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CELL-FREE SYNTHESIS OF EPOXIDE HYDRASE BY LIVER POLY (A^+)-RNA ISOLATED FROM PHENOBARBITAL TREATED RATS

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SUMMARY

Total liver RNA has been isolated from normal and 8 day phenobarbital treated rats by guanidine thiocyanate β-mercaptoethanol extraction and fractionated by oligo (dT)-cellulose chromatography to yield poly (A^+)-RNA. Poly (A^+)-RNA from normal and phenobarbital treated rats have similar translational activity in the rabbit reticulocyte cell-free system. However major alterations occurred in the polypeptide products directed by these two classes of RNA. The translation products directed by 8 day phenobarbital poly(A^+)-RNA were immunoprecipitated with rabbit IgG prepared against purified rat liver epoxide hydrase. The immunoprecipitate was subjected to SDS-polyacrylamide gel electrophoresis and the radioactive products detected by fluorography. Analysis of the fluorogram revealed that the major immunoprecipitable product co-electrophoresed with purified epoxide hydrase. These data suggest that the primary translation product of epoxide hydrase messenger RNA has the same molecular weight as the mature form of the enzyme.

INTRODUCTION

Recent work from a number of laboratories (cf. Ref. 1 for recent review and 2,3,4) have demonstrated that secretory proteins and proteins of certain organelles are synthesized as high molecular weight precursors which are processed to the mature protein while transversing the membrane. However studies on rat liver cytochrome P-450, an integral membrane protein of the endoplasmic reticulum, have indicated that the major phenobarbital-inducible species is synthesized in cell-free systems as the mature enzyme (5,6,7,8,9). In contrast to these findings is a recent report by Kumar and Padmanaban (9) who found that cytochrome P-448 which is induced by 3-methylchloanthrene appears to be synthesized as a high molecular weight precursor which is processed to the mature form by microsomal membranes. Since both cytochromes P-450 and P-448 are incorporated into the microsomal membrane and form an integral part of the monooxygenase system, it is unclear whether the insertion of integral membrane proteins of the ER proceeds by a common mechanism.

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In order to examine the mechanism by which proteins of the endoplasmic reticulum are assembled into the membrane, we have focused our attention on the biosynthesis of epoxide hydrase. Epoxide hydrase in concert with the cytochrome P-450 system plays a major role in the activation and inactivation of a variety of carcinogens and mutagens (10,11,12,13,14). Since phenobarbital administration increases the level of epoxide hydrase in the endoplasmic reticulum, we have used this compound with the aim of increasing the template activity of epoxide hydrase messenger RNA in rat liver. The purpose of this study is to determine by cell-free translation of poly (A^+)-RNA from phenobarbital treated rats and subsequent immunoprecipitation of the translation products with anti-rat liver epoxide hydrase IgG, whether the biosynthesis of epoxide hydrase proceeds via processing of a high molecular weight precursor or whether the enzyme is synthesized as the mature form.

METHODS

RNA Isolations - Male Sprague-Dawley rats (120-170 g) were used in all experiments. Phenobarbital treated rats were given 1 mg/ml of sodium phenobarbital in their drinking water for a period of 8 days before sacrifice. Control animals were given water ad libitum. Total liver RNA was isolated by the guanidine thiocyanate-cesium chloride procedure described by Chirgwin, et al. (15). Total liver RNA was resuspended in sterile water at 20-40 ml A_{260} units, heated 2 min. at 68°C, rapidly cooled to room temperature and adjusted to 0.5M lithium chloride, 0.5% SDS, 1 mM EDTA and 10 mM Tris-HCl pH 7.4. The RNA was passed through an oligo (dT)-cellulose column (Type T-3, Collaborative Research, Waltham, MA) which had been equilibrated with the same buffer. Bound poly (A^+)-RNA was eluted from the column with 10 mM Tris-HCl pH 7.4, 0.1% SDS and 1 mM EDTA. The poly (A^+)-RNA was adjusted to 0.2M potassium acetate, pH 5.0, and precipitated overnight with two volumes of absolute ethanol.

