Studies on the Synthesis of Cytochrome P-450 and Cytochrome P-448 in Rat Liver*

Ashwani Kumar and G. Padmanaban

From the Department of Biochemistry, Indian Institute of Science, Bangalore-560 012, India

The synthesis of cytochrome P-450 (phenobarbital inducible) and cytochrome P-448 (3-methylcholanthrene inducible) have been studied in rat liver *in vivo* and in the wheat germ cell-free system using anti-cytochrome P-450 and anti-cytochrome P-448 antibodies. The major mature forms synthesized *in vivo* correspond to a molecular weight of 47,000 for cytochrome P-450 and 53,000 for cytochrome P-448. Translation of poly(A)-containing RNA from phenobarbital-treated rats in the wheat germ cell-free system reveals that the cell-free product immunoprecipitated with anti-cytochrome P-450 antibody has a molecular weight close to 47,000. In the case of 3-methylcholanthrene, the cell-free product immunoprecipitated with anti-cytochrome P-448 antibody shows a molecular weight around 59,000. Significant conversion of the 59,000 species to the 53,000 species can be demonstrated when the translation is carried out in the presence of microsomal membranes isolated from rat liver. Phenobarbital and 3-methylcholanthrene enhance the translatable messenger RNA contents for cytochrome P-450 and cytochrome P-448, respectively.

The cytochrome P-450 group constitutes an important class of structural proteins of membranes manifesting enzymatic functions leading to the hydroxylation of a variety of xenobiotics (1, 2). Despite the close similarity of these proteins in terms of their localization, function, and molecular weight range, it is becoming clear that cytochrome P-450 species are different proteins, which perhaps differ in their primary amino acid sequence, substrate specificity, immunological cross-reactivity, and inducibility by different classes of drugs (3–5). Although evidence has been provided for the *de novo* synthesis of the different species of cytochrome P-450 in response to different classes of drugs (6–9), the mechanism by which this is brought about is not clear. Actinomycin D blocks the increase in cytochrome P-450 content and synthesis due to phenobarbital (9, 10). Granting that the drugs induce the messenger RNA synthesis for the cytochrome P-450 group of proteins, the inducer specificity in terms of the induction of one or more species needs to be explained. One possibility is that the different cytochrome P-450 species are encoded in a polyprotein precursor that may undergo cleavage to give rise to the different species. Drugs may be able to influence the processing of the polyprotein precursor giving rise to a preponderance of one or more species. On the other hand the synthesis of the mature forms of cytochrome P-450 may not involve a polyprotein precursor, but may just involve the synthesis and translation of individual messenger RNAs giving rise to the different species with the option that each species may have its own precursor. To distinguish between these possibilities, the synthesis of cytochrome P-450 and cytochrome P-448 has been studied *in vivo* and also *in vitro* using the heterologous cell-free system derived from wheat germ.

EXPERIMENTAL PROCEDURES

Materials—ATP, GTP, creatine phosphate, creatine phosphokinase, and sodium lauryl sulfate were purchased from Sigma Chemical Co. L-[4,5-^{3}H]Leucine (105 Ci/mmol) was purchased from Amersham, Bucks, England. [^{14}C]*Chlorella* protein hydrolysate (27 Ci/matom C) was purchased from Brabra Atomic Research Center, Bombay. Emulgen 911 was a gift from KAO Atlas Co., Ltd., Japan.

Treatments of Animals—Rats (70 to 75 g) were injected with phenobarbital (80 mg/kg of body weight) or 3-methylcholanthrene (40 mg/kg of body weight) to induce cytochrome P-450 or cytochrome P-448, respectively.

Antibodies to Cytochrome P-450 and Cytochrome P-448—Cytochrome P-450 (phenobarbital inducible) and cytochrome P-448 (3-methylcholanthrene inducible) were purified from the livers of rats that received four injections of phenobarbital or 3-methylcholanthrene. The cytochromes were purified by the procedures described by Lu and Levin and Ryan *et al.* (11, 12), and the final purified preparations had a specific content of 15 to 16 nmol/mg of protein. Antibodies for the purified preparations were raised in rabbits, and IgG was prepared as described by Thomas *et al.* (13).

