Induction by phenobarbital of the mRNA for a specific variant of rat liver microsomal cytochrome *P*-450

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The treatment of rats for 4 days with phenobarbital causes an apparent 3-fold increase in the amount of total liver cytochrome *P*-450. By sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, metyrapone binding and immunoprecipitation, this increase was found to be due to a much larger increase in a restricted number of specific cytochrome *P*-450 variants. A radioimmunoassay technique demonstrated that the major phenobarbital-inducible variant, of molecular weight 52 000, is induced 24-fold by phenobarbital. Immunoprecipitation analysis of products of translation *in vitro* with an antibody specific to the 52 000-mol.wt. cytochrome *P*-450 showed that phenobarbital induces the mRNA in polyribosomes for this variant 20-fold. Evidence is presented for the action of phenobarbital at the transcriptional and translational levels.

Cytochromes *P*-450 are among the major constituent proteins of the liver microsomal mixed-function mono-oxygenases. They play a central role in the metabolism of steroids, the detoxification of drugs and xenobiotics and the activation of pro-carcinogens (Conney, 1967; Gelboin, 1967; Estabrook & Lindenlaub, 1979; Coon *et al.*, 1980). Information about the mechanisms involved in regulating these enzymes should lead to a better understanding of the regulation of drug detoxification and carcinogen activation. Furthermore, the process of induction of these enzymes can contribute to an understanding of the regulation of expression of genes coding for membrane-bound enzymes in eukaryotic cells.

Several different variants of cytochrome *P*-450 have been isolated from rat liver, which differ in molecular weight, substrate specificity, immunological reactivity and amino acid composition (Guengerich, 1978; Ryan *et al.*, 1979; Botelho *et al.*, 1979). Thus some of the variants must be different gene products. Cytochromes *P*-450 are induced by a variety of foreign compounds, such as

phenobarbital (Estabrook & Lindenlaub, 1979). The spectrum of enzyme specificity induced varies with the actual inducer used.

One of the most intensively studied systems of cytochrome *P*-450 induction in rat liver is that produced by phenobarbital. This substance induces at least two variants of cytochrome *P*-450: the major induced form, of mol.wt. 52 000, which preferentially hydroxylates testosterone at the 16 α position, and a minor species of mol.wt. 48 000, which catalyses the hydroxylation of testosterone at the 7 α position (Ryan *et al.*, 1979).

The mechanism of induction of cytochrome *P*-450 is not understood (reviewed by Rees, 1979). However, there is evidence indicating that synthesis of the enzymes *de novo* is involved (Dehlinger & Schimke, 1972; Rajamanickam *et al.*, 1975; Haugen *et al.*, 1976; Bhat & Padmanaban, 1978).

Short-term treatment of rats with phenobarbital *in vivo* produces a 2- or 3-fold increase in the amount of the mRNA species coding for cytochromes *P*-450 (Dubois & Waterman, 1979; Colbert *et al.*, 1979; Kumar & Padmanaban, 1980). A major question is whether the induction process specifically promotes the expression of a few genes, which are otherwise repressed, or simply increases the rate of expression of genes which are functioning in uninduced animals.

This paper describes experiments on the mechanism of phenobarbital induction of a specific rat liver microsomal cytochrome *P*-450 variant of mol.wt. 52 000. The results demonstrate that what appears as a small induction of total cytochromes *P*-450 on treatment with phenobarbital is actually due to a

Abbreviations used: SDS, sodium dodecyl sulphate; IgG, immunoglobulin G; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; poly(A)⁺ RNA, poly(A)-containing RNA; 52 K *P*-450, cytochrome *P*-450 of mol.wt. 52 000.

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relatively large induction of the mRNA species for a few specific variants. The information reported here shows that the cytochrome *P*-450 system is highly suitable for detailed investigation of the molecular biology of the induction of a eukaryotic membrane protein.

Materials and methods

Animals

Male Sprague-Dawley rats (200–250 g) were used in these experiments. For long-term induction studies animals were injected intraperitoneally once a day for 4 days with a 4% (w/v) solution of sodium phenobarbital in 0.9% NaCl at a dose of 40 mg/kg. For short-term induction studies, animals received a single intraperitoneal injection of a 10% solution of sodium phenobarbital in 0.9% NaCl (100 mg/kg) 6 h before use. When used, cordycepin (3'-deoxyadenosine; 0.3 mg/kg) was injected intraperitoneally 20 min before injection of phenobarbital. Control animals received injections of 0.9% NaCl. All animals were starved overnight before use.

Isolation of microsomal fractions

Postmitochondrial supernatants, smooth-microsomal vesicles and free polyribosomes were isolated as described by Blyth *et al.* (1971). Total microsomal membrane vesicles were prepared as follows. Postmitochondrial supernatant (23 ml) was layered over 20 ml of 2 M-sucrose/50 mM-Tris/HCl (pH 7.5)/25 mM-KCl/5 mM-MgCl₂. Samples were centrifuged at 120 000 *g* for 3½ h at 4°C. Microsomal vesicles were aspirated from the sucrose interface, diluted to 40 ml with 0.25 M-sucrose/50 mM-Tris/HCl (pH 7.5)/5 mM-MgCl₂ and pelleted by centrifugation at 120 000 *g* for 1 h at 4°C. The pellets were re-suspended in 0.25 M-sucrose/50 mM-Tris/HCl (pH 7.5)/5 mM-MgCl₂ and stored at -78°C.

Solubilization of microsomal membrane vesicles

For immunoprecipitation of cytochrome *P*-450 from membrane fractions, the microsomal fraction was solubilized in buffered sodium cholate by the method of Lu & Levin (1974). For immunoprecipitation competition experiments and radio-immunoassay, the microsomal membrane vesicles were solubilized by the method of Warner *et al.* (1978), except for the substitution of Renex 690 (Kao-Atlas Co., Tokyo, Japan) for Emulgen 911.

Determination of cytochrome *P*-450 content

Cytochrome *P*-450 was assayed by the CO-reduced difference-spectra method of Omura & Sato (1964). Protein concentrations were determined by the method of Lowry *et al.* (1951).

