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INDUCTION OF CYTOCHROME P-450 BY PHENOBARBITAL IS MEDIATED AT THE LEVEL OF TRANSCRIPTION

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Abstract—We have previously shown that the 43-fold induction by phenobarbital of the major phenobarbital-inducible cytochrome P-450 of rat liver microsomal membranes (PB P-450) is mediated by a 20-fold increase in the amount of its mRNA in the cytoplasm. Here we demonstrate that the induction of the mRNA can be almost entirely accounted for by an increase in the rate of transcription of genes coding for PB P-450, and involves little or no change in the rates of processing, transport or degradation of the mRNA. Phenobarbital treatment resulted in no amplification or rearrangement of PB P-450 genes.

The cytochrome P-450-mediated† mixed function monooxygenases are of great importance in the metabolism of steroid hormones and xenobiotics, including many drugs and carcinogens [1, 2]. The enzyme system is present in many tissues in a wide range of organisms [3]. Individual organisms usually possess a multiplicity of cytochromes P-450 [4–6] many of which are selectively inducible by a variety of xenobiotics. Our group is investigating the molecular mechanisms involved in the induction process.

In previous studies we found, by radioimmunoassay techniques, that the 2–3-fold increase in the total content of cytochromes P-450 in rat liver microsomal membranes after phenobarbital treatment is due to a >40-fold increase in the amount of a particular cytochrome P-450 (PB P-450) [7]. The induction is mediated by a 22-fold increase in the amount of translatable mRNA coding for PB P-450 [8]. Using a cloned cDNA [*p*P450(1)] coding for a PB P-450 isozyme designated P-450e(UC) we demonstrated that the induction in the translatable mRNA was due to a difference in the amount of PB P-450 mRNA and not to a change in its translatability [9]. Here we investigate the level at which the induction of PB P-450 mRNA by phenobarbital is controlled.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats (180–200 g body wt) bred at University College Animal Facility were used in these experiments. Treatment of animals with sodium phenobarbital was as described previously [7]. Animals were starved overnight before use and killed by cervical dislocation.

Preparation of nuclear and cytoplasmic RNA. Nuclei were isolated by the method of Derman *et al.* [10] except for the inclusion of 0.05% (w/v) Triton X-100 in the homogenization buffer. Cytoplasmic RNA was extracted from the first supernatant by a phenol/chloroform method [11]. Purified nuclei were resuspended in 0.14 M NaCl, 1.5 mM $MgCl_2$, 10 mM Tris.Cl (pH 8.6), 0.5% (w/v) Nonidet P40 (BDH Chemicals Ltd.) containing 10 mM vanadyl ribonucleoside (BRL) and RNA was extracted by the method of Favaloro *et al.* [12] as modified by Maniatis *et al.* [13]. Oligodeoxyribonucleotides were removed by washing the nucleic acid pellets in 20% sodium acetate. Pellets were resuspended in 50 mM Tris.Cl (pH 7.5), 1 mM EDTA and reprecipitated with ethanol. RNA was resuspended in sterile water.

RNA hybridization analysis. For Northern blot hybridization RNA was denatured in glyoxal, electrophoresed as described by McMaster and Carmichael [14] and transferred to a nitrocellulose filter [15]. For dot hybridization RNA dissolved in H_2O was denatured by heating at 100° for 2 min then chilled in an ice/H_2O mixture [15]. RNA was serially diluted in sterile H_2O and 2 μl samples applied to a dry nitrocellulose filter [15]. Filters were wetted in 5 × SSPE for 5 min and prehybridized for 3–24 hr at 42° in 50% (v/v) formamide, 5 × SSPE, 5 × Denhardt's solution [16], 0.1%(w/v) SDS, denatured salmon sperm DNA (200 μg/ml) (100 μl cm^{-2} of filter). The solution was replaced by hybridization buffer: 50% formamide, 5 × SSPE, 2 × Denhardt's, 0.1% SDS, denatured salmon sperm DNA (100 μg/ml), 9% (w/v) dextran sulphate, (50 μl/cm^2 of filter) and incubation was continued at 42° for 1 hr. The plasmid *p*P450(1), radiolabelled by nick-translation [13] to a specific activity of 10^7–10^8 cpm/μg with [α-^{32}P]*d*ATP (800 Ci/mmole, Amersham International), was denatured and added to a final concentration of 8 ng/ml. Filters were hybridized at 42° for ~40 hr. Filters were washed for 2 × 10 min in each of the following: 2 × SSPE, 0.1% SDS (room

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† Abbreviations: PB P-450, the major phenobarbital-inducible cytochrome P-450 of rat liver microsomal membranes and its immunochemically indistinguishable microheterogeneous variants; SSC, 0.15 M NaCl, 15 mM sodium citrate (pH 7.5); SSPE, 0.18 M NaCl, 10 mM sodium phosphate (pH 7.7), 1 mM EDTA; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

temp.); 0.1 × SSPE, 0.1% SDS (room temp.); 0.1 × SSPE, 0.1% SDS (50°). Filters were autoradiographed at −78° with an intensifying screen.