Cytochrome P-450 and Cytochrome P-448 Synthesis In Vivo—To study cytochrome P-450 (phenobarbital inducible) synthesis *in vivo* animals were injected with phenobarbital, and 12 h after the drug injection the animals were sacrificed. The animals received 30 μCi of [^{14}C]*Chlorella* protein hydrolysate 1 h before killing. A similar schedule was adopted to study cytochrome P-448 synthesis except that 3-methylcholanthrene was the inducer used. Microsomes were isolated from the livers, solubilized, and processed for immunoprecipitation and SDS^1-polyacrylamide gel electrophoresis.

Cell-free Synthesis of Cytochrome P-450 and Cytochrome P-448—For this purpose poly(A)-containing RNA was isolated from the livers of rats which had received a single injection of phenobarbital or 3-methylcholanthrene. The animals were killed 12 h after the drug injection, and polysomes were isolated from the livers by the magnesium precipitation method of Palmiter (14). The polysomes were lysed in 10 mM Tris-HCl buffer, pH 7.4, containing 0.5% SDS and 0.4 M NaCl and then passed directly through an oligo-dT cellulose column equilibrated with the same buffer (15). Poly(A)-containing RNA was eluted with Tris buffer (10 mM; pH 7.4) after extensive washing of the column with the loading buffer and precipitated with 2 volumes of ethanol. The poly(A)-containing RNA was subjected to a second cycle of oligo-dT cellulose chromatography. The final preparation contained about 10% of ribosomal RNA which appeared intact when analyzed on 2.5% polyacrylamide gels.

The poly(A)-containing RNA fractions were translated in a wheat germ cell-free system as described by Roberts and Paterson (16) using [^{3}H]leucine as the labeled amino acid precursor. The incubation mixture in 0.2 ml contained 0.06 ml (1 A_{260} unit) of the S_{30} fraction; 20 mM Hepes, pH 7.6; 2 mM dithiothreitol; 1 mM ATP; 20 μM GTP; 8

^{1} The abbreviations used are: SDS, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

mM creatine phosphate; 8 μg of creatine phosphokinase; 40 μM each of 19 unlabeled amino acids except leucine; 10 μCi of [³H]leucine (105 Ci/mmol); 80 mM KCl; 2.25 mM magnesium acetate; 300 μM spermidine; 40 μM phenylmethylsulfonyl fluoride and rat liver poly(A)-containing RNA. In some cases the endoplasmic reticulum membranes stripped free of ribosomes, prepared from control rat liver microsomes by the procedure described by Blobel and Dobberstein (17), were included at a concentration of 10 A_{260} units/ml of incubation mixture. The mixture was incubated at 25°C for 60 min. At the end of the incubation, 5-μl aliquots were transferred onto filter paper discs to measure incorporation into trichloroacetic acid-precipitable proteins and the rest used for immunoprecipitation after the addition of carrier antigen.

Immunoprecipitation Procedures—Microsomal samples were solubilized in 0.1 M sodium phosphate buffer, pH 7.4, containing 20% glycerol, 1 mM EDTA, and 1% sodium cholate. Samples from *in vitro* protein synthesis were made 1% with respect to cholate, and cold leucine was also added to 10 mM final concentration. The samples were clarified by centrifugation at 2500 rpm for 20 min. Immunoprecipitation was carried out in a final volume of 0.5 ml containing the clarified preparation, a 2-fold excess of anti-cytochrome P-450 or anti-cytochrome P-448 γ-globulin, 0.5% cholate, and 1 mM phenylmethylsulfonyl fluoride. The mixtures were incubated at 4°C overnight. The immunoprecipitates were collected by centrifugation, washed thrice with 1.0 ml of 0.1 M Tris-HCl buffer, pH 7.4, containing 0.05 M NaCl, 1% Triton X-100, and 0.5% deoxycholic acid. The precipitate was finally washed with 0.01 M Tris containing 0.05 M NaCl.

SDS-Polyacrylamide Gel Electrophoresis—The labeled immunoprecipitates were analyzed using 8% gels as described by Laemmli (18).