Metyrapone-binding spectral analysis

Spectra produced on binding of metyrapone [2-methyl-1,2-bis-(pyrid-3-yl)propan-1-one] to cyto-

chrome *P*-450 were determined by the method of Jonen *et al.* (1974) with a Perkin-Elmer model 556 double-wavelength spectrophotometer. Binding constants were calculated from double-reciprocal plots of the difference ($A_{446}-A_{490}$) against metyrapone concentration. The amount of phenobarbital-inducible cytochrome *P*-450 present in microsomal membrane fractions was calculated by using an absorption coefficient of 52 mm⁻¹·cm⁻¹ for the ligand-protein complex of the induced cytochrome (Luu-The *et al.*, 1980). From the linear relationship between the absorbance difference obtained from metyrapone-binding spectra and the absorbance at 450 nm of the CO complex of cytochrome *P*-450 for control microsomal fractions, an absorption coefficient of 21 mm⁻¹·cm⁻¹ was calculated for the metyrapone complex of non-inducible cytochrome *P*-450 [see Luu-The *et al.* (1980) for details of method of calculation]. This coefficient was used to determine the amount of non-inducible cytochrome *P*-450 in the microsomal membrane fraction from phenobarbital-treated and untreated animals.

Preparation of antibodies

52K *P*-450 purified from the total microsomal fractions of phenobarbital-treated rats was provided by Dr. M. B. Cooper and Miss M. Estall (Department of Biochemistry, University College London). The enzyme was used to raise antibodies in male New Zealand rabbits. The IgG fraction was isolated from antiserum by using a column of Bio-Rad DEAE Affi-gel Blue (as described in Bio-Rad Bulletin 1062). Control IgG was isolated from the antiserum of rabbits obtained before injection with 52K *P*-450. Rabbit anti-(rat albumin) serum was purchased from ICN Pharmaceuticals, Cleveland, OH, U.S.A., and the IgG fraction was isolated from it as described above.

Immunoprecipitation of cytochrome *P*-450 from solubilized microsomal-membrane vesicles

Membranes were solubilized as described above and clarified by centrifugation at 105 000 *g* for 60 min at 4°C. Samples of solubilized membrane protein (180 µg) were incubated with various amounts of anti-(25K *P*-450) IgG in the presence of 5% glycerol, 10 mM-potassium phosphate buffer (pH 7.5), 0.9% NaCl and 1 mM-phenylmethane-sulphonyl fluoride. Incubations were carried out at 37°C for 60 min, then at 4°C overnight. The antigen-antibody complex was removed by centrifugation at 10 000 *g* for 15 min. The cytochrome *P*-450 remaining in the supernatant was assayed as described above.

SDS/polyacrylamide-gel electrophoresis

Microsomal samples were diluted to a protein concentration of 1 mg/ml in 1% SDS/10 mM-EDTA/

10 mM-sodium phosphate buffer (pH 7.0)/1% 2-mercaptoethanol/15% (v/v) glycerol and electrophoresed on a 10% polyacrylamide slab gel containing SDS (Laemmli, 1970) as described previously (Phillips *et al.*, 1979). Proteins were stained in Kenacid Blue (BDH) as described previously (Phillips *et al.*, 1979).

Preparation of polyribosomes

The method used was essentially that of Kurtz & Feigelson (1977), except for the replacement of cell sap in the sucrose gradients by human placental ribonuclease inhibitor (isolated by the method of Blackburn, 1979) at a concentration of 0.006 A_{280} unit/ml. Polyribosome pellets were resuspended to a concentration of approx. 200 A_{280} units/ml with a Teflon pestle in 50 mM-Hepes (pH 7.4)/75 mM-KCl/5 mM-MgCl₂/3 mM-dithiothreitol containing 0.006 A_{280} unit of ribonuclease inhibitor/ml. Aggregated material was removed by centrifugation at 4000g for 5 min at 4°C and polyribosomes were stored in 25 μ l portions at -78°C. Storage under these conditions for several months resulted in no loss of translational activity.

RNA isolations

Cytoplasmic RNA. Postmitochondrial supernatants were prepared by a modification of the method of Sala-Trepat *et al.* (1978). Rat liver was minced and rinsed twice in ice-cold homogenization buffer [25 mM-Tris/HCl (pH 7.5)/25 mM-NaCl/5 mM-MgCl₂/140 mM-sucrose/50 mM-NH₄Cl/6 mM-2-mercaptoethanol/heparin (0.5 mg/ml)]. Liver was homogenized with a motor-driven Teflon/glass homogenizer for ten strokes in 10 vol. of homogenization buffer. Then $\frac{1}{3}$ vol. of 10% Triton X-100 was added and the mixture rehomogenized (four strokes). The homogenate was centrifuged at 1500g for 5 min at 4°C. The supernatant was adjusted to give a solution which was 1% with respect to sodium deoxycholate, homogenized by hand (two strokes) and centrifuged at 30000g for 10 min at 4°C. To the supernatant were added $\frac{1}{3}$ vol. of 10% SDS and $\frac{1}{3}$ vol. of 1 M-NaCl/100 mM-sodium acetate/10 mM-EDTA (pH 5.4). RNA was extracted with phenol/chloroform/3-methylbutan-1-ol and precipitated with ethanol as described by Stein *et al.* (1977). Heparin and DNA were removed by washing the precipitates twice with 3 M-sodium acetate (pH 6.0) (Palmiter, 1974). RNA was reprecipitated twice with ethanol from 0.25 M-potassium acetate (pH 7) and the final precipitate washed twice with 70% ethanol. RNA was resuspended to a concentration of approx. 5 mg/ml in 10 mM-Tris/HCl (pH 7.4)/1 mM-EDTA and stored in 10 μ l batches at -78°C. When electrophoresed in 2.7% polyacrylamide gels, RNA extracted by this method gave sharp 28S and 18S

peaks, indicating that little degradation took place during isolation.

Poly(A)⁺ mRNA. This was isolated from total cytoplasmic RNA as described by Bantle *et al.* (1976).

Polyribosomal RNA. Polyribosomes were prepared by the method of Palmiter (1974). RNA was extracted and washed as described above.

Translation in vitro

Polyribosomes, cytoplasmic RNA or poly(A)⁺ mRNA were translated *in vitro* in a rabbit reticulocyte lysate obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). The final composition of the translation incubation mixture was: 80% reticulocyte lysate, [³⁵S]-methionine (1 μ Ci/ μ l) (S.J. 204; The Radiochemical Centre) and either polyribosomes or RNA. Polyribosomes were translated at a final concentration of approx. 10 A_{260} units/ml. The incubation was at 30°C for 90 min. Unfractionated RNA and poly(A)⁺ RNA were translated in the same way, except that immediately before translation the RNA was heated at 68°C for 2 min, then rapidly chilled in a mixture of ice and water. The final concentration for unfractionated RNA was 40 μ g/ml and for poly(A)⁺ RNA 4 μ g/ml (unless stated otherwise).

