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IN VITRO BIOSYNTHESIS OF LIVER CYTOCHROME P450 MATURE PEPTIDE SUB-UNIT BY TRANSLATION OF ISOLATED POLY(A)$^+$ mRNA FROM NORMAL AND PHENOBARBITAL INDUCED RATS

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SUMMARY

The biosynthesis of a cytochrome P_{450} peptide sub-unit by the *in vitro* translation of total hepatic poly(A)$^+$ mRNA in an heterologous cell-free-system is described. The ability of the liver poly(A)$^+$ RNA preparations from normal and phenobarbital induced rats to promote protein synthesis and the identification of *in vitro* synthesized proteins revealed the presence of a cytochrome P_{450} peptide sub-unit presenting the same apparent molecular weight of the native peptide. This fact demonstrates that rat liver poly(A)$^+$ mRNA fraction contains an important amount of cytochrome P_{450} peptide messages. Total poly(A)$^+$ RNA from rats in an early phenobarbital induction stage exhibits a higher cytochrome P_{450} template activity in good agreement with the enhancement of this hemeprotein concomitantly observed *in vivo*, in the liver microsomes, it is also concluded that cytochrome P_{450}, peptide sub-unit, induced in rat liver by phenobarbital, is translated in its mature form.

The administration of PB to rats results in an increased activity of the mixed-function-oxidases of the liver, a proliferation of the endoplasmic reticulum and a net increase in its protein content (1). Although the exact molecular mechanism for enzyme induction remains to be elucidated it is known that inductive process involves the synthesis of increased amounts of membrane proteins as drug metabolizing enzymes (2). Cytochrome P_{450} exists in multiple forms characterised by different spectral properpties, catalic specificities and molecular weights, which peptide sub--units represent more than 5% of total microsomal proteins in

Abbreviations used : PB, phenobarbital; H.S.B., high salt buffer; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

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rat liver, easily attaining a value of 10-15% after PB administration (3). It has been recently demonstrated by RYAN and als (4) that in rat liver, the production of a particular form of cytochrome P_{450}, is stimulated by PB in a rather specific way, similarly with the specific induction of Cytochrome P_{450} LM_2 produced by this agent in rabbit liver (5).

Synthesis of new proteins which form membranes and mixed--functions-oxidases is associated to a marked enhancement in poly(A)$^+$ mRNA in the liver microsomes (6). In the present work we have proceeded to the isolation of the total poly(A)$^+$ RNA pool from control and PB induced rats and to its subsequent translation in an heterologous mRNA dependent cell-free-system in order to search for *in vitro* production of cytochrome P_{450} peptide sub-units.

The detection of the *in vitro* translatable cytochrome P_{450} mRNA in the total rat liver poly(A)$^+$ RNA and the recognition of the translation primary products opens the possibility for further studies on the molecular mechanisms of liver enzyme induction by xenobiotics and proliferation of endoplasmic reticulum membranes.

METHODS

Young male Sprague-Dawley rats were used in the experiments. The animals were starved for 20 hr receiving water *ad lib.* before they were weighed and sacrificed.

Control and PB treated rats were kept under the same experimental conditions within each experiment. PB treated animals were given 80 mg/Kg body weight daily in aqueous solution (16 mg/ /ml) intragastrically and killed at different times afterwards.

24 hr after one single dose PB animals were sacrificed as well as control rats. After decapitation and exsanguination, the livers were quickly removed, weighed and total RNA was obtained by fresh tissue extraction in guanidine thiocyanate and 2-mercaptoethanol according to the method described by HARDING et als (7). Poly(A)$^+$ mRNA was isolated by two successive rounds of oligo (dT)-cellulose chromatography. Total RNA extracted from 1g liver, taken in H.S.B. 0.3M NaCl, 10mM TRIS-HCl, pH 7.5, 5mM EDTA and 0.2% SDS was loaded at 10 A_{260} units/ml on to 0.25g

oligo (dT)-cellulose. The column was washed extensively with the same buffer and the bound RNA was eluted with bi-distilled water. The RNA solution was readjusted to 0.3M NaCl and other H.S.B. components, in half the original volume and rechromatographed on the same column. The adsorbed RNA was eluted with water, U.V. Absorbances at 230, 260 and 280nm measured, and further precipitated with 2 volumes of ethanol at -20°C after adition of sodium acetate to 0.25M.

The integrity of Poly(A)$^+$ RNA molecules was checked in methylmercury agarose gel electrophoresis (8) and the ability of these liver mRNA to promote protein synthesis was tested by incubating 1 µg of each purified mRNA preparation in a reticulocyte lysate cell-free-system in the presence of [^{35}S] Methionine in a 25 µls final volume, at 25°C for 30 min (9). Incorporation of radioactivity into total TCA precipitable protein was determined in each translation test and the neo-synthesized proteins analysed by SDS-PAGE (10) and fluorography (11).