Radioactivity Measurements—Filter discs were counted using a 0.5% 2,5-diphenyloxazole (PPO) in toluene mixture in a Beckman LS-100 scintillation counter. In the case of gels, the slices were digested with H_2O_2 and counted using a 0.5% 2,5-diphenyloxazole in Triton-toluene mixture. Under the conditions used, ¹⁴C and ³H were counted with efficiencies of 35% and 5%, respectively.

RESULTS

Cytochrome P-450 and cytochrome P-448 were purified from microsomes of phenobarbital- and 3-methylcholanthrene-injected rat livers, respectively. SDS-polyacrylamide gel electrophoresis of phenobarbital-induced species showed a major band with a molecular weight of 47,000 along with two minor bands representing other species of cytochrome-P-450 (19, 20). In the case of 3-methylcholanthrene-induced species a major band with a molecular weight of 53,000 and a faint band corresponding to another cytochrome P-450 species were observed (21). Ouchterlony double diffusion analysis of the IgG fractions prepared from rabbit antisera for the two protein species revealed that anti-cytochrome P-450 antibody showed a strong reaction with the antigen and a weak cross-reaction with cytochrome P-448. Anti-cytochrome P-448 antibody reacted only with cytochrome P-448. Titration data revealed that at the equivalence point 1 mg of the IgG fraction of the two antibodies precipitated 45 μg and 32 μg of cytochrome P-450 and P-448, respectively.

Microsomes were labeled *in vivo* with [¹⁴C]*Chlorella* protein hydrolysate after a single injection of phenobarbital or 3-methylcholanthrene as described under "Experimental Procedures." The microsomes were then immunoprecipitated with anti-cytochrome P-450 antibody or anti-cytochrome P-448 antibody depending on the source of microsomes. The results presented in Fig. 1 indicate that in the case of phenobarbital-treated animals, anti-cytochrome P-450 antibody precipitates the 47,000 molecular weight species and a minor species corresponding to 53,000 molecular weight as described by Bhat (19). In the case of 3-methylcholanthrene-treated animals, anti-cytochrome P-448 antibody precipitates the 53,000 species, and there is no other detectable labeling of proteins in the cytochrome P-450 region. There is, however, some labeling of a protein with a molecular weight of 76,000. The reason for the detection of this protein in the immuno-
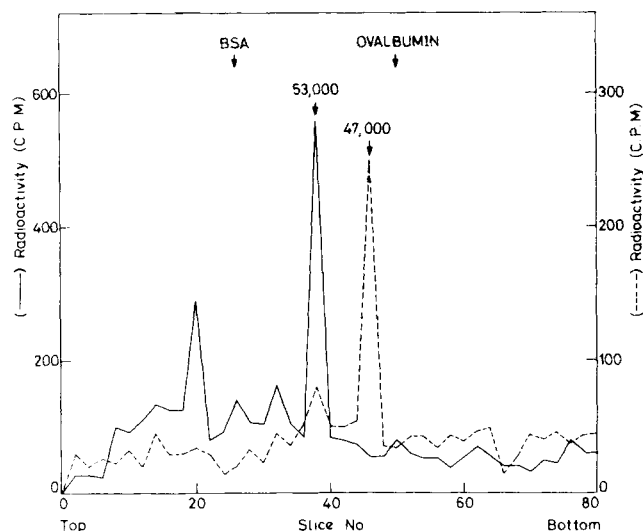

FIG. 1. **SDS-polyacrylamide gel electrophoresis of immunoprecipitates obtained from microsomes labeled *in vivo*.** Microsomes labeled *in vivo* were isolated from phenobarbital- and 3-methylcholanthrene-treated rats and were reacted with anti-cytochrome P-450 and anti-cytochrome P-448 antibody, respectively. The labeled immunoprecipitates from phenobarbital microsomes (- - -) and 3-methylcholanthrene microsomes (———) were subjected to SDS-gel electrophoresis, and the gels were sliced and processed for radioactivity measurements. *BSA*, bovine serum albumin.

precipitate is not clear, and it is unlikely that it is due to nonspecific precipitation. It is a possibility that the 76,000 molecular weight species may represent NADPH-cytochrome *c* reductase which can form a complex with cytochrome P-448, both of which are induced by 3-methylcholanthrene. However, a similar molecular weight species is not immunoprecipitated with the anti-cytochrome P-450 antibody.