Analysis of total translation products

Translation activity was determined by measuring acid-precipitable radioactivity by a modification of the method of Pelham & Jackson (1976), as described in the reticulocyte batch-analysis sheet provided by The Radiochemical Centre. The spectrum of polypeptides synthesized was determined by SDS/polyacrylamide-gel electrophoresis. A volume of the incubation mixture containing 50000 c.p.m. was adjusted to give 2% SDS, 5% 2-mercaptoethanol, 10 mM-EDTA, 30% glycerol and 0.01% Bromophenol Blue. The mixture was heated at 100°C for 3 min. Samples were centrifuged at 10000g for 2 min and electrophoresed on SDS/polyacrylamide gels. After electrophoresis, gels were washed in 40% methanol/10% acetic acid for 1 h and then overnight in 7% acetic acid. Gels were incubated in 3 vol. of Enhance (New England Nuclear) for 1 h, washed in water for 1 h, dried and radioautographed at -78°C with Fuji Rx X-ray film.

Immunoprecipitation

Translation products (5×10^5 c.p.m. unless stated otherwise) were made 10% with respect to sodium cholate, centrifuged at 10000g for 10 min and diluted to 500 μ l with 1% Triton X-100 in NET buffer [150 mM-NaCl/5 mM-EDTA/50 mM-Tris/HCl (pH 7.4)/0.02% NaN₃/2 mM-methionine]. The appropriate antibody, either pre-immune, anti-(serum

albumin or anti-(52 K *P*-450) IgG (30 μ g in 5 μ l), was added and the mixture incubated at 30°C for 30 min, then at 4°C overnight. Fixed *Staphylococcus aureus* cells (50 μ l of a 10% suspension; see below) were added and samples were shaken at room temperature for 60 min. The fixed cells were pelleted by centrifugation at 10000 *g* for 30 s and washed in 3 $\times$ 1 ml of 0.1% SDS/10 mM-Tris/HCl (pH 7.4)/2 mM-EDTA/2 mM-methionine (Maccacchini *et al.*, 1979). The cells were resuspended in 50 μ l of 6% SDS/150 mM-Tris/HCl (pH 6.8)/6 mM-EDTA/30% glycerol/0.01% Bromophenol Blue and heated at 100°C for 3 min. The cells were removed by centrifugation and the supernatants were analysed by electrophoresis on SDS/polyacrylamide gels.

Staph. aureus cells

Fixed cells, prepared as described by Kessler (1975), were obtained from Michelle Ginsberg of the Imperial Cancer Research Fund Laboratories (Lincoln's Inn Fields, London) and stored at -78°C. The cells were pelleted, resuspended in 1% Triton X-100/NET buffer and incubated at room temperature for 15 min. Cells were washed once in 0.05% Nonidet P-40 (Shell Chemical Co.)/NET buffer and resuspended in this buffer containing 2 mM-methionine and ovalbumin (1 mg/ml). Cells were used within 24 h.

Determination of immunoprecipitates

Radioactive bands were located on gels by fluorography. A copy of the fluorogram was placed over the gel and those portions of the gel that corresponded to the radioactive bands of interest were excised and rehydrated in 0.4 ml of water. Soluene-350 (Packard) (1 ml) was added and the slices were incubated at room temperature overnight. Scintillation fluid (15 ml) containing 4 g of 2,5-diphenyloxazole and 0.05 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene/litre of toluene was added and radioactivity was determined by liquid-scintillation spectrometry. The counting efficiency for 35 S was 54%.

Radioimmunoassay of 52 K *P*-450

35 S-labelled 52 K *P*-450 was synthesized by using the reticulocyte-lysate translation system and polyribosomes from phenobarbital-treated animals. Samples of this containing 10⁶ c.p.m. (total acid-precipitable radioactivity) were mixed with various quantities of the detergent-solubilized sample being analysed. An amount of anti-(52 K *P*-450) IgG (3.75 μ g) sufficient to precipitate 50% of the 35 S-labelled 52 K *P*-450 was added to each sample together with 30 μ g of pre-immune IgG. 52 K *P*-450 was immunoprecipitated, electrophoresed and measured as described above. The data are expressed as a percentage of the amount of 35 S-labelled

52 K *P*-450 immunoprecipitated in the absence of any competitor.

Results

Effect of phenobarbital on rat liver microsomal and cytoplasmic proteins

Rat livers from phenobarbital-treated and untreated rats were homogenized and fractionated into postmitochondrial supernatants, total microsomal vesicles, smooth microsomal vesicles and free polyribosomes. The subcellular fractions were analysed by SDS/polyacrylamide-gel electrophoresis (Fig. 1), which showed that phenobarbital treatment does not have a great effect on the general protein complement of the postmitochondrial supernatant (tracks *b* and *c*); however, the amounts of a few proteins are specifically increased by phenobarbital treatment, the most prominent of which are species of 170 000, 52 000 and 50 000 mol.wt. The 170 000-mol.wt. species is not apparently present in

Fig. 1. SDS/polyacrylamide-gel electrophoresis of cytoplasmic and microsomal proteins from livers of phenobarbital-induced and non-induced animals

Subcellular fractions were isolated from the livers of induced and uninduced rats and diluted to a protein concentration of 1 mg/ml in 1% SDS/10 mM-EDTA/10 mM-sodium phosphate buffer (pH 7.0)/1% 2-mercaptoethanol/15% glycerol. After boiling for 3 min, 25 μ g of each sample was electrophoresed on a 10% polyacrylamide gel containing SDS. The gels were stained with Kenacid Blue. (*a*) Molecular-weight markers; (*b*) and (*c*) postmitochondrial supernatant; (*d*) and (*e*) total microsomal-membrane proteins; (*f*) and (*g*) smooth-microsomal-membrane proteins; (*h*) and (*i*) proteins obtained from polyribosomes. Samples in tracks (*b*), (*d*), (*f*) and (*h*) were from untreated animals and those in tracks (*c*), (*e*), (*g*) and (*i*) were from phenobarbital-treated animals. Numbers give molecular weights of the marker proteins ($\times 10^{-3}$). Arrows indicate 170 000- and 52 000-mol.wt. species induced by phenobarbital.

either of the microsomal fractions (tracks *d-g*) or in polyribosomes (tracks *h* and *i*) and is presumably a cell-sap protein. The possibility that it is a nuclear protein en route from its site of biosynthesis in the cytoplasm cannot, of course, be ruled out. The 52000-mol.wt. species is present in all the microsomal fractions (tracks *d-g*), but not in polyribosomes (tracks *h* and *i*), and thus appears to be a microsomal-membrane protein. It corresponds in molecular weight to the major phenobarbital-inducible form of cytochrome P-450 found by ourselves and by Ryan *et al.* (1979). The 50000-mol.wt. species also appears to be a microsomal membrane protein, possibly epoxide hydratase (Pickett *et al.*, 1980).

