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INDUCTION OF TRANSLATIONALLY ACTIVE RAT LIVER GLUTATHIONE S-TRANSFERASE B MESSENGER RNA BY PHENOBARBITAL

Cecil B. Pickett*[1], Wanda Wells*[2] and Anthony Y.H. Lu+
*Department of Biochemical Regulation and +Department of Animal Drug Metabolism and Radiochemistry, Merck Sharp & Dohme Research Laboratories, Rahway, New Jersey 07065
and
Barbara F. Hales
Department of Pharmacology and Therapeutics, McGill University
Montreal, Quebec, Canada H3G 1Y6

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SUMMARY

Liver poly(A^+)-RNA was isolated from untreated and phenobarbital-treated rats and translated in cell-free systems derived from wheat germ and rabbit reticulocyte lysates. The primary translation product of glutathione S-transferase B was comprised of two nonidentically sized subunits which comigrated on SDS-polyacrylamide gels with the purified glutathione S-transferase B subunits. The level of translatable glutathione S-transferase B mRNA in rat liver was elevated approximately 3 to 4-fold by phenobarbital administration. Our data suggest that chronic phenobarbital administration to rats increases the amount of cytosolic glutathione S-transferase B via an increase in the functional mRNA level encoding for the enzyme.

INTRODUCTION

Glutathione S-transferase B, or ligandin, is a major cytosolic protein of rat liver and catalyzes the conjugation of a variety of electrophilic compounds with glutathione as well as binds bilirubin, steroids, azodye carcinogens and many other organic anions (1). Recent work on the characterization of glutathione S-transferase B has demonstrated that the purified enzyme exists as a heterodimer with subunit molecular weights of 22,000 and 25,000 daltons (2,3). Although both subunits have glutathione transferase activity, the high affinity binding site for organic anions is associated with the small molecular weight subunit (3).

When rats are administered phenobarbital the amount of immunoprecipitable glutathione S-transferase B, cytochrome P-450, and epoxide hydrolase in liver is elevated

[1]To whom reprint requests should be sent.

[2]W.W. was a participant in the Merck Sharp & Dohme Research Laboratories summer student program. Her current address is: Department of Chemistry, Atlanta University, Atlanta, Georgia.

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approximately 3-fold (4-6), 15-30 fold (7-9), and 2-3 fold (8) respectively. Recent studies have shown that phenobarbital administration increases the amount of functional mRNA encoding for cytochrome P-450 (10,11), NADPH-cytochrome P-450 reductase (12) and epoxide hydrolase (13,14). On the other hand, Daniel, et al. (15) have reported that the induction of glutathione S-transferase B by phenobarbital is not dependent upon an elevation of translationally active mRNA encoding for the enzyme. These data suggest that the molecular mechanism by which phenobarbital induces glutathione S-transferase B is not at the transcriptional or post-transcriptional level. Therefore it is possible that the molecular mechanism(s) responsible for phenobarbital induction of microsomal and cytosolic enzymes may be completely different. In view of these implications, we feel it is important to re-examine the induction of glutathione S-transferase B by phenobarbital.

During experiments in our laboratory concerning the molecular basis of induction of epoxide hydrolase and cytochrome P-450, we observed that two polypeptides with molecular weights very similar to the two glutathione S-transferase B subunits were synthesized to greater extents when rabbit reticulocyte lysates were programmed with mRNA isolated from phenobarbital-treated rats as opposed to lysates programmed with mRNA isolated from untreated rats (16,17). In the present communication, we have extended our initial observations and have utilized both the wheat germ and rabbit reticulocyte cell-free translation systems along with specific immunoprecipitation to quantitate the level of translationally active glutathione S-transferase B mRNA in rat liver as a function of chronic phenobarbital administration. In contrast to the previous report by Daniel, et al. (15), our data indicate that chronic phenobarbital administration elevates the level of functional glutathione S-transferase B mRNA three to four-fold.

MATERIALS AND METHODS

Purification of Glutathione S-Transferase B - Glutathione S-transferase B was purified from male Sprague-Dawley rats to apparent homogeneity according to the method of Habig, et al. (18) and antiserum against the purified protein was raised in rabbits as previously described (19). Antibody specificity was determined by Ouchterlony double immunodiffusion. Partially purified IgG was prepared from the antiserum by DEAE-Affigel blue chromatography.