Next the poly(A)-containing RNA fractions from phenobarbital- and 3-methylcholanthrene-treated animals were translated in the wheat germ system. The poly(A)-containing RNA fractions used stimulated protein synthesis by 8- to 10-fold. The Mg^{2+} and K^+ concentrations for optimal translation of total and immunoprecipitable products were 2.25 mM and 80 mM, respectively. Addition of exogenous poly(A)-containing RNA elicited a linear response up to a concentration of 50 μg/ml.

Fig. 2, *a* and *b*, depicts the mobilities of the labeled immunoprecipitable products synthesized in the wheat germ cell-free system on addition of poly(A)-containing RNA isolated from phenobarbital- or 3-methylcholanthrene-treated rats. In the case of phenobarbital treatment, a major species with a molecular weight of 47,000 is evident as observed by Bhat (19). In the case of 3-methylcholanthrene treatment, the major species has a molecular weight of 59,000, and there is hardly any labeling of the 53,000 species, which is the mature form of cytochrome P-448. When translation of poly(A)-containing RNA from 3-methylcholanthrene-treated rats is carried out in the presence of stripped microsomal membrane there is a significant decrease in the labeling of the 59,000 molecular weight species with a concomitant significant labeling of the 53,000 molecular weight species.

It may be pointed out that the addition of stripped microsomal membranes to the wheat germ system in the absence of exogenous poly(A)-containing RNA permits the synthesis of about 35% of the total proteins labeled in the presence of near saturating concentrations of poly(A)-containing RNA (50 μg/ml). This is perhaps due to the residual amount of messenger RNA still associated with the stripped membrane preparation. However, in the absence of exogenous poly(A)-containing
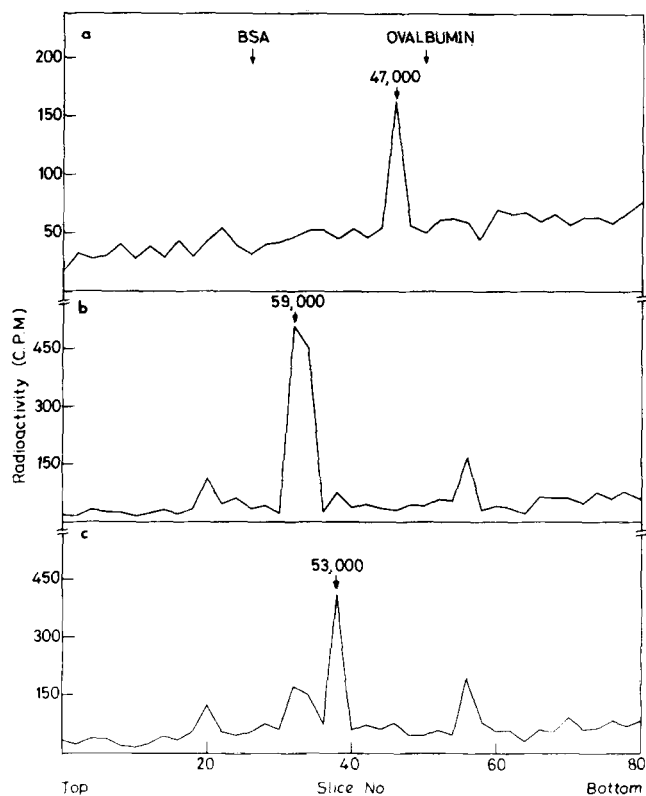
FIG. 2. **Synthesis of cytochromes P-450 and P-448 in the wheat germ cell-free system.** Poly(A)-containing RNA was isolated from liver polysomes obtained from phenobarbital-treated and 3-methylcholanthrene-treated rats. The RNA was translated in the wheat germ cell-free system in the presence and absence of rat liver microsomal membranes. The cell-free products were immunoprecipitated with the respective antibodies and analyzed using SDS-polyacrylamide gels. *BSA*, bovine serum albumin. *a*, poly(A)-containing RNA from phenobarbital-treated rats translated and products immunoprecipitated with anti-cytochrome P-450 antibody. *b*, poly(A)-containing RNA from 3-methylcholanthrene-treated rats translated and products immunoprecipitated with anti-cytochrome P-448 antibody. *c*, same as *b* except that translation was carried out in the presence of rat liver microsomal membranes isolated from control rats.

