Cloning and sequence analysis of a cDNA for a rat liver glutathione S-transferase Y_b subunit

Hui-Chen J.Lai⁺, Gregory Grove and Chen-Pei D.Tu⁺⁺

Department of Molecular and Cell Biology, Pennsylvania State University, University Park,
PA 16802, USA

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ABSTRACT

We have isolated a Y_b-subunit cDNA clone from a GSH S-transferase (GST) cDNA library made from rat liver polysomal poly(A) RNAs. Sequence analysis of one of these cDNA, pGTR200, revealed an open reading frame of 218 amino acids of M_r=25,915. The deduced sequence is in agreement with the 19 NH₂-terminal residues for GST-A. The sequence of pGTR200 differs from another Y_b cDNA, pGTA/C44 by four nucleotides and two amino acids in the coding region, thus revealing sequence microheterogeneity. The cDNA insert in pGTR200 also contains 36 nucleotides in the 5' noncoding region and a complete 3' noncoding region. The Y_b subunit cDNA shares very limited homology with those of the Y_a or Y_c cDNAs, but has relatively higher sequence homology to the placental subunit Y_p clone pGP5. The mRNA of pGTR200 is not expressed abundantly in rat hearts and seminal vesicles. Therefore, the GST subunit sequence of pGTR200 probably represents a basic Y_b subunit. Genomic DNA hybridization patterns showed a complexity consistent with having a multigene family for Y_b subunits. Comparison of the amino acid sequences of the Y_a, Y_b, Y_c, and Y_p subunits revealed significant conservation of amino acids (~ 29%) throughout the coding sequences. These results indicate that the rat GSTs are products of at least four different genes that may constitute a supergene family.

INTRODUCTION

The glutathione S-transferases (GST, EC 2.5.1.18) are a family of multifunctional, dimeric proteins involved in drug biotransformation and xenobiotics metabolism (see Ref. 1 for a review). Several GST isozymes are induced to a higher level by many xenobiotics including phenobarbital, 3-methylcholanthrene, and trans-stilbene oxide (2-5). The expression of rat GSTs is tissue-specific (6-11,19). Historically, rat hepatic GSTs, which are abundant cytosolic proteins, have been the most extensively studied. The rat liver GST subunits can be resolved into five major mobility groups by NaDodSO₄-polyacrylamide gel electrophoresis: Y_c (M_r=28,000), Y_b (M_r=27,000), Y_n (M_r=26,300), Y_a (25,600), and Y_α (M_r=24,400) (10-15). The Y_a, Y_b, and Y_c groups consist of multiple species as determined by analysis of both the purified GST isozymes and GST cDNA sequences (10,11,15-17, H.-C. J. Lai and C.-P. D. Tu, unpublished results). All of the cDNAs for the rat liver GST

subunits have been isolated from phenobarbital-treated rats (10,11,17,18). In this communication, we report the nucleotide sequence of a Y_b subunit cDNA synthesized from normal rat liver poly(A) RNAs. Comparison of the nucleotide and amino acid sequences among Y_c , Y_b , Y_a , and a placental GST cDNA pGP5 (19) revealed significant conservation of amino acid residues throughout the entire polypeptide chains despite very limited homology in the nucleotide sequences.

MATERIALS AND METHODS

Antibodies

Antisera against homogeneous rat hepatic GSTs (affinity chromatography fraction) were raised as previously described (20). The IgG fraction was purified from a protein A-Sepharose (Pharmacia) column and concentrated by ultrafiltration (Minicon-B125 membrane, Amicon Corporation, Danvers, MA) as described by Kraus and Rosenberg (21). Antisera were stored in 50% glycerol and 0.2 mg/ml heparin at -18°C .

Isolation of polysomes

Livers (~ 26 g) from two male Sprague-Dawley rats (body weight ~ 300 g) were homogenized with a Potter-Elvehjem homogenizer in ~ 150 ml (final) of 50 mM Tris-HCl, pH 7.5/25 mM NaCl/5 mM MgCl_2 /0.25 M sucrose, containing bentonite (1 mg/ml), heparin (0.2 mg/ml), and cycloheximide (1 $\mu\text{g/ml}$) in several aliquots to give a 15% (wt/vol) homogenate. Polysomes were isolated exactly according to published procedures (21). The yield was 1389 A_{260} units before dialysis, 1208 A_{260} units after dialysis.

