Cloning and sequence analysis of a cDNA plasmid for one of the rat liver glutathione S-transferase subunits

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ABSTRACT

We describe the construction and characterization of a cDNA plasmid for one of the rat liver glutathione S-transferase subunits. Poly(A)-RNA isolated from rat livers was enriched for glutathione S-transferase mRNA activity and used as templates to synthesize double stranded cDNA. The double stranded cDNAs were annealed to pBR322 through terminal deoxynucleotidyl transferase generated GC-tails followed by transformation into *E. coli*. Several candidate clones were selected by colony hybridization using polynucleotide kinase labeled liver and testis poly(A)-RNA probes. These candidate clones were further characterized by hybrid-selected translation of mRNA followed by immunoprecipitation and SDS gel electrophoresis. The positive clone, pGTR112 was mapped with restriction endonuclease analysis and sequenced by the chemical method of Maxam and Gilbert. The largest open reading frame contains 142 amino acids very rich in Arg and Lys residues. The C-terminal residue phenylalanine of this open reading frame is consistent with what was reported for one of the ligandin subunits by Bhargava *et al.*, (J. Biol. Chem. 253, 4116-4119, 1978). Among the 352 nucleotides covered by both pGTR112 and pGST94 described by Kalinyak and Taylor (J. Biol. Chem. 257, 523-530, 1982), there are only 9 nucleotide differences resulting in four changes of amino acid sequences.

INTRODUCTION

Glutathione S-transferases (GSH-Tr, EC 2.5.1.18) are a group of dimeric, multifunctional detoxification proteins¹ which are induced to a higher level by many xenobiotics including phenobarbital^{2,3}. One of the GSH-Trs is also known as ligandin^{4,5}. This isozyme family has the following activities: (1) catalysis of GSH conjugation to a variety of hydrophobic compounds with electrophilic centers^{6,7}; for example, 1-chloro-2,4-dinitrobenzene, benz(a)-pyrene-7,8-epoxide etc; (2) selenium independent GSH peroxidase (GSH-Px) activity⁸⁻¹⁰ toward organic hydroperoxides such as lipid and steroid hydroperoxides; (3) binding protein(s) for a variety of organic anions such as bilirubin⁴, azo dye carcinogens¹¹, steroid metabolites⁵.

Rat liver GSH-Trs have been reported to exist in multiple forms, however, there is uncertainty regarding the exact number of isozymes and their associated Se-independent GSH-Px activities as well as their functions in the

biosynthesis of leukotrienes, prostaglandins and thromboxanes. Moreover, little is known about the genome organization and control of gene expression for this family of physiologically important isozymes. In this report, we describe our results on the cDNA cloning and nucleotide sequences for the cDNA plasmid (pGTRL12) corresponding to one of the subunits for rat liver GSH-Trs.

MATERIALS AND METHODS

Materials

Chemicals: Sodium Pentobarbital was purchased from A. J. Buck and Son, (Cockeysville, MD.). Guanidinium thiocyanate was obtained from Tridom Chemicals, Inc. (Hauppauge, N.Y.). Freund's complete adjuvant and BCG (M. Tuberculosis Des.) were the products of Difco Laboratory (Detroit MI.). The oligo(dT)-cellulose was purchased from BRL, Inc. (Gaithersburg, MD.). The DPT (diazophenylthioether) paper was obtained from Schleicher and Schuell (Keene, NH). The EN³HANCE used in fluorography was the product of New England Nuclear (Boston, MA). The four dNTP's were purchased from PL Biochemicals. (Milwaukee, WI).

Radioactive Compounds: [γ ³²-P] ATP (specific activity 2000-3000 Ci/mmole) was obtained from ICN (Irvine, CA). The four [α ³²-P] dNTP's (specific activities 600 to 800 Ci/mmole) were purchased from the New England Nuclear. The [³H] dNTP's (specific activities 15 to 40 Ci/mmole) were the products of the Amersham Corporation (Chicago, IL). The ³⁵S-methionine (specific activity 800-1200 Ci/mmole) was purchased from either Amersham or New England Nuclear.