Phenobarbital induction of cytochrome(s) P-450

The total amounts of cytochrome P-450 in liver microsomal vesicles isolated from phenobarbital-treated and untreated animals were assayed by measuring the difference spectra of the CO complexes of the reduced cytochrome (Table 1). Phenobarbital increased the specific content of total cytochromes P-450 2-fold when expressed per mg of microsomal protein and 3-fold when expressed per g of liver. From the concentrations of cytochrome P-450 determined in these two ways it is possible to calculate the phenobarbital induction of microsomal-membrane protein (approx. 1.5-fold) (Table 1). Gel-electrophoretic analysis of microsomal proteins (Fig. 1) indicates that the proliferation of microsomal membranes after phenobarbital treatment probably involves a small, more or less uniform, induction of the majority of microsomal proteins, together with a larger induction of a small number of proteins, of which 52 K P-450 is the most pronounced.

Before investigating the mechanism of cytochrome P-450 induction by phenobarbital, we have used an indirect method to attempt to measure the relative amounts of phenobarbital-inducible and

non-inducible cytochromes P-450 in liver microsomal vesicles of induced and uninduced rats. It has been reported that the cytochrome(s) P-450 induced by phenobarbital in rat liver microsomal vesicles have a greater affinity for metyrapone than do those species of cytochrome P-450 not induced (Kahl *et al.*, 1974; Luu-The *et al.*, 1980). When microsomal vesicles from phenobarbital-treated rats were titrated with low concentrations of metyrapone ($<10\mu\text{M}$), the A_{446} increased with ligand concentration, corresponding to the titration of a limited number of high-affinity sites (Fig. 2*a*). At higher metyrapone concentrations a small secondary increase was observed, which could correspond to binding of lower affinity (Fig. 2*b*). A binding constant of $0.6\mu\text{M}$ was determined for the 'high-affinity' cytochrome(s) P-450 from the initial region of the biphasic binding curve, and a value of $80\mu\text{M}$ was determined for the 'low-affinity' cytochrome(s) P-450 from the secondary region of the curve. The binding constant of $80\mu\text{M}$ is close to that determined by the titration of control microsomal vesicles with metyrapone ($70\mu\text{M}$) (Fig. 2*b*), and it is reasonable to assume that the low-affinity binding derives from the presence of control (i.e. non-induced) cytochrome(s) P-450 in the microsomal vesicles of phenobarbital-treated rats. On the basis of the above observations, two metyrapone concentrations, $10\mu\text{M}$ and 1mM , were selected to estimate the relative amounts of induced and non-induced cytochrome(s) P-450 in the microsomal vesicles of phenobarbital-treated and control animals. In Fig. 3, the magnitude of the difference spectra produced in the presence of $10\mu\text{M}$ -metyrapone is a measure of the phenobarbital-inducible cytochrome(s) P-450, whereas the additional spectral increase observed in the presence of 1mM -metyrapone is a measure of the non-phenobarbital-inducible cytochrome(s) P-450. From these spectral differences it was estimated (see the Materials and methods section) that the phenobarbital-inducible forms of cytochrome P-450 comprised 11% and 53% of total cytochrome P-450 in

Table 1. Induction of total cytochromes P-450 by phenobarbital

Total microsomal membrane vesicles (microsomes) were isolated from the liver of phenobarbital-treated and untreated rats. Cytochrome P-450 was assayed by CO difference spectra. Microsomal membrane protein per g of liver was calculated by dividing the value for the specific content of cytochrome P-450 expressed in nmol/g of liver by that expressed in nmol/mg of protein.

		Total microsomes (control) (A)	Total microsomes (induced) (B)	Induction (B/A)
Specific content of cytochrome P-450	Units			
	nmol/mg of protein	0.82	1.64	2
	nmol/g of liver	12.67	37.4	2.95
Total microsomal membrane protein	mg/g of liver	15.5	22.8	1.47

Fig. 2. Titration with metyrapone of cytochrome P-450 in liver microsomal vesicles isolated from control or phenobarbital-treated rats

The $A_{446} - A_{490}$ produced on binding of low (a) or high (b) concentrations of metyrapone to microsomal membrane vesicles isolated from control (○) or phenobarbital-treated (●) rats is shown. Arrowheads indicate K_d values, which were calculated from double-reciprocal plots of the data in this Figure. These values are $0.6 \mu M$ for high-affinity binding to microsomal vesicles from phenobarbital-treated rats, and $70 \mu M$ and $80 \mu M$ for the low-affinity binding to microsomal vesicles from control and phenobarbital-treated rats respectively.

non-induced and induced animals respectively. Since the total cytochrome P-450 increases 2-fold on induction, there is, on this basis, a 10-fold increase of phenobarbital-inducible cytochrome(s) P-450/mg of membrane protein. Since phenobarbital treatment also results in a 1.5-fold increase in microsomal vesicles, the induction per g of liver of the specific forms is about 15-fold. Thus it would appear that the

Fig. 3. Metyrapone binding to membranes from phenobarbital-induced and non-induced animals

Difference spectra produced on metyrapone binding to cytochrome P-450 in the microsomal vesicles from induced (a) and control (b) animals were determined in the presence of either (—) $10 \mu M$ - or (---) $1 mM$ -metyrapone. Dashed line (---) represents the difference spectra in the absence of metyrapone.

effect of phenobarbital is to increase the amounts of specific variants of the enzymes about 15-fold, and this gives rise to a 3-fold increase in the total cytochrome(s) P-450. The sum of the amounts of inducible and non-inducible cytochrome(s) P-450 measured by metyrapone binding was 96% of the total amount of cytochrome P-450 measured by the CO-difference spectra. Thus internally consistent results are obtained by the use of metyrapone to measure the amount of the various forms of cytochrome P-450 in microsomal-membrane fractions.