Immobilization of polysome-antibody complexes and elution of specific mRNA

We recovered 1130 A_{260} units of polysomes for immunoadsorption with anti-GST IgG (7.1 mg). Protein A-Sepharose affinity chromatography and elution of bound RNAs were carried out as described by Kraus and Rosenberg (21). The eluted RNAs were immediately adjusted to 0.5 M NaCl and 0.5% NaDodSO_4 and purified further by oligo(dT)-cellulose column (22,23). The purified poly(A) RNAs were assayed by in vitro translation and immunoprecipitation (20,24) before cDNA synthesis. The immunoprecipitated materials were separated on NaDodSO_4 -polyacrylamide gel and visualized after fluorography (25,26).

Isolation of GST cDNA clones

cDNA synthesis was performed according to the method of Okayama and Berg (27) as modified by Gubler and Hoffman (28) with some further modifications. Approximately 100-500 ng of poly(A)⁺ RNA from immunoprecipitated polysomes were used for cDNA synthesis. Reverse transcription of the mRNA into cDNA was

carried out in 40 μ l containing 50 mM Tris-HCl (pH 8.3), 100 mM NaCl, 10 mM $MgCl_2$, 10 mM DTT, 4 mM sodium pyrophosphate, 1.25 mM of the four dNTPs, 1800 U/ml RNAsin (Promega Biotec), 100 μ g/ml oligo(dT)₁₂₋₁₈ (Pharmacia/PL), and 3000 U/ml AMV Reverse Transcriptase (Molecular Genetic Resources). The reaction was incubated 25 min at 43°C, then terminated by adding 2 μ l of 0.5 M EDTA. The reaction was extracted with 1 volume of phenol:chloroform, and the organic phase back-extracted with one-half volume of TE buffer (pH 7.9). The combined aqueous phases were extracted with one volume of chloroform and again the organic phase back-extracted with TE buffer. To the combined aqueous phases, 1 volume of 4M Ammonium acetate was added and the nucleic acid was precipitated by adding 2 volumes of ethanol and chilling on dry ice powder for 20-30 min. The solution was then warmed to room temperature for 5 min and centrifuged for 15 min in an Eppendorf microfuge at 4°C. The resulting pellet was dissolved in 25 μ l of TE buffer. Ammonium acetate (25 μ l of 4M) and 100 μ l of ethanol were added and the nucleic acid precipitated and recovered as before. The pellet was washed with 70% ethanol, then dried and dissolved in 20 μ l of H₂O.

Replacement of the mRNA strand of the mRNA-cDNA hybrid was carried out in 50 μ l containing 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 100 mM KCl, 0.15 mM β -NAD, 0.04 mM of the four dNTPs, 20 μ l of the first strand product, 10-20 μ Ci ³²P-dATP, 10 U/ml E. coli DNA ligase (New England BioLabs), 230 U/ml DNA polymerase (Boehringer Mannheim), and 8.5 U/ml E. coli RNase H (Pharmacia/PL). The reaction was incubated 90 min at 12-14°C, then 1 hour at room temperature. The double-stranded cDNA was purified by phenol:chloroform extraction and recovered by ethanol precipitation exactly as described for the first strand product. The dried pellet was dissolved in a final volume of 20 μ l H₂O.

Ten μ l of the double-stranded cDNA were tailed with dCTP in a total volume of 20 μ l containing 100 mM potassium cacodylate (pH 7.0), 1 mM $CoCl_2$, 0.2 mM DTT, 0.1 mM dCTP, and 500 U/ml terminal deoxynucleotidyl transferase (Pharmacia/PL). The reaction was incubated 1-2 min at 37°C after which 20 μ l of 4 mM EDTA were added and the enzyme heat inactivated by incubation at 65°C for 10 min.

Pst I-digested, dG-tailed pBR322 (BRL, Inc.) was added in approximately a 1:1 molar ratio to the dC-tailed, double-stranded cDNA (i.e., about 5-10 fold excess by molecular weight of vector over the estimated amount of cDNA). The DNA solution (cDNA + vector) was diluted to give a final total DNA concentration of 0.5-2.0 ng/ μ l in the presence of 10 mM Tris-HCl (pH 7.5), 1

mM EDTA, and 150 mM NaCl. The mixture was incubated for 5 min at 65°C, then the DNA was annealed at 55-58°C for 90 min.