Enzymes: Restriction endonucleases were obtained from the following companies: BRL, Inc., Boehringer Mannheim (Indianapolis, IN); New England Biolabs (Beverly, MA). The AMV reverse transcriptase was obtained from J. Beard, Life Sciences, Inc., (St. Petersburg, FL). DNA polymerase I and the klenow fragment were purchased from Boehringer Mannheim. The terminal deoxynucleotidyl transferase was purchased from PL Biochemicals or Boehringer Mannheim. T4 polynucleotide kinase was obtained from Biogenics Research Corporation (Chardon, OH). Rabbit reticulocyte lysates were the product of BRL, Inc.

Experimental Animals: Male Sprague-Dawley rats and female New Zealand white rabbits (6 month old) were obtained from Hilltop labs (Scottsdale, PA) and Dutchland labs (Denver, PA) respectively.

METHODS

Isolation of Poly(A)-RNA from Rat Livers: Male rats weighing 250 to

300 g were provided with 1mg/ml pentobarbital in drinking water (freshly prepared each day) for 10 to 14 days¹². Livers and other organs were extricated and processed immediately. They were minced in guanidinium thiocyanate denaturing solution¹³ and homogenized with a motor driven Teflon pestle tissue grinder. The nucleic acids were fractionated by CsCl gradient centrifugation in 60Ti rotor at 140,000 x g for 20 hours¹⁴. The total RNA (lowest band of the gradient) was collected from the side of the cellulose nitrate centrifuge tube with a syringe and diluted four fold with diethylpyrocarbonate treated H₂O. The RNA was precipitated with ethanol and washed twice with 70% ethanol. The RNA pellets were dissolved in binding buffer and passed through oligo(dT)-cellulose column twice according to published procedures^{15,16}.

Sucrose Gradient Sedimentation to Enrich for GSH-Tr mRNAs: A linear sucrose gradient (5% to 20%) was used in a SW41 Ti rotor at 200,000 x g for 12 hours¹⁷. Fractions were collected from the bottom of the gradient and assayed for GSH-Tr mRNA activities by in vitro translation in the rabbit reticulocyte lysate system¹⁸ coupled with immunoprecipitation and SDS gel electrophoresis.

Purification of Rat Liver GSH-Trs: Livers were obtained from male Sprague-Dawley rats weighing 400-450 g and homogenized with four volumes of buffer A (25 mM Tris-HCl, pH 8.0, 0.25 M sucrose). Subcellular fractionation was carried out as previously described¹⁹. The 105,000 x g supernate was concentrated to a minimum volume (~15 ml) by ultrafiltration. The subsequent steps involved gel filtration on a Sephadex G150 column, affinity chromatography on a S-hexylglutathione linked Sepharose 6B column, and CM-cellulose column chromatography. Seven major activity peaks (assayed with 1-chloro-2, 4,-dinitrobenzene as substrate) can be resolved through the CM-cellulose column. The detailed purification procedure and characterization of each major activity peaks will be published elsewhere. Each GSH-Tr isozyme peak is at least 95% pure as judged by SDS gel electrophoresis analysis.

Preparation of Antiserum Against Mixtures of GSH-Tr Isozymes: Equal concentrations of each major isozyme activity peak from the CM-cellulose column fractions were mixed as the source of antigen at a final protein concentration of 0.41 mg per ml. This antigen preparation was mixed with an equal volume of Freund's complete adjuvant containing additional 10 mg/ml of BCG organisms²⁰. This antigen suspension was emulsified thoroughly before being injected subcutaneously into rabbits. Each rabbit received 2 ml of the emulsified mixture at 6 to 7 sites on the back (~0.1 ml per site) and the

four leg tendon region (0.1 ml each) as well as the neck region. Further immunization was carried out on days 7 and 14. One week after the last immunization, prepared sera from all the immunized rabbits were found to contain high titers of antibody against liver GSH-Trs as determined by diffusion²¹ analysis in agar, whereas control sera demonstrated no reactivity against GSH-Trs.

Plasmid DNA Purification: The rapid alkaline extraction procedure of Birnboim and Doly²² was used for quick analysis of cDNA clones. For large scale covalently closed circular DNA (cccDNA) preparation, the cleared-lysate, CsCl-ethidium bromide gradient method²³ was used as previously described²⁴.

Synthesis of Double Stranded cDNA (dscDNA): The first strand cDNA synthesis was carried out with AMV reverse transcriptase according to the procedure of Friedman and Rosbash²⁵. The second strand synthesis was carried out with DNA polymerase I according to Wickens *et al.*²⁶. Hairpin structure was removed by S1 nuclease treatment according to the procedure of Goodman and MacDonald¹⁴.