Although the results obtained from metyrapone-binding spectra indicate that phenobarbital induces a specific variant(s) of cytochrome P-450, we cannot be certain of the number of different proteins that contribute to the spectral changes on metyrapone

binding and we cannot relate the results directly to the induction of a specific variant of cytochrome P-450. As a first step towards further characterization we prepared an IgG fraction that was specific for the 52 000-mol.wt. cytochrome P-450 induced by phenobarbital (52 K P-450). Immunotitration experiments showed that at equivalence 1 mg of anti-(52 K P-450) IgG precipitated 220 µg of purified 52 K P-450. The extent of induction of the 52 K P-450 was estimated by determining the percentage of total cytochrome P-450 immunoprecipitated by anti-(52 K P-450) IgG from the liver microsomal vesicles of phenobarbital-treated and untreated animals. Solubilized microsomal vesicles were incubated with increasing amounts of anti-(52 K P-450) IgG, and the quantity of cytochrome P-450 remaining in the supernatants was determined by CO difference-spectral analysis (Table 2). Approx. 55% of the total cytochromes P-450 was immunoprecipitable by the anti-(52 K P-450) IgG in microsomal vesicles from phenobarbital-treated animals, whereas the same quantity of IgG precipitated only about 10% of the cytochromes P-450 from control microsomal vesicles. No cytochrome P-450 was precipitated from either of the microsomal preparations by control IgG from non-immunized rabbits. These results are consistent with those obtained by metyrapone binding and suggest that phenobarbital induces a 52 000-mol.wt. variant of cytochrome P-450 which is present in only low amounts in microsomal vesicles from untreated animals. These results are confirmed below by rigorous quantification using radioimmunoassay.

Effect of phenobarbital on translation in vitro

As a first step towards investigating the induction

process, we examined the effect of phenobarbital on the translatable mRNA species in polyribosomes. The total population of polyribosomes was isolated from the livers of phenobarbital-treated and untreated rats. Phenobarbital treatment for 4 days resulted in a large increase in liver weight (55%), but the amount of polyribosomal RNA per g of liver increased only slightly (12%). Translation of the polyribosomes in a reticulocyte cell-free system showed that the relative translational efficiencies of the polyribosomes were 3.5×10^5 and 2.6×10^5 d.p.m./µg of RNA from induced and non-induced animals respectively. Using a shorter period of phenobarbital treatment, Colbert *et al.* (1979) and Gonzalez & Kasper (1980) also found an increase in the translational efficiency of polyribosomes *in vitro*.

To determine if phenobarbital treatment resulted in a specific increase in the amount of translatable mRNA for individual proteins, the translation products were electrophoresed on SDS/polyacrylamide gels. Fig. 4 shows a radioautograph obtained by fluorography of the dried gel. The overall patterns of protein synthesis directed by polyribosomes from control and induced animals are very similar. Both sets of polyribosomes contain mRNA coding for a great number of polypeptides of up to 200 000 mol.wt. However, the messengers for certain specific polypeptides are clearly induced by phenobarbital. The most prominent of these are labelled on the right-hand side of the gel and correspond to molecular weights of 27 000, 52 000 and 170 000. The 52 000-mol.wt. species synthesized *in vitro* corresponds in molecular weight to the major form of cytochrome P-450 induced by phenobarbital (see Fig. 1). The identity of the other induced proteins is not known. Although the 27 000-mol.wt. species has

Table 2. Immunoprecipitation of 52 K P-450 from solubilized total microsomal membrane vesicles

Solubilized liver microsomal membrane vesicles (microsomes; 180 µg of protein) from either phenobarbital-treated or untreated rats were incubated with various amounts of anti-(52 K P-450) IgG. The anti-(52 K P-450) IgG used in this experiment was not isolated from the same rabbit as that used for all other experiments. The antigen-antibody complex was removed by centrifugation and the cytochrome P-450 remaining in the supernatant was determined by CO difference spectra. The results are expressed as a percentage, with 100% representing the amount of cytochrome P-450 remaining in the supernatant in the absence of antibody. Abbreviation: ND, not determined.

	Cytochrome P-450 (% remaining in supernatant)	
	Total microsomes (control)	Total microsomes (induced)
Anti-(52 K P-450) IgG (mg)		
0	100	100
3.9	98	ND
6.1	91	95
7.5	40	100
10.0	51	88
Non-immune IgG (mg)		
10.0	100	100

Fig. 4. SDS/polyacrylamide-gel electrophoresis demonstrating the induction of translatable 52K P-450 mRNA in rat liver polyribosomes by phenobarbital

Total polyribosomes were isolated from the livers of phenobarbital-treated and untreated rats. Polyribosomes were incubated at a concentration of $10 A_{260}$ units/ml in a rabbit reticulocyte-lysate translation system (The Radiochemical Centre) containing [35 S]methionine. For analysis of total translation products (tracks *h* and *i*) samples containing 5×10^4 acid-precipitable c.p.m. were adjusted to 2% SDS, 5% 2-mercaptoethanol, 10 mM-EDTA, 30% glycerol, 0.01% Bromophenol Blue and electrophoresed on a 10% polyacrylamide gel containing SDS. For immunoprecipitation analysis (tracks *a-f*) samples containing 5×10^5 acid-precipitable c.p.m. were incubated with 30 μ g (5 μ l) of pre-immune IgG (tracks *a* and *b*), anti-(serum albumin) IgG (tracks *c* and *d*) or anti-(52K P-450) IgG (tracks *e* and *f*). Antibody-antigen complexes were bound to *Staph. aureus* cells, eluted and electrophoresed; the gel was dried and fluorographed. Translation products were from polyribosomes isolated from control (tracks *a*, *c*, *e* and *h*) or induced (tracks *b*, *d*, *f* and *i*) animals. Molecular-weight markers were run in track (*g*): numbers give molecular weights ($\times 10^{-3}$).

a molecular weight similar to that of ligandin (a protein induced by phenobarbital; Fleischner *et al.*, 1972), it has been reported that the induction of ligandin does not occur via an increase in the amount of its mRNA (Daniel *et al.*, 1977). From Figs. 1 and 4 it is clear that the effect of phenobarbital on the polypeptide composition of the postmitochondrial supernatant *in vivo* corresponds closely to its effect on amounts of translatable polyribosomal mRNA assayed *in vitro*, suggesting that the induction of the 170 000- and 52 000-mol.wt. polypeptides is accompanied by an increase

in the amounts of their mRNA molecules in polyribosomes.