RNA Isolations - Total liver RNA was isolated from untreated and phenobarbital-treated rats by the guanidine-thiocyanate method of Chirgwin, et al. (20) as previously described by our laboratory (16). Animals treated with phenobarbital received 1 mg/ml of sodium phenobarbital in their drinking water for 8 days. Poly(A^+)-RNA was isolated from total liver RNA preparations by oligo(dT)-cellulose affinity chromatography (16).

Cell-Free Protein Synthesis - Poly(A^+) RNA was translated for 60 or 90 min. at 30°C in the micrococcal-nuclease treated rabbit reticulocyte lysate as well as the wheat germ lysate. Incubation conditions for translation of liver poly(A^+)-RNA in the rabbit reticulocyte lysate has been published (14,16). Both in vitro translation systems were purchased from Bethesda Research Laboratories. The reaction mixture (90-180 µl) for the wheat germ lysate contained 30-60 µl of lysate, 20 mM Hepes, 63 mM K acetate, 33 mM KCl, 2.37 mM Mg acetate, 1.2 mM ATP, 0.1 mM GTP, 5.5 mM creatine phosphate, 0.2 mg/ml creatine kinase, 80 µM spermidine phosphate and 50 µM 19 amino acids (-methionine), 20 µCi [^{35}S] methionine and 10-30 µg/ml of poly(A^+)-RNA. To assess the incorporation of [^{35}S] methionine into protein, 2 µl of the reaction mixture was spotted on Whatman 3 MM paper and placed in cold 10% trichloroacetic acid. The filter papers were boiled for 15 min. in 5% trichloroacetic acid, rinsed thoroughly with cold 5% trichloroacetic acid, followed by absolute ethanol, ethanol-ether (1:1) and finally ether. The filter papers were dried, placed in scintillation vials and 500 µl of Protosol (New England Nuclear) was added to each vial. The vials were then incubated at 55° for 30 min. then allowed to cool to room temperature. Ten ml of Econofluor was added and total radioactivity determined by liquid scintillation counting.

Immunoprecipitation and Quantitation of Glutathione S-Transferase B -Glutathione S-transferase B was recovered from the translation mixtures by indirect immunoprecipitation utilizing protein A-sepharose. Translation mixtures were diluted 1:4 with 190 mM NaCl, 50 mM Tris-HCl pH 7.5, 6 mM EDTA, 1.0% Triton X100 and 1.0% Na deoxycholate and centrifuged at 100,000 x g for 1 hour to pellet ribosomes. Fifteen microliters of rabbit anti-glutathione S-transferase B IgG was added to the ribosomal-free supernatant followed by 200 µl of protein A-sepharose. The total mixture was then incubated overnight with end to end rotation. The immune complex-protein A sepharose suspension was filtered through cellulose membranes utilizing the MF-1 microfilter assembly (Bioanalytical Systems Inc., West Lafayete, Indiana). The immune complex was collected on the cellulose filter and washed 5 times with phosphate buffered saline containing 0.1% SDS. The antigen was eluted by boiling in SDS sample buffer containing 10 mM dithiothreitol. The eluted antigen was subjected to SDS-polyacrylamide gel electrophoresis (21) and radiolabeled glutathione S-transferase B identified in the gel by fluorography (22) using EN^{3}HANCE (New England Nuclear). In order to quantitate the level of translatable glutathione S-transferase B mRNA, the region of the gel corresponding to purified glutathione S-transferase B was excised and dissolved in 60% perchloric acid -30% hydrogen peroxide at 60°C. Aqualsol 2 was added to the solubilized gel slices and total radioactivity was determined by liquid scintillation counting. Background radioactivity was determined by excising gel regions above and below the radiolabeled glutathione S-transferase B and solubilizing the slices as described above. The values obtained were then subtracted from the radioactivity obtained in the slices containing glutathione S-transferase B.