Homopolymer Tailing with Terminal Deoxynucleotidyl Transferase: The dscDNA was tailed with dCTP and *Pst*I linearized pBR322 was tailed with dGTP by terminal transferase. The conditions used were those described by Deng and Wu²⁷ for the addition of approximately 20 residues per 3'end.

Transformation into *E. coli*: The (dC)_n tailed dscDNA and the (dG)_n tailed pBR322 were annealed at a molar ratio of 1 : 1 overnight before transformation into *E. coli* strain MC294²⁸ as previously described²⁹.

End-Labeling of RNA and DNA Fragments: Poly(A)-RNA from liver or testis enriched for GSH-Tr mRNA activities was partially hydrolyzed with NaOH according to Schibler *et al.*³⁰ before labeling with T4 polynucleotide kinase and [γ -³²P]ATP. DNA fragments containing 5' protruding ends were end-labeled with an appropriate [α -³²P]dNTP and AMV reverse transcriptase as previously described²⁴.

DNA Sequence Analysis: The chemical method of Maxam and Gilbert³¹ was used throughout this work. The following reactions were performed on 3'end-labeled DNA fragments: G, A > C, C and C + T.

Hybrid-Selected Translation of mRNA: The cccDNA (e. g. pGTR112) was denatured and partially cleaved with 0.3 N NaOH at 100°C for 7 min. The solution was quickly chilled to 4° and neutralized by the addition of HCl and sodium phosphate (pH 6) at a final concentration of 0.3 N and 0.1 M respectively. The denatured DNA fragments were covalently linked to DPT-paper

(1 x 1 cm) as described by Alwine *et al.*³² and Goldberg *et al.*³³. The derivatized paper was mixed with 40 µg of total poly(A)-RNA isolated from pentobarbital treated rat liver at 37°. The samples were incubated for 12 to 16 hours in a final concentration of 50% formamide, 0.6 M NaCl, 75 mM sodium citrate, 100 µM sodium phosphate pH 7.0, 0.1% SDS, 200 µg/ml poly-(adenylate) and 200 µg/ml tRNA in 0.4 ml. The DNA-DPT papers were prehybridized for 1 hour at 37° before the addition of poly(A)-RNA. After hybridization, the papers were washed in 15 ml of 50% formamide, 0.2 M NaCl, 8 mM sodium citrate, 0.2% SDS four times at 37° at 10 minute intervals. The hybridized RNA was released by boiling in 0.5 ml of 10 mM EDTA, 0.1% SDS for 3 minutes followed by repeated ethanol precipitation with 10 µg tRNA carrier. The recovered RNA was used to program *in vitro* translation with the rabbit reticulocyte lysate system and ³⁵S-methionine. Translation products were analyzed by SDS-polyacrylamide gel electrophoresis³⁴ before and after immunoprecipitation. The SDS gels were dried with a LKB slab gel drier and processed for fluorography with EN³HANCE according to the manufacturer's procedure.

Immunoprecipitation: The procedure we used was a modification of the one described by Kessler³⁵ according to Gilman *et al.*³⁶.

The *in vitro* translation products (30 µl) were mixed with an equal volume of 2X RIPA buffer (1X RIPA buffer contains 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM MgCl₂, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 1% Aprotinin) and 6 to 12 µl of antiserum against total liver GSH-Trs. After overnight incubation at 4°C, 100 µl of a 10% suspension of formalin fixed, heat killed *S. aureus* (Cowan I) was added to the mixture. The *S. aureus* cells (containing protein A) were collected by centrifugation in a Microfuge B (Beckman) after 30 minutes at room temperature. The pellet was resuspended in 500 µl 1X RIPA buffer and sonicated for 5 minutes in an ultrasonic cleaner. After two more identical washes with RIPA buffer, the cells were similarly washed with 500 µl of 62.5 mM Tris-HCl pH 6.8 and concentrated by microfuge B. The pellet was resuspended in 50 µl 62.5 mM Tris-HCl pH 6.8, 2% SDS and 5% 2-mercaptoethanol. The immunoprecipitated polypeptides bound to the protein A containing cells were eluted from the cell by heating at 100° for 5 minutes. After centrifugation the supernate was collected and a small aliquot (~5 µl) was counted for ³⁵S radiation. The remaining solution was mixed with 8 µl 100% glycerol and 1 µl of 1% bromophenol blue, heated to 100° for 5 minutes and separated by SDS-polyacrylamide gel electrophoresis (11% gel) according to Laemmli³⁴.