Phenobarbital induction of translatable mRNA for 52K P-450 in polyribosomes

To determine whether the 52K P-450 is synthesized by the translation system *in vitro*, the translation products were incubated with anti-(52K P-450) IgG and the antigen-antibody complex was bound to *Staph. aureus* cells. The bound antigen was eluted from the cells and electrophoresed in an SDS/polyacrylamide gel. Anti-(52K P-450) IgG precipitated only one polypeptide species (Fig. 4, track *f*) from the many synthesized by polyribosomes from phenobarbital-induced animals (Fig. 4, track *i*), and this has a mol.wt. of 52 000, strongly suggesting that the precipitated species is indeed 52K P-450. This suggestion is supported by several control experiments. The 52 000-mol.wt. protein was not precipitated from translational products of polyribosomes from phenobarbital-treated animals by either pre-immune IgG or by anti-(serum albumin) IgG (Fig. 4, tracks *b* and *d*). Purified 52K P-450 competed with the translation product for the anti-(52K P-450) IgG binding sites, whereas no competition was observed in controls with ovalbumin (Fig. 5) or with serum albumin. Thus the 52 000-mol.wt. protein has antigenic determinants common to those of 52K P-450. It was also found that proteins from smooth vesicles from phenobarbital-treated rats competed with the 52 000-mol.wt. protein for anti-(52K P-450) IgG far more efficiently than did proteins from untreated animals (Fig. 6). This confirms that the 52K P-450 that is immunoprecipitated from translation products *in vitro* by anti-(52K P-450) IgG is also induced *in vivo* by phenobarbital treatment.

Having established that the mRNA for 52K P-450 is present in polyribosomes isolated from the livers of phenobarbital-treated rats and is translated in a translation system *in vitro*, the specific anti-(52K P-450) IgG/*Staph. aureus* immunoprecipitation method was used to measure the amounts of this mRNA in the liver polyribosomes of phenobarbital-treated and untreated animals. 52K P-450 was immunoprecipitated from the translation products of polyribosomes from phenobarbital-treated and control rats and electrophoresed in an SDS/polyacrylamide gel. The radiolabelled protein was detected on the gel by fluorography (Fig. 4, tracks *e* and *f*). Fig. 4 shows that phenobarbital treatment resulted in a large induction of mRNA for 52K P-450 in rat liver polyribosomes. To quantify this induction, the band of radioactivity corresponding to the 52 000-mol.wt. protein was excised from the gel and the radioactivity was determined by liquid-scintillation spectrometry. The amount of radioactivity in the 52 000-mol.wt. region of a gel of

Fig. 5. Immunoprecipitation of 52 K *P*-450 from translation products *in vitro* in the presence of ovalbumin or purified cytochrome *P*-450

Polyribosomes from the livers of phenobarbital-treated rats were incubated in a reticulocyte-lysate system containing [35 S]methionine. Samples containing 5×10^5 acid-precipitable c.p.m. were mixed with stated quantities of purified 52 K *P*-450 (●) or ovalbumin (○) and incubated with 30 μg (5 μl) of anti-(52 K *P*-450) IgG. Immunoprecipitates were isolated by the *Staph. aureus* method and analysed by SDS/polyacrylamide-gel electrophoresis and fluorography. Regions of the gel corresponding to 52 000 mol.wt. were excised and radioactivity was determined. Results are expressed as a percentage of the radioactivity present in this region, with 100% representing the value in the absence of any added competitor.

translation products immunoprecipitated with pre-immune-serum IgG was taken as background, and this value was subtracted from those obtained with anti-(52 K *P*-450) IgG. The radioactivity incorporated into the 52 K *P*-450 is assumed equivalent to the amount of its translatable mRNA present in the polyribosomes. Calculation of the amount of 52 K *P*-450 mRNA as a percentage of the total mRNA (measured by acid-precipitable radioactivity in the total translation products) enables the quantification of the specific induction of this mRNA. 52 K *P*-450 represents 0.03% of the proteins synthesized by control polyribosomes and 0.53% of those synthesized by polyribosomes from phenobarbital-induced animals. Thus the amount of 52 K *P*-450 mRNA in polyribosomes was specifically induced 18-fold by phenobarbital treatment. Phenobarbital treatment had no effect on the amount of serum albumin mRNA on polyribosomes (Fig. 4, tracks *c* and *d*), in agreement with the results of Gonzalez & Kasper (1980).

The determination of the amount of 52 K *P*-450

Fig. 6. SDS/polyacrylamide-gel electrophoresis of translation products *in vitro* immunoprecipitated by anti-(52 K *P*-450) IgG in the presence and absence of proteins derived from smooth microsomal vesicles of induced and non-induced animals

Polyribosomes from the livers of induced rats were incubated in a reticulocyte-lysate system containing [35 S]methionine. Samples containing 5×10^5 acid-precipitable c.p.m. were mixed with 5 (*a* and *h*), 10 (*b* and *i*), 25 (*c* and *j*), 50 (*d* and *k*), 100 (*e* and *l*) or 200 μg (*f*) of solubilized liver microsomal-membrane proteins from control (*a*–*f*) or phenobarbital-treated (*h*–*l*) rats. No competitor was added to sample in track (*g*). Protein mixtures were incubated with 30 μg of anti-(52 K *P*-450) IgG. Immunoprecipitates were isolated by the *Staph. aureus* method and analysed by SDS/polyacrylamide-gel electrophoresis and fluorography. Molecular-weight markers were run in track (*m*); numbers give molecular weights ($\times 10^{-3}$).

mRNA as a percentage of total mRNA depends on the assumption that the relative proportion of methionine in 52 K *P*-450 and in the total translation products is similar. The percentage of the amino acids in 52 K *P*-450 induced by phenobarbital that are methionine is 2.4% (Botelho *et al.*, 1979) and this is similar to both the average content of methionine found in sequenced mammalian proteins and that predicted from codon frequencies (King & Jukes, 1969). Even if this is not the case, the calculated induction of 52 K *P*-450 mRNA probably would not differ, owing to the similarity of the total translation products of mRNA from control and phenobarbital-treated animals. A similar extent of induction of this mRNA was found when [3 H]leucine was used as the radiolabel.

Quantification of 52 K *P*-450 mRNA in cytoplasmic poly(A) $^{+}$ RNA

The large induction of translatable 52 K *P*-450 mRNA found in polyribosomes could be due, in

part, to a difference in the translational efficiency of the mRNA in intact polyribosomes. There is the further possibility that 52 K *P*-450 mRNA could be present in the post-polyribosomal cytoplasmic fraction of untreated liver cells and not detected. To investigate these possibilities, the amount of translatable 52 K *P*-450 mRNA in cytoplasmic poly(A)⁺ RNA was assayed.

