Rat Liver Glutathione S-Transferases

DNA SEQUENCE ANALYSIS OF A Yb₂ cDNA CLONE AND REGULATION OF THE Yb₁ AND Yb₂ mRNAs BY PHENOBARBITAL*

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We have constructed a cDNA clone, pGTA/C48, which is complementary to the rat liver glutathione S-transferase Yb₂ mRNA. Recombinant clone pGTA/C48 contains a cDNA insert of 845 base pairs which overlaps nucleotides 108–952 of the Yb₁ cDNA clone, pGTA/C44, described previously by our laboratory (Ding, J.-F., Lu, A. Y. H., and Pickett, C. B. (1985) J. Biol. Chem. 260, 13268–13271). Over the protein coding region of the Yb₁ and Yb₂ cDNA clones there is an 84% nucleotide sequence homology, whereas the 3′ untranslated regions are only 32% homologous. The complete amino acid sequence of the Yb₂ subunit has been determined from a combination of DNA sequence analysis of pGTA/C48 and conventional protein sequence analysis of the glutathione S-transferase Yb₁ Yb₂ heterodimer. The Yb₂ subunit is comprised of 218 amino acids with a molecular weight of 25,705 and has an amino acid sequence which is 79% homologous to the sequence of the Yb₁ subunit. We have utilized the divergent 3′ untranslated regions of three rat liver glutathione S-transferase cDNA clones as specific probes to determine the effect of phenobarbital on the level of Yb₁, Yb₂, and Yc mRNAs. Our results clearly show that the Yb₁ and Yb₂ mRNAs are elevated 5–6-fold by phenobarbital administration; whereas the Yc mRNA is only modestly elevated by this xenobiotic. Finally, our data suggest that the Yb₂ subunit is encoded by a gene(s) which is distinct from the Yb₁ gene(s) and provides direct evidence for the existence of multiple glutathione S-transferase Yb genes in the rat.

The rat liver glutathione S-transferases are a family of enzymes which catalyze the conjugation of the reduced sulfhydryl group of glutathione with electrophiles. In addition the transferases bind with high affinity various exogenous hydrophobic compounds as well as potentially toxic compounds such as bilirubin and heme (1–3). The enzymes are comprised of binary combinations of at least six major subunits, Yα, Yα, Yb₁, Yb₂, Yc, and Yn which can be separated by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (4–6).

Our laboratory has reported recently the construction and characterization of Ya, Yc, and Yb₁ cDNA clones (7–9). We have used these clones in RNA blot hybridization and nuclear run-off assays to demonstrate that the rat liver glutathione S-transferase genes are transcriptionally activated by phenobarbital and 3-methylcholanthrene (7, 10). DNA sequence analysis has indicated that the Ya and Yc mRNAs have a 75% sequence homology in the protein coding region; however, the 5′ and 3′ untranslated regions are divergent (8). Based on the extent of sequence homology between the Ya and Yc mRNAs, we have concluded that the Ya and Yc genes are members of the same glutathione S-transferase gene family.

In contrast, however, we have found that the nucleotide sequence of the Yb₁ cDNA clone, pGTA/C44, is not homologous to the nucleotide sequence of the Ya or Yc cDNA clones (9). These data indicate that the Yb₂ subunit is encoded by a glutathione S-transferase gene family which is distinct from the Ya-Yc gene family. In the present study, we have extended our work on the Yb gene family and have isolated and characterized a new Yb cDNA clone, pGTA/C48, which is complementary to the glutathione S-transferase Yb₃ mRNA. Conventional protein sequence analysis of glutathione S-transferase C, a Yb₁ Yb₂ heterodimer, has confirmed the identity of pGTA/C48 as a Yb₂ cDNA clone. Since nucleotide sequence analysis has allowed us to ascertain regions between the glutathione S-transferase cDNA clones which lack sequence homology, we have used the divergent regions to examine the degree of induction of specific glutathione S-transferase mRNAs by phenobarbital. Both the Yb₁ and Yb₂ mRNAs are elevated in response to xenobiotic administration; whereas the Yc mRNA is only modestly elevated by this xenobiotic.