The annealed cDNA:vector was transformed into *E. coli* MM294 using 5 µl of DNA per 200 µl aliquot of competent cells (29). The control transformation frequency was $1-2 \times 10^8$ transformants per µg supercoiled pBR322. The transformed cells were plated on LM plates (without Mg⁺⁺) containing 17 µg/ml tetracycline (29). The resulting colonies were tested for ampicillin sensitivity. Those tetracycline-resistant colonies that were sensitive to ampicillin (~ 50%) were picked for further analysis.

RESULTS AND DISCUSSION

Identification of a Y_b GST cDNA clone, pGTR200

Plasmid DNAs were purified from 354 ampicillin-sensitive transformants (30) and digested with *Pst*I to determine cDNA insert sizes. Among them, 134 contained visible cDNA inserts by agarose gel electrophoresis. Those with inserts greater than 800 nucleotides (24 clones) were analyzed further by Southern blot hybridization with Y_a (pGTR261) and Y_c (pGTR262) probes (10,11,31). Twelve clones that did not hybridize to these probes were then characterized further by hybrid-selected in vitro translation (32). One of the negative clones was designated pGTR200. Rat liver poly(A) RNAs selected by pGTR200 DNA immobilized on activated aminophenylthioether cellulose (APT-paper) were eluted at 65°, 70°, 75°, 80°, 85°, 90°, and 100° and used to program in vitro translation in the rabbit reticulocyte lysate system. The in vitro translation products were divided into two halves and immunoprecipitated separately by antisera against either the anionic GSTs (peak I, ref. 16) or the total rat liver GSTs and then analyzed by NaDodSO₄-polyacrylamide gel electrophoresis. The fluorogram shown in Figure 1 indicates that the immunoprecipitated product(s) was indeed of Y_b mobility (M_r=27,000) (10,12,15) and that no mRNA of other classes of subunits was selected by pGTR200. This result is consistent with the selection scheme described above, since earlier hybrid-selected in vitro translation experiments with Y_a and Y_c clones did not reveal any Y_b subunit products (10,11).

Five distinct Y_b-containing isozymes are expressed in rat livers. Antisera preparations against these five different isozymes demonstrated different efficiency and specificity in immunoprecipitations of the human poly(A) RNA in vitro translation products (33). Therefore, these five Y_b subunits may be products of at least five separate but related genes. A bimodal distribution of the immunoprecipitation products (Y_b subunits) was

Figure 1. In vitro translation of pGTR200 DNA-selected rat liver poly(A) RNAs. The in vitro translation products were divided into two halves and immunoprecipitated by antiserum against anionic Y_b (peak I) GST (3) of rat liver (Panel A) or by antiserum against mixtures of rat liver GSTs (Panel B), fractionated by NaDodSO₄-polyacrylamide gel electrophoresis, and processed for fluorography. The rat liver RNA samples used in each lane in Panels A and B are as follows. Lane 1, total poly(A) RNAs from phenobarbital-treated rat liver. Lane 2, Y_b subunit marker. Lanes 3 to 9, poly(A) RNAs selected by immobilized pGTR200 DNA and eluted at 65° (lane 3), 70° (lane 4), 75° (lane 5), 80° (lane 6), 85° (lane 7), 90° (lane 8), or 95° to 100° (lane 9).

observed with each panel in Figure 1. A species of Y_b mRNA was eluted from pGTR200 DNA maximally at 70°, whose in vitro translation product(s) can be precipitated by the antiserum against anionic GSTs (panel A) with similar efficiency to the antiserum against total rat liver GSTs (panel B). More interestingly, the mRNA(s) for anionic GSTs were eluted from immobilized pGTR200 DNA maximally at 85° (panel A), whereas the majority of the Y_b mRNAs were eluted from the pGTR200 DNA at 90° (panel B). In summary, pGTR200 DNA may contain a basic Y_b subunit sequence that is not expressed abundantly in rat livers relative to the anionic Y_b subunits (16). Compared with the protein

Figure 2. Sequencing strategy for the pGTR200 cDNA insert. The cDNA fragments (rectangle) generated by the various restriction endonuclease cleavages were labeled at the 3' ends(*). Arrows indicate extents of DNA sequence information for each experiment. Sequence determination was repeated at least once.

purification results (16), it is likely that pGTR200 may correspond to the Y_b subunit of peak VIII isozyme, which may be the GST-A of Jakoby *et al.* (2).