RESULTS

Colony Hybridization of Tetracycline Resistant (Tc^R) and Ampicillin Sensitive (Ap^S) Transformants with Liver and Testis Poly(A)-RNA Enriched for GSH-Tr mRNAs.

Approximately 1500 $Tc^R Ap^S$ transformants were obtained from the cDNA cloning experiment. Duplicate nitrocellulose filters containing these colonies were prepared with chloramphenicol amplification³⁷. They were processed for hybridization according to the procedure of Grunstein and Hogness³⁸. Due to the abundance of tissue-specific mRNA in the poly(A)-RNA fraction enriched for GSH-Tr mRNAs, hybridization results using liver RNA probes alone did not lead to the easy identification of GSH-Tr cDNA clones. We therefore used both the enriched liver poly(A)-RNA probe and enriched testis poly(A)-RNA probe in the colony hybridization. Colonies which gave positive signals with both probes were candidates for further characterization by hybrid-selected translation. One such pair of colony hybridization results is shown in Figure 1.

Hybrid-Selected Translation: The cccDNA was purified from each of the candidate clones that were identified by colony hybridization. Approximately 40-50 μ g of each cccDNA was bound to DPT-paper according to published proce-

Figure 1 Colony hybridization of rat liver cDNA plasmids with RNA fractions enriched for GSH-Tr mRNA activities and labeled *in vitro* with ^{32}P . (a) liver RNA probe; (b) testis RNA probe. Colony located at the upper left corner is clone number 1. The rest of the colonies are numbered from left to right and top to bottom. Position 95 did not contain any colony. The strong signals in part (a) represented cDNA clones for abundant liver specific poly(A)-RNAs. As a result, colonies of strong hybridization signals with both RNA probes were selected for further analysis by hybrid-selected translation. The arrows indicate positions of our final clone pGTR112.

dures³². The selected poly(A)-RNAs were purified by repeated ethanol precipitation. They were used to program in vitro translation in the rabbit reticulocyte lysate system¹⁸. Total translation products were fractionated on SDS gels. From the fluorogram in Figure 2(a), clones 19, 27, 73, 101, 139, and possibly 132 did not select mRNA for any polypeptides in the molecular weight range of 24 to 27.5K (i.e., GSH-Tr subunits). The RNAs selected by these clones apparently directed the synthesis of ~16K polypeptide(s). However, clone 112 selected poly(A)-RNA which programmed the synthesis of polypeptides in the range of 24 to 27.5K. The fluorogram in Figure 2(b) shows that two major bands of 26K and 24K molecular weight were precipitated by GSH-Tr specific antiserum. These results clearly demonstrate that clone 112 (now designated as pGTR112) contains nucleotide sequences for one or both of these GSH-Tr subunits. The reason why pGTR112 selected poly(A)-RNAs for the synthesis of two polypeptides became clear after we obtained the DNA sequencing results.

Restriction Endonuclease Mapping and DNA Sequence Analysis of pGTR112:

The pGTR112 plasmid was mapped with various restriction endonucleases before sequence analysis. Figure 3 summarized a restriction map which was

Figure 2 In vitro translation of hybrid-selected poly(A)-RNAs with clones 19, 27, 73, 101, 112, 132 and 139. (a) Translation products before immunoprecipitation, (b) translation products after immunoprecipitation with GSH-Trs specific antiserum. C lanes represented control translation experiments. T lane represented total translation products of liver poly(A)-RNAs. M lane represented ¹⁴C-labeled molecular weight markers (Amersham Corp.). Position of 30K marker is marked by an arrow in panel (b). The upper band selected by clone 112 has a molecular weight of 26K. The band below it corresponds to a molecular weight of 24K.