MATERIALS AND METHODS

Preparation of cDNA and Construction of Recombinant Plasmids—In order to prepare double-stranded cDNA, the glutathione S-transferase Yb mRNAs were purified by polysomal immunabsorption techniques as described previously (8). Both the first and second strands of the cDNA were synthesized as described by Gubler and Hoffman (11) and tailed with dCTP using terminal deoxynucleotidyltransferase as described previously (8).

Hybrid-select Translation—Hybrid-select translations were carried out according to the procedure described by Cleveland et al. (12) as outlined in a previous publication from our laboratory (7).

In Vitro Labeling of cDNAs—cDNAs were labeled in vitro either with [³²P]dCTP by nick translation, at the 5′ end with [³²P]ATP by T4 polynucleotide kinase, or at the 3′ end with [³²P]dideoxy-ATP by terminal deoxynucleotidyltransferase (13).

Restriction Endonuclease Mapping of pGTA/C48—A restriction map of the cDNA insert in pGTA/C48 was constructed by the method of Smith and Birnstiel (14) using 5′ end-labeled fragments. The sizes of the end-labeled fragments generated by the partial restriction endonuclease digestion were determined on 5% polyacrylamide gels.

Nucleotide Sequence Analysis—The chemical method of Maxam and Gilbert was used for DNA sequence analysis (15). Appropriate

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restriction fragments were 5' and 3' end-labeled and subjected to DNA sequence analysis.

RNA Slot Blot Analysis—Total rat liver RNA was isolated from the livers of 3–4 rats (male Sprague-Dawley) by the procedure of Chirgwin et al. (16). Poly(A)* RNA was isolated from total RNA as described previously (17). Various concentrations of poly(A)* RNA were spotted onto nitrocellulose sheets using a minifold II-slot blot apparatus (Schleicher & Schuell). Prehybridization and hybridization of the radiolabeled cDNA probes were done as described by Thomas (18). Autoradiographs of the x-ray films were scanned with a densitometer (Bio-Rad).

Amino Acid Sequence Analysis—Five nmol of the rat liver glutathione S-transferase Yb, Yb heterodimer were sequenced in the Applied Biosystems gas phase sequenator (model 870-A) according to the manufacturer's specifications. High performance liquid chromatography was used to quantitate the phenylthiohydantoin derivatives produced at each step (19).

RESULTS

Construction and Characterization of a cDNA Clone Complementary to Rat Liver Glutathione S-Transferase Yb2 mRNA—In previous work from our laboratory, we reported the isolation and characterization of a nearly full-length cDNA clone, pGTA/C44, which is complementary to the Yb2 mRNA. During the screening of the Yb cDNA library, a second clone, pGTA/C48, was identified that had a restriction endonuclease map distinct from pGTA/C44 (Fig. 1). However, in hybrid-select translation experiments, pGTA/C48 hybridized to a mRNA that upon translation yielded a polypeptide with the same electrophoretic mobility as the Yb2 polypeptide (Fig. 2). Since this clone cross-hybridizes with pGTA/C44, we felt it would be a likely candidate for a Yb2 cDNA clone.

DNA Sequence Analysis of pGTA/C48—Nucleotide se-
Glutathione S-Transferase Yb₂ mRNA

The protein coding regions of both cDNA clones there is an 845 bp minus the dC tails. The sequence homologous to the Ybl subunit. The complete amino acid sequence of the Ybz subunit along with the Yb₁ subunit is (amino acids 25-218). The molecular weight of the Yb₁ subunit is 25,705. The carboxyl-terminal sequence, Pro-Lys, agrees with one possible carboxyl terminal sequence deduced from purified glutathione S-transferase Yb₂ dimers (20).