The apparent molecular weight of the neo-synthesized peptide chains was evaluated by their relative mobilities in the SDS-PAGE which was simultaneously calibrated with proteins of known molecular weight.

Cytochrome P_{450} was purified from steady-state induced rats, sacrificed after 3 days PB treatment. Liver microsomes were prepared and extracted with 0.1M sodium pyrophosphate buffer containing 1.0mM EDTA. The pyrophosphate-treated microsomes were solubilized in the presence of glycerol. The purification procedure was followed by collecting the 8-10% polyethylene glycol fraction made 0.5% (v/v) in Renex 690 and applied to a DEAE--cellulose column as described by HAUGEN and COON (5).

The partially purified cytochrome P_{450} eluted from the DEAE--cellulose column was used as a standard of PB inducible form adopting the criterion that has been proposed to characterize the multiple forms of this cytochrome, i.e. the apparent molecular weight of their peptide sub-unit determined in a SDS-PAGE by measuring the relative mobilities of the bands seen upon staining with coomassie blue.

RESULTS

24 hr after a single PB administration liver/body weight ratio was already significantly enhanced and a marked rise in cytochrome P_{450} was observed, indicating a good inductive response to this agent. Concomitantly the amount of total poly(A)$^+$ RNA per gram liver was found to be higher in the induced livers, as shown in Table I.

Total undegraded poly(A)$^+$ mRNA extracted from normal and PB induced liver, with 260/280nm Absorbance ratios of 1.98 and 1.85 respectively, showed equaly good activities, 700.000 c.p.m. [^{35}S] methionine incorporation per ug RNA per hr into total TCA precipitable protein.

Table I : Effect of 24 hr PB treatment on cytochrome P-450 and poly(A)$^+$RNA in rat liver. Cytochrome P-450 determined in total microsomes from CO difference spectrum. Total poly(A)$^+$RNA evaluated by the U.V. absorbance as described in Methods.

ANIMALS	g liver per 100 g body wt.	Cyt. P-450 nmoles per mg prot.	260 nm A.Units Poly(A)$^+$RNA per g liver	Poly(A)$^+$ RNA / Total x100
CONTROL	2.336	.99	7.1	1.62%
PB	2.744 (117,5%)	1.98 (200%)	10.4 (147%)	2.08%

The presence of a peptide band exhibiting the same apparent molecular weight of a P_{450} peptide sub-unit in the fluorogram — as compared to partially purified protein, 13.3 nmol cyt. P_{450}/ /mg protein, obtained from PB induced rat liver (Fig. I) — was

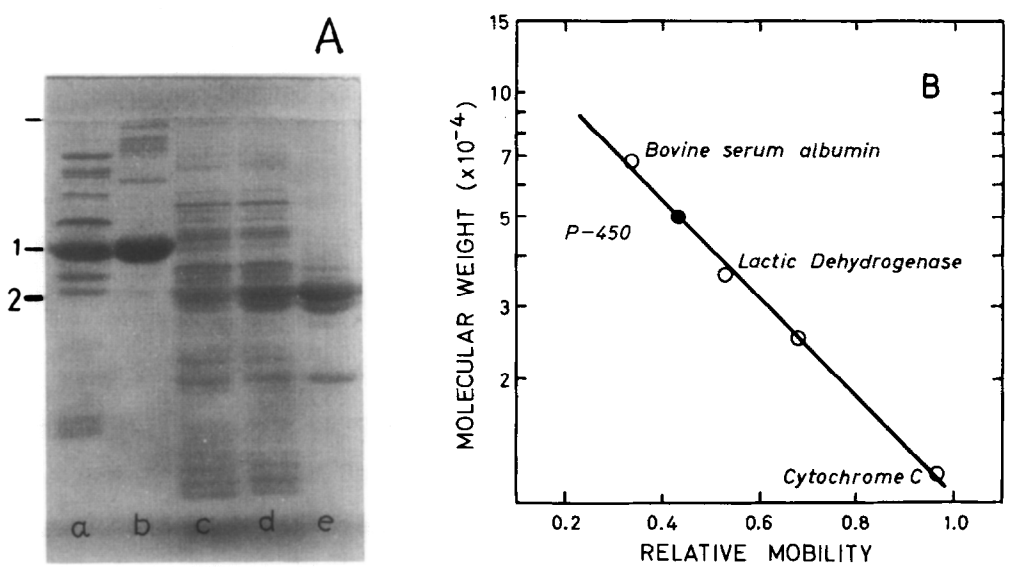

Fig. I - A - 10% Polyacrylamide slab gel electrophoresis in a discontinuous buffer system. Migration was *top* to *bottom*. Samples a - rat blood serum, b - bovine serum albumin, c and d - microsomes from control and PB induced livers, e - partially purified cytochrome P-450 prepared as described in Methods.