Figure 3 Restriction map of pGTR112 cDNA insert. The open rectangular box (drawn to scale) represents the cDNA insert of GSH-Tr sequences (500 bp). Dashed lines cover the GC-homopolymeric regions. The solid lines represent pBR322 DNA sequences. A 1.05 kb DdeI fragment (see text) containing the insert and a 380 bp PstI fragment were purified as the starting material for restriction mapping. Positions of AvaI, BglII, HinfI, PstI, SmaI and TaqI were mapped by double digestion with end-labeled fragments. The rest of the restriction sites were mapped from DNA sequencing results. The lower portion of the figure describes our sequencing strategy. The *'s denote the sites used for 3'-end labeling. The associated arrows cover the length of nucleotide sequences obtained from each end-labeled fragment. The dotted portion represents nucleotide sequences missing from a particular sequencing experiment. (1) The PstI fragment was cleaved with Sau3A, labeled at the 3'ends and separated on 3.5% polyacrylamide gel before sequence analysis. (2) The DdeI fragment was cleaved with TaqI and labeled at the 3'ends before sequence analysis. (3) The DdeI fragment was cleaved with HinfI and labeled at the 3'ends. Only the shorter fragment was sequenced. (4) The pGTR112 DNA was linearized with BglII, labeled at the 3'ends and cleaved with PstI to obtain single-end labeled fragments for sequence analysis. (5) The PstI fragment was cleaved with AvaI and labeled at the 3'ends before sequence analysis.

useful in developing our DNA sequencing strategies. This clone has an insert of approximately 500 nucleotides. There is an internal PstI site as well as single cleavage sites for AluI, AvaI, AvaII, BglII, HinfI, SmaI and TaqI. For DNA sequence analysis, 3'-end-labeling was used in all the experiments. Since pBR322 did not contain any BglII site, sequence analysis around the BglII site was carried out using circular pGTR112 DNA. For sequencing from the other sites, a unique DdeI fragment (1.05kb) containing portions of pBR322 DNA was purified from pGTR112 DNA and used as the starting material. The chemical method of Maxam and Gilbert³¹ was used throughout this analysis. Most of the sequences were confirmed by sequencing one of the two complementary strands of the insert in pGTR112 more than once. The 493 nucleotides as determined is shown in Figure 4. The longest reading frame deduced from either strand is the one shown with 142 amino acid sequences in Figure 4. It has phenylalanine at the C-terminus and a lysine at the