Translation of poly(A)⁺ mRNA in the reticulocyte lysate was linear with respect to RNA concentration up to 8 µg/µl and the translational efficiency was 6.25×10^6 d.p.m./µg of RNA. Translation products were incubated with anti-(52 K *P*-450) IgG and *Staph. aureus* cells, and the immunoprecipitate was electrophoresed in an SDS/polyacrylamide gel. Fig. 7 shows that anti-(52 K *P*-450) IgG precipitated a 52000-mol.wt. polypeptide from the translation products of poly(A)⁺ mRNA isolated from induced, but not from control, animals. No proteins were precipitated by control IgG (Fig. 7, tracks *b* and *k*). Varying the amount of

poly(A)⁺ mRNA caused parallel changes in the amount of immunoprecipitated 52 K *P*-450 (Fig. 7, tracks *g*–*j*) and total translation products. Thus the ratio of 52 K *P*-450 synthesis to total protein synthesis remained constant (0.5%) over the range of poly(A)⁺ mRNA concentrations tested.

These results confirm that the difference observed in the amount of 52 K *P*-450 produced by translation of polyribosomes from phenobarbital-treated and untreated animals is due to different amounts of the specific mRNA for 52 K *P*-450. It is also clear that the poly(A)⁺ mRNA for this protein is not present in the cytoplasm of untreated cells in the form of stored, untranslated ribonucleoprotein complexes at significant amounts.

Quantification of 52 K P-450 mRNA in unfractionated polyribosomal and cytoplasmic RNA

It is also necessary to exclude the possibility of differential recovery of poly(A)⁺ 52 K *P*-450 mRNA [e.g. because of variation in poly(A) chain length]. To check this, unfractionated polyribosomal and cytoplasmic RNA species were isolated from the livers of induced and control animals, translated in the reticulocyte cell-free system and the translation products immunoprecipitated as described above. As found for the products of translation *in vitro* of polyribosomes and poly(A)⁺ mRNA, control IgG did not precipitate any proteins, and anti-(52 K *P*-450) IgG precipitated only the 52000-mol.wt. protein from the translation products of RNA from phenobarbital-treated, but not untreated, animals (Fig. 8). Thus the observed induction of 52 K *P*-450 mRNA is not an artifact caused by differential poly(A)⁺ mRNA recovery, and therefore no (or very little) 52 K *P*-450 mRNA (in a form that is translatable *in vitro*) is present in the cytoplasm or polyribosomes of control rat liver cells. Phenobarbital treatment results in the specific substantial induction of this mRNA.

Effect of an inhibitor of transcription on the phenobarbital induction of 52 K P-450 mRNA in polyribosomes

We examined the ability of cordycepin (3'-deoxyadenosine), a terminator of mRNA synthesis (Darnell *et al.*, 1971; Beach & Ross, 1978), to block the initial induction of 52 K *P*-450 mRNA. The injection of cordycepin 20 min before phenobarbital caused the 52 K *P*-450 mRNA present 6 h after phenobarbital injection to decrease from 0.08% to 0.06% of the total polyribosomal mRNA (a decrease of 25%, results not shown). This difference was found to be reproducible for several immunoprecipitation experiments. Cordycepin had no effect on the translational efficiency of polyribosomes *in vitro*, nor on the amount of translatable serum albumin mRNA. Even though optimal experimental con-

Fig. 7. SDS/polyacrylamide-gel electrophoresis of immunoprecipitated translation products demonstrating the induction by phenobarbital of 52 K *P*-450 mRNA in isolated rat liver cytoplasmic poly(A)⁺ RNA

Poly(A)⁺ mRNA was isolated from the cytoplasmic RNA of the livers of induced and uninduced rats. It was incubated in a reticulocyte lysate system containing [³⁵S]methionine at RNA concentrations of 0.05 (*c* and *g*), 0.1 (*d* and *h*), 0.2 (*b*, *e*, *i* and *k*) or 0.3 µg/ml (*f* and *j*). Translation products were from cytoplasmic poly(A)⁺ RNA obtained from control (tracks *b*–*f*) or induced (*g*–*k*) rats, or from globin mRNA (Miles) (track *l*). A 20 µl portion of translation products was incubated with 30 µg of pre-immune IgG (tracks *b* and *k*) or anti-(52 K *P*-450) IgG (tracks *c*–*j* and *l*). Immunoprecipitates were isolated by the *Staph. aureus* method and analysed by SDS/polyacrylamide-gel electrophoresis and fluorography. Molecular-weight markers were run in tracks (*a*) and (*m*); numbers give molecular weights of the marker proteins ($\times 10^{-3}$).

Fig. 8. SDS/polyacrylamide-gel electrophoresis of immunoprecipitated translation products demonstrating the induction by phenobarbital of 52K *P*-450 mRNA in total polyribosomal and cytoplasmic RNA

RNA was extracted from the cytoplasmic (tracks *a* and *b*) or polyribosome (tracks *c*–*e*) fraction of the livers from control (tracks *a* and *c*) or induced (tracks *b*, *d* and *e*) rats. RNA was incubated in a reticulocyte-lysate translation system containing [35 S]methionine; 3×10^5 acid-precipitable c.p.m. were incubated with 30 μ g of pre-immune IgG (track *e*) or anti-(52K *P*-450) IgG (tracks *a*–*d*). Immunoprecipitates were isolated by the *Staph. aureus* method and analysed by SDS/polyacrylamide-gel electrophoresis and fluorography. Molecular-weight markers were run in track (*f*); numbers give molecular weights of markers ($\times 10^{-3}$).

Fig. 9. Radioimmunoassay of 52K *P*-450 in rat liver smooth-membrane vesicles isolated from phenobarbital-treated and untreated animals

Translation products (1×10^6 acid-precipitable c.p.m.) of polyribosomes isolated from the livers of phenobarbital-treated rats were mixed with stated quantities of unlabelled purified 52K *P*-450 (●), solubilized smooth-microsomal-membrane proteins isolated from the livers of phenobarbital-treated (○) or untreated (■) rats, or ovalbumin (Sigma) (□). Protein mixtures were incubated in a final volume of 500 μ l with an amount of anti-(52K *P*-450) IgG (3.75 μ g) sufficient to precipitate 50% of the labelled protein in the absence of competitor. Immunoprecipitates were isolated by the *Staph. aureus* method and analysed by SDS/polyacrylamide gel electrophoresis and fluorography. The 52000-mol.wt. bands were removed from the gel and radioactivity was determined. The data are expressed as the percentage of the 35 S-labelled 52K *P*-450 precipitated in the absence of competitor.

ditions for the cordycepin experiment were not established, the results demonstrate that the induced increase in the translatable 52K *P*-450 mRNA is due, at least in part, to events at the level of transcription. The extent of the mRNA induction 6h after a single injection of phenobarbital was only approx. 3-fold (0.03 to 0.08% of total mRNA), compared with the 18-fold induction obtained after four daily injections, and is similar to the extent of induction after short-term treatment found by Colbert *et al.* (1979) and Kumar & Padmanaban (1980).