RESULTS AND DISCUSSION

Characterization of Glutathione S-Transferase B - The purified glutathione S-transferase B preparation utilized in this study has been characterized in detail in a previous study (19). When the purified enzyme was subjected to SDS-polyacrylamide

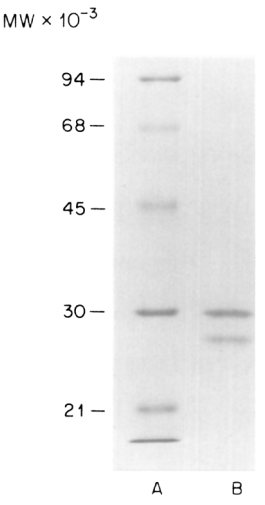

Figure 1. SDS-polyacrylamide gel electrophoresis of purified glutathione S-transferase B (lane B, 10 µg). Lane A represents molecular weight markers: phosphorylase B (94,000), bovine serum albumin (68,000), ovalbumin (45,000), carbonic anhydrase (30,000) and soybean trypsin inhibitor (21,000).

gel electrophoresis utilizing a 10% gel a subunit heterogeneity was noted (Fig. 1). Based on the migration of specific marker proteins, we have calculated the molecular weights of the two subunits to be 29,000 and 27,000 daltons. Our data confirm the results from other laboratories (2,3,15) which have indicated that purified glutathione S-transferase B exists as a heterodimer. However, the molecular weights we have calculated for the two subunits are higher than the values of 25,000 and 22,000 daltons which have been reported previously. The difference may in part reside in the use of different SDS-polyacrylamide gel electrophoresis systems as well as different molecular weight markers.

Rabbit antibody against the purified enzyme was prepared and characterized in a previous publication (19). The antibody produced a single precipitin band on Ouchterlony double immunodiffusion gels when reacted against either the purified enzyme or a 100,000 x g supernatant fraction isolated from rat liver (19). Furthermore the precipitin band generated by the purified enzyme and the form present in the 100,000 x g supernatant fused indicating immunochemical identity between the purified enzyme and the form present in the high speed supernatant.

Cell-Free Translation and Quantitation of Glutathione S-Transferase B mRNA

During previous studies in our laboratory we noted that when rabbit reticulocyte lysates were programmed with liver poly(A^+)-RNA isolated from phenobarbital-treated rats polypeptides with molecular weights similar to glutathione S-transferase B were synthesized to greater extents as compared to translation experiments carried out with poly(A^+)-RNA isolated from uninduced rats (16,17). Similar observations were also made using a cell-free system derived from wheat germ extracts. Since the amount of immunoprecipitable glutathione S-transferase B in rat liver had been shown to be elevated by phenobarbital treatment, we felt that the alterations in the total translation products we had observed might indicate that translationally active mRNA encoding for glutathione S-transferase B was also induced by phenobarbital. Therefore we wanted initially to determine the size and number of subunits of the primary translation product of glutathione S-transferase B and then to quantitate the translatable mRNA level as a function of chronic phenobarbital treatment. In order to determine if the primary translation product of glutathione S-transferase B was a heterodimer, total liver poly(A^+)-RNA isolated from untreated rats was translated in the wheat germ cell-free protein synthesizing system using [^{35}S]-methionine as the labeled amino acid. The translation mixture was subjected to immunoprecipitation utilizing anti-glutathione S-transferase B IgG. The immunoprecipitable product was subjected to SDS-polyacrylamide gel electrophoresis and identified by fluorography. The immunoprecipitated translation product (Fig. 2, lane B) existed as two polypeptides with subunit molecular weights identical to the purified enzyme. When pre-immune IgG was used in place of the immune IgG, no immunoprecipitable product was obtained. As can be seen in Fig. 2, lane B, the intensity of labeling of the small subunit was several-fold higher than that observed for the large subunit. Bhargava, et al. (3) have recently determined the amino acid composition of the purified subunits and reported that the methionine content of the two subunits were very similar. Therefore it is unlikely that the difference we observe in [^{35}S]-methionine incorporation into the two subunits is due to a difference in methionine content between them. However we feel there are several possibilities which might account for our observations. Firstly, the mRNA