**NH₂-terminal Sequence Analysis of the Rat Liver Glutathione S-Transferase Yb₂ Yb₂ Heterodimer—**Since the Yb₂ cDNA clone was truncated and did not contain sequences complementary to the first 24 amino acids of the Yb₁ subunit that had been published by Frey et al. (21), we confirmed the identity of the clone by subjecting the Yb₁ Yb₂ heterodimer to conventional protein sequence analysis. The sequence of the first 46 amino acids of the Yb₁ Yb₂ heterodimer is presented in Table I. Over the first 46 amino acids, we found 13 positions where two amino acids were identified at each step. These positions are (Thr-Ile), 8 (Asp-Asn), 9 (Ile-Val), 13 (Ala-Thr), 15 (Ala-Pro), 19 (Phe-Leu), 24 (Thr-Ser), and 33 (Ser-Ala). The amino acid sequence of one of the monomers starting at amino acid residue 25 agrees with the sequence deduced from nucleotide sequence analysis of pGTA/C48.

Amino acids corresponding to residues 2-24 are not represented in the truncated Yb₁ cDNA clone, pGTA/C48. From pGTA/C48 is presented together for comparison. The nucleotide sequence of Yb₂ cDNA clone, pGTA/C44, are homologous to pGTA/C48. The nucleotide sequence of pGTA/C48 was carried out using the chemical sequencing procedure of Maxam and Gilbert (15). The entire nucleotide sequence of pGTA/C48 along with the nucleotide sequence of pGTA/C44 is presented in Fig. 3. Dashed lines in the pGTA/C48 sequence represent nucleotides present in Fig. 4 for comparison. The amino acid sequence analysis of the Yb₁ cDNA clone, pGTA/C44, is presented in Fig. 4 for comparison. The amino acid sequence analysis of the Yb₁ cDNA clone, pGTA/C44, is presented in Table I. Over the first 46 amino acids, we found 13 positions where two amino acids were identified at each step. These positions are (Thr-Ile), 8 (Asp-Asn), 9 (Ile-Val), 13 (Ala-Thr), 15 (Ala-Pro), 19 (Phe-Leu), 24 (Thr-Ser), and 33 (Ser-Ala). The amino acid sequence of one of the monomers starting at amino acid residue 25 agrees with the sequence deduced from nucleotide sequence analysis of pGTA/C48.

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Amino acids corresponding to residues 2-24 are not represented in the truncated Yb₁ cDNA clone, pGTA/C48. From

**Fig. 3. DNA sequence analysis of pGTA/C48.** The nucleotide sequence of the Yb₂ cDNA clone, pGTA/C48, and the Yb₁ cDNA clone, pGTA/C44, are presented together for comparison. The dashed lines represent bases in pGTA/C48 which are homologous to those in pGTA/C44.
position 2–46 the amino acid sequence of the Yb subunit agrees with the sequence deduced from nucleotide sequence analysis of pGTA/C48, the Yb cDNA clone (9) with two exceptions. These are at position 29 where Asp was identified for both the Yb and Yb subunits and we predicted Glu for the Yb subunit. The Glu at position 29 may not have been identified because of overlap from the Glu at position 28. The inability to detect Arg at position 31 is due to the low yield of this amino acid this far into the sequencing run.

**FIG. 4. Amino acid sequence of the rat liver glutathione S-transferase Yb subunit.** The amino acid sequence of the Yb subunit was determined from a combination of conventional protein sequencing techniques and DNA sequence analysis of pGTA/C48 (residues 25–218).

**DISCUSSION**

We have constructed and characterized a cDNA clone, pGTA/C48, which is complementary to the Yb mRNA. In the protein coding region the nucleotide sequence of pGTA/C48 is 84% homologous to the nucleotide sequence of the Yb cDNA clone, pGTA/C44, described previously by our laboratory (9). However, the 3' untranslated regions of the Yb and the Yb clones are only 32% homologous. These data suggest that the Yb and Yb polypeptides are encoded by different genes rather than being generated by alternative processing of a single gene.

