek *et al.*, 1984). These subunit 1/2 like genes were shown to be numerous, to occur in tandem and therefore to constitute a multigene family.

Summary

In conclusion, we have shown immunological evidence that GSH transferase subunits synthesized *in vitro* exhibit unexpected complexity and also considerable tissue-specific variation. These conclusions have been supported by Northern blot analysis of polyadenylated RNA isolated from a number of tissues. In addition, Southern blot analysis of restriction enzyme digests of genomic DNA demonstrates that there are a number of loci which are complementary to a subunit 1 cDNA clone, a result in keeping with the existence of a multigenic GSH transferase family. The identification of tissues which express high levels of mRNAs corresponding to novel subunits will assist the characterization of specific probes in order to study the regulation and number of genes encoding this complex family of proteins.

This work was supported by the Cancer Research Campaign of which B.K. is a Life Fellow. During part of the work, S.P. was in receipt of the Countess of Lisburne scholarship from the Middlesex Hospital. We would also like to thank Dr. David Meyer and Ms. Lucia Christodoulides for providing the antibodies.

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Received 29 January 1986; accepted 1 May 1986