Cell-Free Protein Synthesis - Poly (A^+)-RNA was translated for 60 minutes in a micrococcal nuclease treated rabbit reticulocyte lysate (16) purchased from Bethesda Research Laboratories. The reaction mixture (90 µl) contained 30 µl of lysate, 25 mM Hepes pH 7.4, 10 mM creatine phosphate, 48 mM KCl, 87 mM K acetate, 1.2 mM $MgCl_2$, 50 mM 19 amino acids (-methionine), 20 µCi [^{35}S]-methionine and 1-2 µg 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 10 min. in 5% trichloroacetic acid, rinsed thoroughly with cold 5% trichloroacetic acid, followed by 100% ethanol, ethanol-ether (1:1) and finally ether. The filter papers were allowed to dry before being placed in Aquasol 2 and counted. Aliquots of the translation mixtures were mixed with an equal volume of 5.0 mM Tris-HCl pH 6.8, 2% SDS, 2% β-mercaptoethanol, and 20% glycerol, boiled for 2 min., then subjected to SDS-polyacrylamide gel electrophoresis (17). The radioactive products were visualized by autoradiography. Autoradiograms were scanned with a Helena Quik Scan Densitometer.

Preparation of Antibodies Against Purified Epoxide Hydrase - Phenobarbital-induced epoxide hydrase was purified by the method of Knowles and Burchell (18) as modified by Guengerich, et al. (19). The purified protein gave a single band when subjected to SDS-polyacrylamide gel electrophoresis. Antibody against rat liver epoxide hydrase

was raised in rabbits by intradermal injections (8 sites) of 200 µg of purified enzyme mixed with Freud's complete adjuvant. Booster injections consisting of 70 µg of epoxide hydrase mixed with Freud's incomplete adjuvant were given at approximately weekly intervals. Rabbit IgG was partially purified from the antiserum by DEAE-Affi gel blue affinity chromatography. Antibodies against epoxide hydrase gave a single sharp immunoprecipition band in Ouchterlony immunodiffusion gels when reacted against either purified epoxide hydrase or sodium cholate solubilized microsomes isolated from phenobarbital treated rats. The antibody did not cross react against either purified cytochrome P-450 from phenobarbital treated rats or cytochrome P-448 from 3 methylcholanthrene treated rats.

Immunoprecipitation of Epoxide Hydrase - After a 60 min. incubation period the translation mixtures (270 µl) were adjusted to 5% SDS, boiled for 3 mins. and diluted 1:10 with 2.5% Triton X-100, 190 mM NaCl, 50 mM Tris-HCl pH 7.4 and 6 mM EDTA. The mixture was preincubated for 5 min. with heat-inactivated formaldehyde fixed S. aureus cells (20 µl) to decrease non-specific binding during immunoprecipitation (20). The cells were removed by centrifugation and twenty microliters of anti-rat liver epoxide hydrase IgG was added to the supernatant. The mixture was incubated at room temperature for 1 hr. followed by overnight incubation at 4°C. Antigen-antibody complexes were precipitated by the addition of 100 µl of S. aureus cells. The cells were washed three times with 0.1% SDS, 10 mM Tris-HCl pH 7.4, 2 mM EDTA then boiled for 3 min. in SDS sample buffer containing 5% SDS and 100 mM DTT. The S. aureus cells were removed by centrifugation and the supernatant containing the immunoprecipitated translation product was analyzed by SDS-polyacrylamide gel electrophoresis. The radioactive product was detected by fluorography (21) using EN^{3}HANCE purchased from New England Nuclear.

RESULTS AND DISCUSSION

In order to assess the translational activity of poly (A^+)-RNA from normal and phenobarbital treated rats in the rabbit reticulocyte system, equal concentrations of both fractions of poly (A^+)-RNA were translated in the cell-free system and the total trichloroacetic acid insoluble [^{35}S] radioactivity determined on aliquots of the mixture at various times during the 60 minute incubation period. Both poly (A^+)-RNA from control and phenobarbital treated rats stimulated total protein synthesis to a similar extent (Fig. 1).