Nucleotide sequence of the pGTR200 cDNA insert

The DNA sequence of pGTR200 was determined according to the strategy in Figure 2 by the chemical method of Maxam and Gilbert (35). The sequence shown in Figure 3 has an open reading frame (nucleotides #37 to 690) of 218 amino acids with the NH_2 -terminal 19 residues identical to those determined for GST-A by Frey *et al.* (36). The Y_b coding sequence of pGTR200 differs from that of another Y_b cDNA clone, pGTA/C44 (18), in four nucleotides: G→A at position 327; T→C at position 600; T→G at position 633; and T→A at position 634 of pGTR200. The latter two substitutions result in changes of two amino acids: Asn→Lys and Cys→Ser in pGTR200. Other sequences of these two clones, including the 5' and 3' noncoding sequences, are identical except that pGTR200 contains the complete 3' noncoding sequence of the mRNA and is longer than pGTA/C44 by 18 nucleotides before the poly(A) residues.

The Y_b subunits are encoded by a multigene family. Genomic blotting patterns showed multiple hybridization signals with the complete cDNA probe. The 3' noncoding sequence probe has revealed a major band and one or two other weaker bands (Figure 4), again strongly supporting the notion of a gene family.

Expression of pGTR200 sequences in various rat tissues

Rat GST expression has been shown to be tissue-specific (6). Among the rat tissues examined (Figure 5), only the kidney lacks Y_b subunit expression as determined by protein purification and *in vitro* translation (6,9). Using

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      10      20      30      40      50      60
CTGAAGCCAAATTGAGAAGCCACAGCGCCAGAACCATGCCTATGATCTGGGATACTGG
      70      80      90     100     110     120
AACGTCGCGGGCTGACACACCCGATCGCCTGCTCCTGGAATACACAGACTCAAGCTAT
N V R G L T H P I R L L L E Y T D S S Y
      130     140     150     160     170     180
GAGGAGAAGAGATACGCCATGGGCGACGCTCCGACTATGACAGAAGCCAGTGGCTGAAT
E E K R Y A M G D A P D Y D R S Q W L N
      190     200     210     220     230     240
GAGAAGTTCAAAGTGGGCTGGACTTCCCAATCTGCCCTACTTAATTGATGGATCGGCG
E K F K L G L D F P N L P Y L I D G S R
      250     260     270     280     290     300
AAGATTACCCAGAGCAATGCCATAATGCGCTACCTGGCCGCAAGCACCACCTGTGTGGA
K I T Q S N A I M R Y L A R K H H L C G
      310     320     330     340     350     360
GAGACAGAGGAGGAGCGGATTGCTGCAGACATTGTGGAAACAGGTCATGGACAACCGC
E T E E R I R A D I V E N Q V M D N R
      370     380     390     400     410     420
ATGCAGCTCATCATGCTTTGTTACAACCCGACTTTGAGAAGCAGAAGCCAGAGTCTCTG
M Q L I M L C Y N P D F E K Q K P E F L
      430     440     450     460     470     480
AAGACCATCCCTGAGAAGATGAAGCTCTACTCTGAGTTCTGGGCAAGCGACCATGCTTT
K T I P E K M K L Y S E F L G K R P W F
      490     500     510     520     530     540
GCAGGGGACAAGGTACCTATGTGGATTTCCTTGCTTATGACATTCTTGACCAGTACCA
A G D K V T Y V D F L A Y D I L D Q Y H
      550     560     570     580     590     600
ATTTTGAAGCCCAAGTGCCTGGACGCTTCCCAAACCTGAAGGACTTCTGCGCCGCTTC
I F E P K C L D A F P N L K D F L A R F
      610     620     630     640     650     660
GAGGGCTGAAGAAGATCTCTGCCTACATGAAGAGCAGCGCTACCTCTCAACACCTATA
E G L K K I S A Y M K S S R Y L S T P I
      670     680     690     700     710     720
TTTTGCAAGTTGGCCCAATGGAGTAACAAGTAGGCCCTTGCTACACTGGCACTCACAGAG
F S K L A Q W S N K *
      730     740     750     760     770     780
AGGACCTGTCCACATTGGATCCTGCAGGCACCCTGGCCTTCTGCACTGTGGTTCTCTCTC
      790     800     810     820     830     840
CTTCTGCTCCCTTCTCCAGCTTTGTGCAGCCCATCTCCTCAACCTCACCCAGTCATGC
      850     860     870     880     890     900
CCACATAGTCTTCATTCTCCCACTTTCTTTTCATAGTGGTCCCCTTCTTTATTGACACT
      910     920     930     940     950     960
TAACACAACCTCACAGTCTTTTCTGTGATTTGAGGTCTGCCCTGAAGTCAAGTCTCCCTA
      970     980     990     1000    1010    1020
GACTTACCCCAAAATGTAACACTGTCTCAGTGCCAGCCTGTTCTGCTGGGGAGCTGCC
      1030    1040    1050    1060    1070
CAGGCTGTCTCATCTTTAATAAGCCTGAACACAAAAAAAAAAAAAAAAAAAA