**Nucleotide sequence analysis of the Yb and Yb cDNA clones enabled us to identify regions of poor homology in the 3' untranslated regions of the two mRNAs.** We have used these regions as specific probes in RNA slot blot analysis to demonstrate that both the Yb and Yb mRNAs are elevated by phenobarbital administration. These data are in contrast with previous reports indicating that only the glutathione S-transferase Yb subunit is elevated by phenobarbital administration (4, 20, 22, 23). The reason for this discrepancy is unclear. However, ideally, the effect of phenobarbital on the Yb subunit should be examined using immunochemical procedures. Unfortunately, the existent polyclonal antibodies are not specific enough to distinguish different Yb subunits. It is unclear whether the changes in the Yb mRNA correlate with an increase in Yb protein.
obtained from RNA slot blot hybridizations with a densitometer. The
mRNA of 3-4 rats. Yb, Yc, and Yn mRNAs were determined by scanning the x-ray films
unaffected by phenobarbital treatment or only slightly ele-
vated (17, 24, 25). However these data were based exclusively
of the rat liver glutathione S-transferase (27) is homologous to the
peptide with an isoelectric point of 9.3, it shares significant
sequence homology with a second mouse liver glutathione S-
transferase mRNA encoding a polypeptide with an isoelectric point of 8.7. The rat liver Yb subunit may represent the
analogue of the mouse liver glutathione S-transferase 8.7 sub-
unit. The NH2-terminal sequence of a human glutathione S-
transferase (27) is homologous to the NH2-terminal sequence of the rat liver Yb and Yb subunits. Although it is unknown
whether the sequence homology between the human liver glutathione S-transferase and the rat liver glutathione S-
transferases will be conserved throughout the entire amino
acid sequence, the similarity in the NH2-terminal sequences
suggests that these glutathione S-transferases have evolved
from a common ancestral gene.

The existence of two Yb mRNAs in rat liver may represent the
minimal number present. Tu and Reddy (28) have indicated that five different Yb homodimers or heterodimers can be isolated from rat liver cytosol. Interestingly, Hayes (6) has
characterized a glutathione S-transferase subunit, designated
Yn, which can form a heterodimer with the Yb or Yb subunit. The Yn subunit shares a number of common tryptic peptides with the Yb and Yb subunits, suggesting they have
elements of amino acid sequence homology. We have not iden-
tified a third cDNA clone in our cDNA library that is com-
plementary to the Yb mRNA; however, this is not surprising
given the very low level of the Yn subunit and presumably
the mRNA in rat liver (6).

The isolation and characterization of a cDNA clone com-
plementary to the Yb mRNA provide further evidence for
heterogeneity in the rat liver glutathione S-transferase multi-
gene family. The Yb cDNA clone will allow us to isolate the
structural gene(s) encoding the glutathione S-transferase Yb subunit as well as elucidate the mechanism(s) by which this
gene is regulated by xenobiotics.

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### Table I

| Hours after phenobarbital treatment | Relative mRNA levels |
|------------------------------------|----------------------|
| 0                                  | Yb1  | Yb2  | Yc   |
| 4                                  | 2.7  | 2.7  | 1.0  |
| 8                                  | 2.5  | 4.2  | 1.0  |
| 16                                 | 2.5  | 4.9  | 2.0  |
| 24                                 | 5.8  | 4.7  | 1.6  |

Although the Ya mRNA is elevated ~8-fold by phenobarbital administration (7), the Yc mRNA is reported to be
unaffected by phenobarbital treatment or only slightly ele-
vated (17, 24, 25). However these data were based exclusively
on in vitro translation analysis and may have been due to differences in translational efficiencies of the Yc mRNA
versus the Ya mRNA. Interestingly, our hybridization assay
with a specific Yc probe showed that phenobarbital resulted in
a very modest elevation in the Yc mRNA, which is consis-
tent with our previous in vitro translation and immunopre-
cipitation experiments (24). Therefore, only the Ya, Yb, and
Yb2 mRNAs are elevated significantly by phenobarbital. This
elevation is due in part to transcriptional activation of the Ya
and Yb genes (10).

The rat liver Yb cDNA clone, pGTA/C44, shares signif-
icant nucleotide sequence homology with a mouse liver glutathione S-transferase cDNA clone, pGT55, characterized by
Pearson et al. (26). Although pGTB55 is complementary to a
mouse liver glutathione S-transferase mRNA encoding a polypeptide with an isoelectric point of 9.3, it shares significant
sequence homology with a second mouse liver glutathione S-
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