TABLE I

Effect of phenobarbital and 3-methylcholanthrene on the translatable messenger RNA contents for cytochrome P-450 and cytochrome P-448, respectively

Poly(A)-containing RNA (20 μg/ml) from normal, phenobarbital-treated, and 3-methylcholanthrene-treated rats was translated in the wheat germ cell-free system, and the products were immunoprecipitated with the appropriate antibodies, and the immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis. The area under the radioactivity peaks was calculated and given as a measure of synthesis of cytochrome P-450 or cytochrome P-448.

Source of poly(A)-containing RNA	Protein synthesis (cpm/0.5 ml of reaction mixture)		
	Total ($\times$ 10^{-4})	Cytochrome P-450	Cytochrome P-448
Control	20.0	2000	Not detectable
Phenobarbital treated	21.0	8000	
3-Methylcholanthrene treated	21.5		6000

RNA, the messenger RNA associated with the stripped membrane prepared from control rats does not contribute to detectable labeling of cytochrome P-448 or its precursor in the immunoprecipitate. The labeling of total trichloroacetic acid-precipitable proteins observed in the presence of exogenous

poly(A)-containing RNA plus the stripped membranes (38 $\times$ 10^4 cpm/ml) is close to that observed with exogenous poly(A)-containing RNA alone (40 $\times$ 10^4 cpm/ml). The lack of an additive effect in the former case may be due to the exogenous poly(A)-containing RNA being already present at a near saturating concentration rather than to any inhibitory effect of the stripped membranes on translation in the wheat germ system. It has also been found that the presence of phenylmethylsulfonyl fluoride is essential to obtain reproducible results in these studies.

Finally, the translatable cytochrome P-450 and cytochrome P-448 messenger RNA contents have been estimated using the wheat germ system under optimum conditions of translation. The results presented in Table I indicate that at an equivalent poly(A)-containing RNA concentration of 20 μg/ml, the RNA preparation from phenobarbital-treated animals stimulates cytochrome P-450 synthesis by almost 4-fold as observed by Bhat (19). There is hardly any detectable synthesis of cytochrome P-448 precursor when poly(A)-containing RNA from control animals was used. Therefore, the increase of cytochrome P-448 messenger RNA content after 3-methylcholanthrene treatment is at least several-fold.

DISCUSSION

Recent studies on the biosynthesis of a variety of "exportable" proteins, exported out of the cell or to other organelles within the cell, have shown the existence of precursor proteins which are processed to the mature forms while traversing the membranes (22). The cytochrome P-450 group of proteins in the endoplasmic reticulum falls into a different category, where the proteins synthesized on the ribosomes can have direct access to the endoplasmic reticulum membrane and become an integral part of the membrane. Since the heterologous cell-free systems lack the processing machinery, many of the precursor forms have been identified. The present results indicate that the major phenobarbital-inducible 47,000 molecular weight species is synthesized, perhaps directly, as the mature form. The 3-methylcholanthrene-inducible cytochrome P-448 species appears to be synthesized as a precursor of molecular weight 59,000. The ability of microsomal membranes to process this form into the mature 53,000 molecular weight species confirms the existence of the precursor. It has been observed that the NH_2-terminal region of cytochrome P-450 (23, 24) is quite similar to that of the signal peptide and perhaps this region need not be cleaved as the protein is retained in the endoplasmic reticulum. However, it is also likely that the hydrophobic NH_2-terminal region of cytochrome P-448 precursor may be long enough so that the signal peptide-like region is retained in the mature form even after the cleavage process. Processing of the integral proteins of the endoplasmic reticulum membrane would indicate that these proteins may not be integrated only at the site of their synthesis but may actually undergo lateral movement and become incorporated elsewhere in the membrane. This would mean that the endoplasmic reticulum would show an asymmetric regional distribution of the cytochrome P-450 group of proteins. Again, it cannot be categorically stated that the phenobarbital-inducible species does not involve the processing of a precursor, since it has been reported that heterologous cell-free systems such as the wheat germ and reticulocyte lysates may have a weak processing machinery (25). However, the present results clearly rule out the existence of a polyprotein precursor from which the different cytochrome P-450 group of proteins may arise by drug-mediated differential processing.