Radioimmunoassay of 52K *P*-450

The highly specific immunoprecipitation of radio-labelled 52K *P*-450 from the translation products of polyribosomes from induced animals (see above) permitted the direct use of this system for the quantification of the 52K *P*-450 by a radioimmunoassay. The assay was capable of detecting 52K *P*-450 in amounts as low as 2 ng (35 fmol) and was sensitive up to 200 ng (3.5 pmol) (Fig. 9). This assay was used to quantify 52K *P*-450 in smooth-microsomal vesicles isolated from livers of induced and unin-

duced animals (Fig. 9). This form of cytochrome *P*-450 represented 0.6% of the protein and 11% of the total cytochrome *P*-450 of the control membranes, and 9.6% of the protein and 86% of the total cytochrome *P*-450 of the membranes from phenobarbital-treated animals. There is thus approximately a 16-fold induction of the 52K *P*-450 (expressed per unit of microsomal-membrane protein). If the increase in microsomal proteins caused by phenobarbital (1.5-fold) is also taken into account, the total induction of the 52K *P*-450 (per g of liver) is about 24-fold. This confirms the results reported above, based on metyrapone binding to membrane vesicles and antibody titration of solubilized microsomal proteins. Clearly the major phenobarbital-inducible form of cytochrome *P*-450 is present in relatively low amounts in microsomal vesicles-derived from the livers of untreated rats. It is also clear that the protein precipitated from the translation products *in vitro* corresponds to a microsomal-membrane protein that is induced by phenobarbital *in vivo*. The 24-fold induction by phenobarbital of the 52K *P*-450 protein *in vivo* corresponds well to the 18-fold induction of the

mRNA coding for this protein (Fig. 4). Thus the induction of this protein by phenobarbital is due to an increase in the amount of its mRNA in the cytoplasm.

Discussion

The 3-fold induction of rat liver microsomal total cytochrome *P*-450 observed after administration of phenobarbital for 4 days was found to be due to relatively large increases in the amounts of specific variants of the enzyme, together with a small general increase of total microsomal-membrane proteins. The latter finding is in agreement with results obtained from electron microscopy (Ernster & Orrenius, 1965; Orrenius *et al.*, 1965; Stäubli *et al.*, 1969). The major phenobarbital-inducible cytochrome *P*-450 variant is a species of mol.wt. 52000. By using a radioimmunoassay, the amount of this variant was found to be increased 24-fold by phenobarbital. This assay is more sensitive than radial immunodiffusion, the only other method used so far to quantify accurately specific cytochromes *P*-450 (Thomas *et al.*, 1979), and provides a generally applicable, easy, rapid, sensitive and accurate method for the determination of specific cytochromes *P*-450.

The results obtained from SDS/polyacrylamide-gel electrophoresis, metyrapone binding and radioimmunoassay suggest that the phenobarbital induction of 52 K *P*-450 involves enzyme synthesis *de novo*. A similar conclusion has been reached by Dehlinger & Schimke (1972), Rajamanickam *et al.* (1975), Haugen *et al.* (1976) and Bhat & Padmanaban (1978), using labelling studies *in vivo*. Immunoprecipitation analysis of the translation products *in vitro* of polyribosomes isolated from the livers of phenobarbital-treated rats showed that the phenobarbital-inducible 52 K *P*-450 is probably synthesized as the mature polypeptide, which does not require to undergo extensive post-translational modifications. This is consistent with the location of this variant on the cytosolic face of the membrane (Bar-Nun *et al.*, 1980). Similar results have been found for phenobarbital-induced cytochromes *P*-450 (Dubois & Waterman, 1979; Bhat & Padmanaban, 1979; Colbert *et al.*, 1979; Bar-Nun *et al.*, 1980) and for other induced microsomal membrane proteins such as epoxide hydratase (Pickett *et al.*, 1980) and NADPH-cytochrome *c* (*P*-450) oxidoreductase (Gonzalez & Kasper, 1980). Our data do not preclude the possible presence of a retained 'signal' peptide sequence on the *N*-terminus of the 52 K *P*-450 molecule.

The 24-fold induction of 52 K *P*-450 after prolonged treatment with phenobarbital occurs through a 20-fold increase in its mRNA in polyribosomes. This is a much larger increase than that

observed by ourselves and others (Dubois & Waterman, 1979; Colbert *et al.*, 1979; Kumar & Padmanaban, 1980) after short-term treatment with phenobarbital. The induction is highly selective, and there is no effect on the amount of serum albumin mRNA (in agreement with the results of Gonzalez & Kasper, 1980). No, or very little, 52 K *P*-450 mRNA was found in the poly(A)⁺ RNA or unfractionated RNA isolated from control animals. This virtually eliminates the possibility that this mRNA is present in the cytoplasm of control cells as a stored ribonucleoprotein particle from which it is released on phenobarbital treatment. Although we cannot completely rule out the possibility that the induction of the 52 K *P*-450 mRNA may be due, in part, to a change in the translational efficiency of this mRNA relative to that of total mRNA (perhaps through some structural change such as modification of the 5' non-coding region), our evidence strongly indicates that induction is at the level of transcription and/or processing of mRNA. This is consistent with previous studies *in vivo* using inhibitors of transcription (Conney & Gilman, 1963; Orrenius *et al.*, 1965; Nebert & Gielen, 1971; Vainio *et al.*, 1974; Jacob *et al.*, 1974; Bhat & Padmanaban, 1978). Although there is evidence that phenobarbital does not increase the activity of RNA polymerase II (Lindell *et al.*, 1977), an increased activity of this enzyme would not be required for a selective change in transcription.

There are also effects of phenobarbital which operate at the translational level, including an increase in the number of cytoplasmic ribosomes and in the general efficiency of protein synthesis measured *in vitro*. A similar increase in protein synthesis caused by phenobarbital was reported *in vivo* by Venkatesan & Steele (1975). It has also been reported that phenobarbital may increase the stability of mRNA (Cohen & Ruddon, 1971; Venkatesan & Steele, 1975; Lindell *et al.*, 1977). Thus phenobarbital probably acts at several different levels to increase the synthesis of 52 K *P*-450. The net result of these different actions is the selective induction of a small number of proteins (both microsomal and non-microsomal), and there must be considerable specificity in the control mechanisms. All our evidence indicates that one aspect of the action of phenobarbital on the rat liver is to promote the expression of a small number of genes that are otherwise only minimally expressed. Further studies of the mechanisms of induction of 52 K *P*-450 require the availability of a cloned complementary-DNA probe for the mRNA.

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