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Figure 3. Nucleotide sequence of pGTR200 cDNA insert. The single letter code of amino acids is used for the 218 residue open reading frame. The poly(A) addition signal AATAAA is underlined.

the 3' noncoding PstI fragment of pGTR200 as the probe in RNA blot hybridization, we found that the pGTR200 or related mRNA sequences is expressed in the various rat tissues with the following relative intensity: 12.4 (brain):1.0 (heart):13.2 (kidney):155 (liver):15.3 (lung):1.0 (spleen): 1.3 (seminal vesicles):20.3 (testis). Rat tissues that do not express the cationic GSTs well, such as the heart (7) or seminal vesicles, did not have strong hybridization signals to the pGTR200 sequence probe. To explain the signal in rat kidney poly(A) RNAs, we propose that some of the Y_a mobility

Figure 4. The rat genomic DNA blot hybridization pattern with the 32 P-cDNA probe of pGTR200 (Y_b).

The rat liver genomic DNA (15 μ g each), isolated according to published procedures (44), was digested with BamH-1 (lane 1), EcoRI (lane 2), HindIII (lane 3), PstI (lane 4), and PvuII (lane 5); separated by electrophoresis on an 0.8% agarose gel, transferred to a Pall BiodyneTM membrane, and hybridized with nick-translated 32 P-cDNA fragment of pGTR200 in the presence of 50% formamide and 10% dextran sulfate at 42° for 16 hours. The membrane was washed extensively in 2X SSC, 0.1% SDS and 0.1X SSC, 0.1% SDS before exposure at -70° with intensifying screen (Lightning Plus, DuPont) for a week. Panel A, DNA restriction patterns stained with EtBr; panel B, autoradiogram revealed by the 32 P-cDNA probe; panel C, autoradiogram revealed by the 3' noncoding (small PstI fragment) sequence probe of pGTR200. Numbers on the side of the panels are size markers in Kb units.

(and/or Y_c mobility) subunits of rat kidneys may contain Y_b -related sequences, similar in composition to the H_a subunits of human liver GSTs (33), which responded well to Y_b -subunit antisera in the immunoprecipitation experiments (33).

Rat GSTs are encoded by a supergene family

We compared nucleotide sequences between pairs of the various clones of rat GSTs using Zweig's Dot Matrix program (34). The results in Figure 6 show

Figure 5. Rat tissue RNA blot hybridization with ^{32}P -labelled 3' noncoding sequence of pGTR200 cDNA insert.

Rat tissue poly(A) RNAs (10 μg each) were fractionated by electrophoresis in 1.5% agarose gel in the presence of formaldehyde according to published procedures (42). The RNAs were transferred to Pall BiodyneTM membrane, hybridized with ^{32}P -labelled pGTR200 3' noncoding region in the presence of 50% formamide at 42° according to distributor's procedure. The membrane was thoroughly washed after hybridization before autoradiography. The RNAs in each lane were isolated from control rat tissues and purified through an oligo(dT)-cellulose column twice: lane 1, brain; lane 2, heart; lane 3, kidney; lane 4, liver; lane 5, lung; lane 6, spleen; lane 7, seminal vesicles; lane 8, testis. Each poly(A) RNA preparation has been tested by the *in vitro* translation assay. The 28S and 18S rRNA markers were from the rabbit embryo.

three regions of partial sequence homology between the Y_b and Y_a (10) or the Y_b and Y_c (17) coding sequences, and four regions of sequence homology between the Y_b subunit and the placental subunit Y_p (pGP5) (19). The presence of these conserved regions among the four classes of GST subunits (i.e. Y_a , Y_b , Y_c , and Y_p) suggests that these four subunits probably evolved from a common ancestral GST gene through gene duplication and subsequent divergence. The limited extent of sequence homology, however, indicates that they may have diverged very early in their evolution.