The results also indicate that both phenobarbital and 3-methylcholanthrene enhance the translatable messenger

RNA contents for the respective cytochrome P-450 species. While the phenobarbital-inducible messenger RNA species is detectable in normal microsomes, the 3-methylcholanthrene-inducible messenger species is not detectable by the technique used. On the basis that actinomycin D blocks the increase in the rate of synthesis of cytochrome P-450 due to phenobarbital (9), it appears reasonable to conclude that the drugs act at the level of transcription. It would be of considerable interest to know about the organization of the different cytochrome P-450 genes and to investigate whether the individual cytochrome P-450 genes are noncontiguous.

REFERENCES

1. Conney, A. H. (1967) *Pharmacol. Rev.* **19**, 317–366
2. Alvares, A. P., Parli, C. J., and Mannering, G. J. (1973) *Biochem. Pharmacol.* **22**, 1037–1045
3. Imai, Y., and Sato, R. (1966) *Biochem. Biophys. Res. Commun.* **23**, 5–11
4. Thomas, P. E., Lu, A. Y. H., Ryan, D., West, S. B., Kawalek, J., and Levin, W. (1978) *Mol. Pharmacol.* **12**, 746–758
5. Alvares, A. P., Schilling, G., Levin, W., and Kuntzman, R. (1967) *Biochem. Biophys. Res. Commun.* **29**, 521–526
6. Dehlinger, P. J., and Schimke, R. T. (1972) *J. Biol. Chem.* **247**, 1257–1264
7. Rajamanickam, C., Rao, M. R. S., and Padmanaban, G. (1975) *J. Biol. Chem.* **250**, 2305–2310
8. Haugen, D. A., Coon, M. J., and Nebert, D. W. (1976) *J. Biol. Chem.* **251**, 1817–1827
9. Bhat, K. S., and Padmanaban, G. (1978) *FEBS Lett.* **89**, 337–340
10. Marver, H. S. (1969) in *Microsomes and Drug Oxidations* (Gillette, J. R., Conney, A. H., Cosmides, G. J. Estabrook, E. W., Fouts, J. R., and Mannering, G. J., eds) pp. 495–515, Academic Press, New York
11. Lu, A. Y. H., and Levin, W. (1972) *Biochem. Biophys. Res. Commun.* **46**, 1334–1339
12. Ryan, D., Lu, A. Y. H., Kawalek, J., West, S. B., and Levin, W. (1975) *Biochem. Biophys. Res. Commun.* **64**, 1134–1141
13. Thomas, P. E., Lu, A. Y. H., Ryan, D., West, S. B., Kawalek, J., and Levin, W. (1976) *J. Biol. Chem.* **251**, 1385–1391
14. Palmiter, R. D. (1974) *Biochemistry* **13**, 3606–3615
15. Krystosek, A., Cawthon, M. L., and Kabat, D. (1975) *J. Biol. Chem.* **250**, 6077–6084
16. Roberts, B. E., and Paterson, B. M. (1973) *Proc. Natl. Acad. Sci. U. S. A.* **70**, 2330–2334
17. Blobel, G., and Dobberstein, B. (1975) *J. Cell Biol.* **67**, 852–862
18. Laemmli, U. K. (1970) *Nature* **227**, 680–685
19. Bhat, K. S. (1979) Ph.D. thesis, Indian Institute of Science, Bangalore
20. Craft, J. A., Cooper, M. B., and Rabin, B. R. (1978) *FEBS Lett.* **88**, 62–66
21. Guengrich, F. P. (1977) *J. Biol. Chem.* **252**, 3970–3979
22. Katz, F. N., Rothman, J. E., Lingappa, V. R., Blobel, G., and Lodish, H. F. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74**, 3278–3282
23. Haugen, D. A., Armes, L. G., Yasunobu, K. T., and Coon, M. J. (1977) *Biochem. Biophys. Res. Commun.* **77**, 967–973
24. Ozols, J., Gerard, C., Imai, Y., and Sato, R. (1978) *Fed. Proc.* **37**, 1759
25. Goldman, B. M., and Blobel, G. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75**, 5066–5070