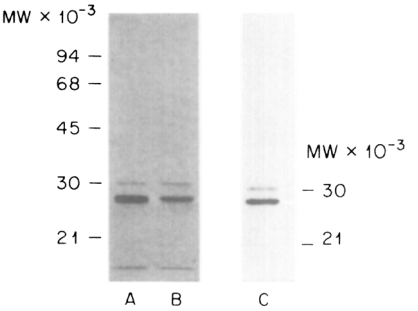

Figure 2. Fluorogram of SDS-polyacrylamide gel electrophoresis of glutathione S-transferase B immunoprecipitated from wheat germ translation mixtures programmed with poly(A^+)-RNA isolated from phenobarbital-treated (lane A) and untreated (lane B) rats. Equal aliquots of the immunoprecipitate were layered on the 10% SDS-polyacrylamide gel. Lanes A and B represent a 72 hour fluorogram. Lane C represents a 24 hour fluorogram of glutathione S-transferase B which had been immunoprecipitated from rabbit reticulocyte lysates programmed with poly(A^+)-RNA isolated from untreated rats. Molecular weight markers are identical to those described in Figure 1.

encoding the small subunit might be translated by the wheat germ system more efficiently than the mRNA encoding the large subunit. Secondly, a higher concentration of functional mRNA encoding the small subunit might be present in the total messenger RNA population. Thirdly, the antibody preparation utilized in this study might not be quantitatively immunoprecipitating both subunits from the in vitro translation system. Although we have not determined which of the above possibilities might be correct, we believe that the difference in [^{35}S]-methionine incorporation is probably not due to different translational efficiencies for the separate subunits in the wheat germ system. When total liver poly(A^+)-RNA isolated from untreated rats was translated in the rabbit reticulocyte cell-free system identical results were obtained (Fig. 2, lane C).

The level of mRNA specific for glutathione S-transferase B was quantitated by determining the amount of immunoprecipitated radioactivity which comigrated on 10% SDS-polyacrylamide gels with the purified enzyme. Figure 2, lane A, represents immunoprecipitated glutathione S-transferase B from wheat germ translation systems programmed with total liver poly(A^+)-RNA isolated from rats treated chronically with phenobarbital. A three to four-fold increase of translationally active glutathione S-transferase B mRNA was detected in poly(A^+)-RNA isolated from phenobarbital-

TABLE 1

Induction of Glutathione S-Transferase B mRNA by Phenobarbital

	Treatment	Radioactivity in Total Protein[a] cpm x 10^{-6}	Radioactivity in Immunoprecipitated Glutathione S-Transferase B[b] cpm x 10^{-3}
Exp. 1	None	1.9	1.0
	Phenobarbital	1.9	3.6
Exp. 2	None	2.5	1.7
	Phenobarbital	2.5	7.1

a. Radioactivity in total protein was determined as described in the Methods section. The values presented have been corrected for endogenous protein synthesis which occurs in the wheat germ lysate in the absence of exogenous mRNA. The in vitro translation studies reported above were carried out at a final poly(A^+)RNA concentration of 20 µg/ml for either 60 min. (Exp. 1) or 90 min. (Exp. 2). Incorporation was linear up to poly(A^+)-RNA concentrations of 50 µg/mL

b. The amount of radioactivity in the immunoprecipitated glutathione S-transferase B was determined as described in the Methods section.

treated rats (Table I). Also note from Fig. 2, lane A (as compared to lane B) that the increase in [^{35}S]-methionine incorporation occurs in the small molecular weight subunit not both subunits. Identical results were obtained using the rabbit reticulocyte cell-free system. Therefore, it appears that phenobarbital induces translationally active glutathione S-transferase B mRNA, cytochrome P-450 mRNA (10,11), epoxide hydrolase mRNA (13,14) and NADPH cytochrome P-450 reductase mRNA (12) to very similar extents.

In conclusion we have found that the primary translation product of rat liver glutathione S-transferase B mRNA exists as two distinct polypeptides and in contrast to a previous publication the amount of translationally active mRNA encoding the enzyme is induced 3-4 fold by chronic phenobarbital administration. Furthermore, our results indicate it is unnecessary to postulate that the large subunit is the primary gene product and is proteolytically converted to the small subunit (3). Although we cannot completely rule out the possibility that the large subunit is being converted to

the small subunit during cell-free translation, we believe it is unlikely that both translation systems possess such a novel processing activity. Finally our results suggest that the mRNAs encoding the two subunits may be present in the total mRNA population at unequal concentrations and that phenobarbital selectively increases the concentration of the mRNA encoding the small molecular subunit. Interestingly, it is the small subunit which has the high affinity binding site for organic anions (3). Definitive proof of these latter possibilities however must await further experimentation.

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