The amino acid sequences of the four polypeptides are significantly more conserved than are the DNA sequences. Using the conserved DNA sequences as references for alignments, we found 32 identical amino acids scattered throughout the four different classes of GST subunits (Figure 7). If we consider the amino acids in five groups of side chains as proposed by Swenson

Figure 6. Dot Matrix analyses of pGTR200 (Y_b) sequence against pGTR261 (Y_a) (10), pGTB42 (Y_c) (17), and pGP5 (Y_p) (19) sequences. Numbers indicate nucleotide positions of published sequences. The condition for the homology search was 20 nucleotide matching in 25 nucleotides.

(37) and Doolittle (38)—small nonpolar (T,A,P,C), large nonpolar (V,I,L,M,F), small polar (S,G,D,N), large polar (E,Q,K,R) and intermediately polar (Y,H,W)—the extent of sequence conservation among these four GST subunits is increased to 29% (63 amino acids out of an average of 217 residues in the GST coding sequences). The overall amino acid conservation can be as high as 60% if we consider matching of amino acid groupings in three of the four different classes of rat GST subunits. Furthermore, the hydropathicity plot (Figure 8) of the Y_b (pGTR200), Y_c (pGTB42), and Y_a (pGTR261) showed more similarity than the primary amino acid sequences (39).

The cDNA clone pGTR200 contains a coding sequence for one of the Y_b subunits. Its NH_2 -terminal sequence is identical to that determined for GST-A by Frey *et al.* (35). Because of the existence of multiple Y_b isozymes, identity of N-terminal sequences is not sufficient to assign cDNA sequence to

Figure 7. Comparison of amino acids among the four classes of rat GST subunits, Y_c (pGTB42), Y_a (pGTR261), Y_p (pGP5), and Y_b (pGTR200). Identical amino acids are in boxes. Amino acids of the same polarity group among these four subunits are indicated by "*." The dagger (†) marks positions where amino acid conservation occurs in at least three different classes of GST subunits. Neighboring amino acids of different groups are counted as the same group (e.g., serine and threonine).

any Y_b proteins; in particular, the complete sequence of pGTR200 differs from that deduced for pGTA/C44, Y_{b1} (18) by only two amino acids. It is not clear whether the sequence of pGTR200 or that of pGTA/C44 represents the Y_{b1} (1) subunit(s) purified from rat livers. More likely, the "Y_{b1}" subunit of GST isozyme(s) may be a mixture of microheterogeneous sequences represented by the coding sequences in pGTR200 and pGTA/C44. Similar sequence microheterogeneity in GST isozymes has been proposed for the Y_a subunit-containing isozymes of rat livers (10,39) and the Y_b-containing isozymes of rat hearts (7). This sequence microheterogeneity may be due to allelic polymorphism or to multiple genes. In any event, it is important to determine whether the two

Figure 8. Hydropathicity plots for the rat liver GST Y_a (10), Y_b , and Y_c (17) subunits. The plots were generated by the Hydropathicity Program of Bionet™ (43) using the values of Kyte and Doolittle (39). All other parameters were set to their default values. Positive values indicate hydrophobic regions, which are often located on the interior of proteins. Arrows point to the translation termination codon of the coding sequences.

Y_b subunit amino acid sequences represented by the pGTR200 and pGTA/C44 sequence have different catalytic specificity or efficiency. We are currently pursuing purification of Y_b subunits expressed from cDNA sequences in *E. coli*.

Published results from our laboratory and others (10,11,15,17-19) clearly indicate that rat GSTs are products of at least four different genes that may constitute a supergene family (40).

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+Intercollege Program of Genetics.

*To whom correspondence should be addressed

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