B - Calibration plot of peptides molecular weight *versus* electrophoretic relative mobilities. Molecular weight of cytochrome P-450 peptide sub-unit induced by PB, 50.000 daltons.

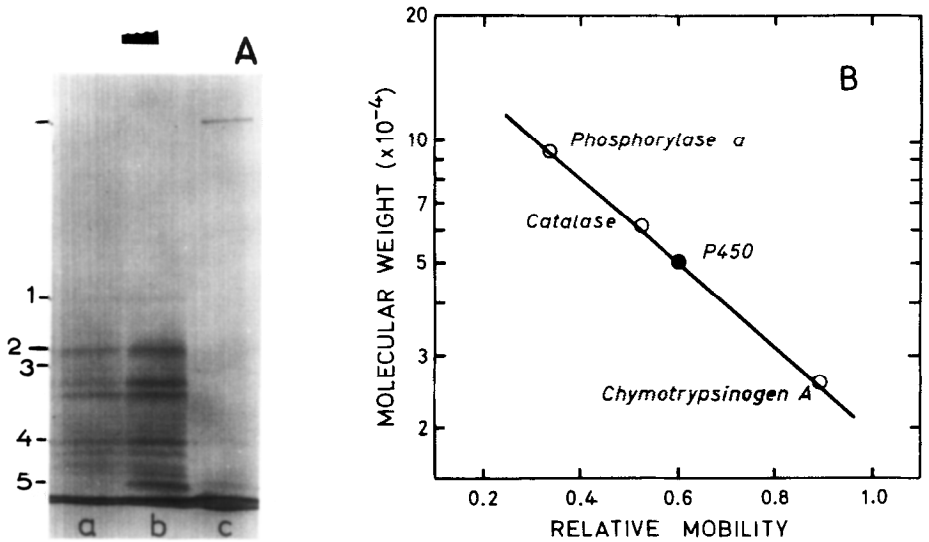

Fig. II - A - Polypeptides synthesized in rabbit reticulocyte lysate in response to rat liver poly(A)$^+$RNA's. One µg RNA incubated in the presence of radioactive-methionine at 25°C for 30 m. Aliquots of each reaction (100.000 c.p.m. TCA precipitable protein) resolved on a slab SDS-PAGE and fluorographed as described in Methods. Slot a and b - mRNA from control and PB induced rat liver, c - bands detected in the absence of exogenous RNA. The main regions of radioactivity are numbered in slot (a), band 2 contains *in vitro* synthesized cytochrome P-450 peptide sub-unit.
B - Calibration plot of peptides molecular weight in the fluorogram. *In vitro* synthesized cytochrome P-450 in the slab fluorogram by its relative mobility and molecular weight 50.000 daltons.

detected in the *in vitro* synthesized peptides, in higher amount when poly(A)$^+$ mRNA from PB induced rats was used as a template (Fig. II).

DISCUSSION

The production of a cytochrome P_{450} peptide sub-unit by *in vitro* translation of total poly(A)$^+$ RNA from normal rat liver, in a heterologous rabbit reticulocyte cell-free-system, and the enhancement in template activity for this peptide in PB induced livers now described demonstrate that cytochrome P_{450} mRNA exists in rat liver as a polyadenilated active form.

The fact that PB enhances the amount of this specific message translation in rat liver, both *in vivo* and *in vitro* can be

related to the increase in microsomal total poly(A)$^+$ mRNA previously described (6). Actually if the *in vivo* new-synthesized mRNA contains a higher amount of messages for the inducible form of this hemeprotein after PB administration the well known rise in its synthesis induced by this agent could be explained as a direct consequence of the increase in the number of active messages in the cell.

The fact that inducible form of cytochrome P_{450} is identified as a peptide with a molecular weight of 50.000, when synthesized in the cell-free-system, can be interpreted as an evidence for the primary translation product of the mRNA being the mature form of this peptide sub-unit.

The possibility of isolating cytochrome P_{450} peptide specific mRNA, now opened, will allow new experimental aproaches to the study of the molecular mechanisms of microsomal enzyme induction and endoplasmic reticulum membranes proliferation depending on gene expression and regulation mechanisms in the liver.

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