The translation products directed by control and phenobarbital poly (A^+)-RNA were subjected to SDS-polyacrylamide gel electrophoresis and the radioactive polypeptides visualized by autoradiography. Analysis of the autoradiogram (Fig. 2a) or the densitometric scans of these autoradiograms (Fig. 2b) revealed that liver poly (A^+)-RNA from phenobarbital treated rats directed the synthesis of at least 3 polypeptides (molecular weights, 50-52,000, 29,000 and 27,000) which appeared either to be not synthesized by control poly (A^+)-RNA or synthesized in greatly reduced amounts. We have identified by cell-free translation and immunoprecipitation that one of the

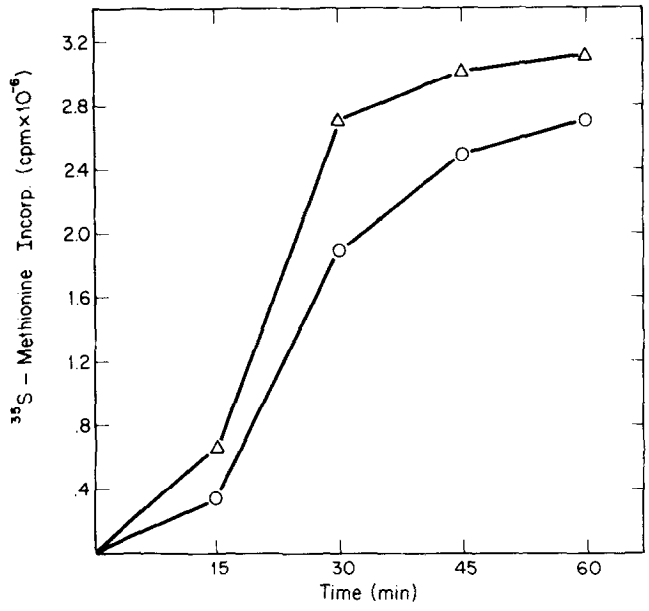

Figure 1 Poly (A^+)-RNA from control (○) and 8 day phenobarbital treated rats (△) were translated in vitro using the rabbit reticulocyte cell-free system. During the incubation, 2 µl aliquots were removed and counted as described in the Methods section to assess the incorporation of [^{35}S]-methionine into protein. Incorporation of [^{35}S]-methionine into protein is based on the total translation mixture (90 µl).

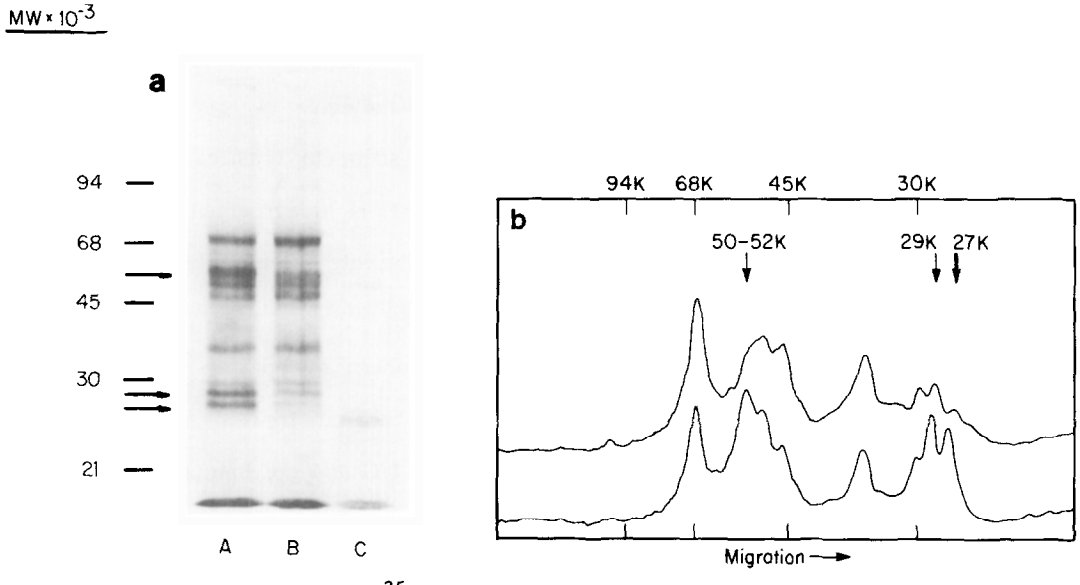

Figure 2a Autoradiogram of the [^{35}S] labeled polypeptides directed by liver poly (A^+)-RNA from phenobarbital treated rats (A) control rats (B) and by the rabbit reticulocyte lysate in the absence of exogenous poly (A^+)-RNA (C). The arrows represent the major alterations which occur in the polypeptides directed by poly (A^+)-RNA from phenobarbital treated animals. The molecular weight markers were phosphorylase B (94K), BSA (68K), ovalbumin (45K), carbonic anhydrase (30K) and soybean trypsin inhibitor (21K).