				10					20					30					40					50					60
(G _n)C	CTC	TAT	GGG	AAG	GAC	ATG	AAG	GAG	AGA	GCC	CTG	ATT	GAC	ATG	TAT	TCA	GAG	GGT	ATT	TTA									
	<i>leu</i>	<i>tyr</i>	<i>gly</i>	<i>lys</i>	<i>asp</i>	<i>met</i>	<i>lys</i>	<i>glu</i>	<i>arg</i>	<i>ala</i>	<i>leu</i>	<i>ile</i>	<i>asp</i>	<i>met</i>	<i>tyr</i>	<i>ser</i>	<i>glu</i>	<i>gly</i>	<i>ile</i>	<i>leu</i>									
				70					80					90					100					110					120
				↓					↓					↓					↓				↓					↓	
	GAT	CTG	ACT	GAA	ATG	ATT	ATC	CAA	TTG	GTA	ATA	TGT	CCC	CCA	GAC	CAA	AGA	GAA	GCC	AAG									
	<i>asp</i>	<i>leu</i>	<i>thr</i>	<i>glu</i>	<i>met</i>	<i>ile</i>	<i>ile</i>	<i>gln</i>	<i>leu</i>	<i>val</i>	<i>ile</i>	<i>cys</i>	<i>pro</i>	<i>pro</i>	<i>asp</i>	<i>gln</i>	<i>arg</i>	<i>glu</i>	<i>ala</i>	<i>lys</i>									
				130					140					150					160					170					180
				↓					↓					↓					↓				↓					↓	
	ACC	GCC	TTG	GCA	AAA	GAC	AGG	ACC	AAA	AAC	CGG	TAC	TTG	CCT	GCC	TTT	GAA	AAG	GTG	TTG									
	<i>thr</i>	<i>ala</i>	<i>leu</i>	<i>ala</i>	<i>lys</i>	<i>asp</i>	<i>arg</i>	<i>thr</i>	<i>lys</i>	<i>asn</i>	<i>arg</i>	<i>tyr</i>	<i>leu</i>	<i>pro</i>	<i>ala</i>	<i>phe</i>	<i>glu</i>	<i>lys</i>	<i>val</i>	<i>leu</i>									
				190					200					210					220					230					240
				↓					↓					↓					↓				↓					↓	
	AAG	AGC	CAT	GGC	CAA	GAC	TAC	CTT	GTA	GGT	AAC	AGG	CTG	ACC	CGG	GTA	GAC	ATC	CAC	CTG									
	<i>lys</i>	<i>ser</i>	<i>his</i>	<i>gly</i>	<i>gln</i>	<i>asp</i>	<i>tyr</i>	<i>leu</i>	<i>val</i>	<i>gly</i>	<i>asn</i>	<i>arg</i>	<i>leu</i>	<i>thr</i>	<i>arg</i>	<i>val</i>	<i>asp</i>	<i>ile</i>	<i>his</i>	<i>leu</i>									
				250					260					270					280					290					300
				↓					↓					↓					↓				↓					↓	
	CTG	GAA	CTT	CTC	CTC	TAT	GTT	GAA	GAG	TTT	GAT	GCC	AGC	CTT	CTG	ACC	TCT	TTC	CCT	CTG									
	<i>leu</i>	<i>glu</i>	<i>leu</i>	<i>leu</i>	<i>leu</i>	<i>tyr</i>	<i>val</i>	<i>glu</i>	<i>glu</i>	<i>phe</i>	<i>asp</i>	<i>ala</i>	<i>ser</i>	<i>leu</i>	<i>leu</i>	<i>thr</i>	<i>ser</i>	<i>phe</i>	<i>pro</i>	<i>leu</i>									
				310					320					330					340					350					360
				↓					↓					↓					↓				↓					↓	
	CTG	AAG	GCC	TTC	AAG	AGC	AGA	ATC	AGC	AGC	CTC	CCC	AAT	GTG	AAG	AAG	TTC	CTG	CAG	CCT									
	<i>leu</i>	<i>lys</i>	<i>ala</i>	<i>phe</i>	<i>lys</i>	<i>ser</i>	<i>arg</i>	<i>ile</i>	<i>ser</i>	<i>ser</i>	<i>leu</i>	<i>pro</i>	<i>asn</i>	<i>val</i>	<i>lys</i>	<i>lys</i>	<i>phe</i>	<i>leu</i>	<i>gln</i>	<i>pro</i>									
				370					380					390					400					410				420	
				↓					↓					↓					↓				↓				↓		
	GGC	AGT	CAG	AGA	AAG	CCA	GCC	ATG	GAT	GCA	AAA	CAA	ATC	GAA	GAA	GCA	AGG	AAG	GTT	TTC									
	<i>gly</i>	<i>ser</i>	<i>gln</i>	<i>arg</i>	<i>lys</i>	<i>pro</i>	<i>ala</i>	<i>met</i>	<i>asp</i>	<i>ala</i>	<i>lys</i>	<i>gln</i>	<i>ile</i>	<i>glu</i>	<i>glu</i>	<i>ala</i>	<i>arg</i>	<i>lys</i>	<i>val</i>	<i>phe</i>									
				430					440					450					460					470				480	
				↓					↓					↓					↓				↓				↓		
	AAG	TTT	TAG	CGG	AGC	TGC	ACT	GTC	CAA	TTT	CTT	GTA	ATC	CAG	GCT	CTG	ATG	TTT	TGC	AAA									
	<i>lys</i>	<i>phe</i>																											
	AAA	TGA	GAA	GCA	(C _n)																								

Figure 4 Nucleotide sequences of pGTR112 cDNA insert and their corresponding amino acid sequences. The deduced amino acid sequence is the largest one among the six possible reading frames. Nucleotide 1 corresponds to the first C of the leu codon in the first amino acid deduced. This nucleotide numbering system is also used in Table 1.

penultimate position. In an earlier report, Bhargava *et al.*³⁹ determined the C-terminal amino acid sequences of ligandin subunits (one of the isozymes of GSH-Trs) and found one of the subunits has phenylalanine at the C-terminus followed by a basic amino acid. Their results are consistent with what we deduced from the DNA sequencing results. Our deduced amino acid sequences are very rich in basic amino acids lysine and arginine (total of 24). At least one of the possible CNBr peptides from this sequence (nucleotide #43 to 76) should be acidic.

DISCUSSION

We have constructed and characterized a cDNA plasmid (pGTR112) containing a partial sequence for one of the rat liver GSH-Tr subunits starting from poly(A)-RNA purified from pentobarbital treated male Sprague-Dawley rats. We identified the clone pGTR112 by the hybrid-selected translation procedure followed by immunoprecipitation using antiserum specific for the mixture of total rat liver GSH-Trs. From the DNA sequence of pGTR112, we have deduced

an open reading frame of 142 codons with codons for phenylalanine at the C-terminus and a basic amino acid (lysine) at the penultimate position. This sequence is highly rich in basic amino acids (Figure 4) and one of the possible CNBr fragment contains charged amino acid residues with acidic side chains only. These observations are consistent with the peptide mapping and terminal amino acid analysis of ligandin. This particular isozyme of GSH-Trs is known to be a heterodimer with blocked N-termini³⁹. Bhargava *et al.*³⁹ treated ligandin with carboxypeptidase A and found that only phenylalanine was released even after extended incubation. Since the other C-terminal residue was known to be arginine by carboxypeptidase B analysis, they suggested that the penultimate residue to phenylalanine should be a basic amino acid. They also reported that while most of the CNBr peptides have isoelectric points between pH 7 and 10, two of them were found in the acidic range (pH 3-5). The total number of tryptic peptides for ligandin was between 28 and 31 reflecting an abundance of lysine and arginine residues. Based on these related results, our cDNA plasmid pGTR112 probably contains partial coding sequences for one of the ligandin subunits.

In addition to selecting the poly(A)-RNA for the 26K subunit, the pGTR112 DNA also selected to a lesser extent a poly(A)-RNA species coding for the 24K subunit (Figure 2b). This suggests to us that extensive sequence homology may exist between these two poly(A)-RNA species. The supporting evidence came from the comparison of DNA sequences between pGTR112 and another GSH-Tr cDNA plasmid, pGST94, isolated by Kalinyak and Taylor⁴⁰. They reported the characterization of pGST94 as coding for part of a GSH-Tr subunit also on the basis of hybrid-selected translation and immunoprecipitation. The major poly(A)-RNA species selected by pGST94 DNA programmed the *in vitro* synthesis of the 24K polypeptide. However, pGST94 DNA also selected poly(A)-RNA coding for the 26K subunit to a lesser extent. As shown in Table 1, among the 352 nucleotides covered by both pGTR112 and pGST94, there were only 9 nucleotide differences resulting in only four changes of amino acid sequences. Moreover, most changes have retained the original polarity of amino acid residues (e.g. Ser¹⁶ vs Thr, Ile²⁷ vs Met, and Arg³⁷ vs Lys). These few changes in nucleotide sequences alone are not sufficient to explain the observed difference in relative intensity of the 24K and the 26K GSH-Tr subunit in the two independent hybrid-selected translation experiments from two different laboratories. The major diversity in nucleotide sequences may very well reside in the N-terminal portions or in the C-terminal portions of these two RNA species. However, the N-terminal portion was missing in both pGST94 and

Table 1. Comparison of DNA Sequences between pGTR112 and pGST94 with maximum overlap.

Position in pGTR112 (Figure 4)	Nucleotide Sequence		Amino Acid Sequence	
	pGTR112	pGST94	pGTR112	pGST94*
45	T	C	Tyr	Tyr
46	T	A	Ser	Thr
48	A	C		
51	G	A	Glu	Glu
54	T	C	Gly	Gly
81	C	G	Ile	Met
110	G	A	Arg	Lys
352	C	T	Leu	Ser
353	T	C		

* Nucleotide sequences of pGST94 were those reported by Kalinyak and Taylor⁴⁰.

pGTR112 and the C-terminal portion was available only for pGTR112, so the final answer will come when full length clones of both subunits become available.

All the previously reported in vitro translation experiments^{41,42} of GSH-Tr mRNAs from different laboratories resulted in a pair of polypeptide products of similar molecular weights of different intensity after immunoprecipitation with antisera against ligandin. Earlier explanations⁴¹ that proteolytic cleavage (post-translational modification) of the larger ligandin subunit at the C-terminus generated the smaller ligandin subunit now seem to be much less satisfactory on the basis of DNA sequence analysis of pGTR112 by us and pGST94 by Kalinyak and Taylor⁴⁰. This striking DNA sequence homology is therefore sufficient evidence that the two ligandin subunits are not identical but highly homologous to each other. From the hybrid-selected translation results with pGTR112 (this work) and pGST94⁴⁰, it seems obvious that pGTR112 contains partial coding sequence for the large subunit of ligandin (26K) whereas pGST94 contains partial coding sequence for the small subunit of ligandin (24K). Genetic variation therefore has significant contribution to the sequence diversity of GSH-Tr isozymes. Different GSH-Tr subunits are most likely coded for by different mRNA populations. Studies of the genomic structure and control of gene expression for this family of isozymes will be all the more interesting.

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