Figure 2b Densitometric scans of lane A (lower scan) and lane B (upper scan) of figure 2. Arrows point to the molecular weight range where the major changes occur in the translation products directed by poly (A^+)-RNA from phenobarbital treated animals.

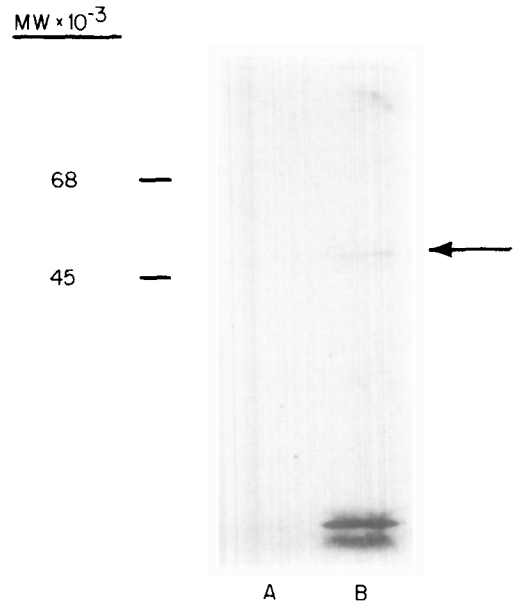

Figure 3 Fluorogram of [^{35}S]-labeled epoxide hydrase immunoprecipitated from translation products directed by poly (A^+)-RNA isolated from phenobarbital treated rats. Lane A represents the immunoprecipitation using pre-immune IgG. Lane B represents immunoprecipitation using anti-epoxide hydrase IgG. Co-electrophoresis of unlabeled authentic epoxide hydrase is indicated by the arrow.

polypeptides in the 50-52,000 molecular weight range is cytochrome P-450 (unpublished observations). The identity of the 29,000 and 27,000 molecular weight polypeptides remains unknown. Although phenobarbital does not seem to drastically alter the template activity of total liver messenger RNA which is in agreement with other studies (6), it does appear that this compound selectively increases the level of translatable messenger RNA for several polypeptides.

In order to detect the primary translation product of epoxide hydrase, the polypeptides directed by poly (A^+)-RNA from phenobarbital treated animals were immunoprecipitated with anti-rat liver epoxide hydrase IgG and the immunoprecipitate run on 10% SDS-polyacrylamide gels. The immunoprecipitable product migrated as a single radioactive band and had the same electrophoretic mobility as purified epoxide hydrase (Fig. 3, lane B; the arrow represents the position of purified epoxide hydrase). Identical results were obtained when the immunoprecipitates were run on 7.5% polyacrylamide gels. When pre-immune serum or IgG was used, no immunoprecipitable product was detected (Fig. 3, lane A). Since the primary translation product of epoxide hydrase

messenger RNA appears to have the same molecular weight, 50,000 daltons, as the mature form of the enzyme, we suggest that the biosynthesis of this enzyme does not proceed via processing of a high molecular weight precursor; thereby resembling the biosynthesis of the phenobarbital-inducible species of cytochrome P-450 rather than cytochrome P-448.

Since our cell-free translation experiments demonstrate that epoxide hydrase is synthesized as the mature form of the protein, we feel it might contain a sequence which is the functional equivalent to the signal or insertion sequence of secretory proteins (1). Dubois, et al. (22) have sequenced the NH_2-terminal of purified rat liver epoxide hydrase and found that it resembles the signal sequence of many secretory proteins with regard to hydrophobicity and the presence of methionine as the NH_2-terminal residue. It is probable, therefore, that the NH_2-terminal of epoxide hydrase serves as a signal for insertion of the protein into ER but unlike the signal sequence of most secretory proteins, it is not removed by the membrane associated protease. The mechanism by which this protein is inserted into microsomal membranes is currently under investigation by our laboratory.

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