Transcription of nuclei in vitro. To minimise the possibility of introducing nicks into DNA, nuclei were isolated by a method that utilizes buffers containing no divalent cations [17]. Levels of endogenous mRNA sequences were reduced by treatment of crude nuclei with RNase A before purification [17]. Purified nuclei were incubated at 26° in a reaction volume of 50–100 μl containing 100 mM Tris.Cl (pH 7.9), 50 mM NaCl, 0.4 mM EDTA, 0.1 mM phenylmethylsulphonylfluoride, 1.2 mM dithiothreitol, 4 mM $MnCl_2$, 1 mM GTP, 1 mM ATP, 1 mM CTP, 25% (v/v) glycerol, 10 mM creatine phosphate, human placental RNase inhibitor [18] (0.006 A_{280} units/ml) and 2.4 mM [^{3}H]UTP (42 Ci/mmole) or 2.5 μM [α-^{32}P]UTP (760 Ci/mmole, New England Nuclear). Concentration of nuclei and length of incubation were as indicated in the figure and table legends. Reactions were terminated by addition of DNase I to 20 μg/ml and further incubation at 26° for 5 min. Transcriptional activity was determined by measuring incorporation of radioactivity into trichloroacetic acid-precipitable material. RNA was isolated by the method of Groudine *et al.* [19].

Hybridization of nuclear transcripts to immobilized DNA. Plasmid DNAs were isolated [20] and purified by CsCl density gradient centrifugation [13]. Plasmid DNA, in 2 M NaCl, 0.2 M NaOH, was heated at 100° for 1 min and chilled quickly on ice. Denatured DNA (1 μg in 3 μl) was spotted on to a 40 mm "Zeta probe" blotting membrane (Bio Rad) that had been presoaked first in H_2O, then in 20 × SSC and finally dried. Following application of DNA, filters were air dried then baked *in vacuo* at 80° for 2 hr. Three filters containing immobilized *p*AT153, *p*P450(1) or *p*9F1 were placed in the same 2.5 ml polypropylene tube (Nunc). The filters were prehybridized, and hybridized at 42° for 72 hr in a volume of 200 μl to ^{32}P-labelled RNA isolated from nuclei transcribed *in vitro*. Buffers were as described in the section "RNA hybridization analysis". Filters were then washed individually in 2 ml of each of the following: 2 × SSPE, 0.1% SDS (2 × 10 min at room temp.); 2 × SSPE (2 × 10 min at room temp.); 2 × SSPE, RNase A (10 μg/ml), RNase T1 (1 μg/ml) (30 min at 37°); 0.1 × SSPE, 0.1% SDS (2 × 10 min at room temp., 2 × 10 min at 50°). Hybridized RNA was eluted by incubating filters in 2 × 200 μl of H_2O at 90–100° for 10 min. The radioactivity of the eluted RNA was determined by liquid scintillation spectrometry after addition of 10 ml of Aquasol-2 scintillation fluid (New England Nuclear).

DNA hybridization analysis. Livers were excised, minced and frozen in liquid nitrogen. Frozen liver (~1 g) was mixed with pellets of solid CO_2 and ground to a fine powder in an electric coffee grinder. High mol. wt DNA was isolated essentially by the method of Blin and Stafford [21]. Southern blotting was performed using the method of Southern [22]. For dot blots DNA was denatured and applied to a nitrocellulose filter as described by Collins and Groudine [23]. Filters were prehybridized, hybridized, washed and autoradiographed as described in the section "RNA hybridization analysis".

RESULTS

Induction by phenobarbital of the steady state concentration of PB P-450 mRNA sequences in rat liver nuclei

Analysis by Northern blot hybridization demonstrated that in the cytoplasm of rat liver cells PB P-450 mRNA is encoded by a single mRNA size class of ~2100 nucleotides (Fig. 1, lane a) [24]. The mature mRNA is also present in rat liver nuclei isolated from phenobarbital-treated animals (Fig. 1, lanes b and c). Although the major PB P-450 mRNA species present in nuclei corresponds in size to the mature cytoplasmic mRNA, PB P-450 coding sequences are also contained in both larger and smaller RNA species. Presumably these RNAs are nuclear precursors to PB P-450 mRNA or breakdown products of the mature mRNA. The PB P-450 mRNA species are far less abundant in nuclei isolated from the livers of untreated rats (Fig. 1, lanes d and e).

The extent of induction by phenobarbital of PB P-450 mRNA species in the nuclei was determined by RNA dot hybridization. Various amounts of RNA extracted from nuclei isolated from livers of phenobarbital-treated or untreated rats were hybridized to [^{32}P] *p*P450(1) as described in "Materials and Methods" (Fig. 2). The intensity of the hybridization signals was measured by Scanning densitometry (Joyce Loebl Chromoscan 3) and plotted against the amount of RNA. The hybridization signal was linear with respect to the amount of total RNA applied to the filter. A comparison of the slopes of the lines generated showed that phenobarbital treatment resulted in a 20-fold induction of PB P-450 mRNA sequences in rat liver nuclei (Fig. 2B). This is the same extent of induction by phenobarbital of PB P-450 mRNA in the cytoplasm, measured by either RNA dot hybridization (Fig. 2D) or solution hybridization [9].

Effect of phenobarbital treatment on the transcription of PB P-450 genes

Nuclei were isolated from the livers of phenobarbital-treated or untreated rats and transcribed *in vitro* in a buffer containing [^{3}H]UTP as outlined in "Materials and Methods". The time course of incorporation of [^{3}H]UTP into trichloroacetic acid-precipitable material was very similar for both preparations of nuclei (Fig. 3). Transcription was directly proportional to the concentration of nuclei over the entire time course. The transcriptional activity of nuclei isolated in buffers devoid of divalent cations was 10-fold greater than that of nuclei isolated by a standard procedure [10] that involved buffers containing divalent cations (data not shown). Thus, for subsequent experiments, nuclei prepared by the former method were used.

To determine whether the induction by phenobarbital of PB P-450 mRNA sequences in rat liver nuclei involved an increase in the transcription of genes coding for PB P-450, we estimated the relative transcription of PB P-450 genes in nuclei isolated from the livers of phenobarbital treated or untreated rats. This was done by comparing the concentration of nascent PB P-450 transcripts in these nuclei. Nascent transcripts were radiolabelled with [^{32}P]

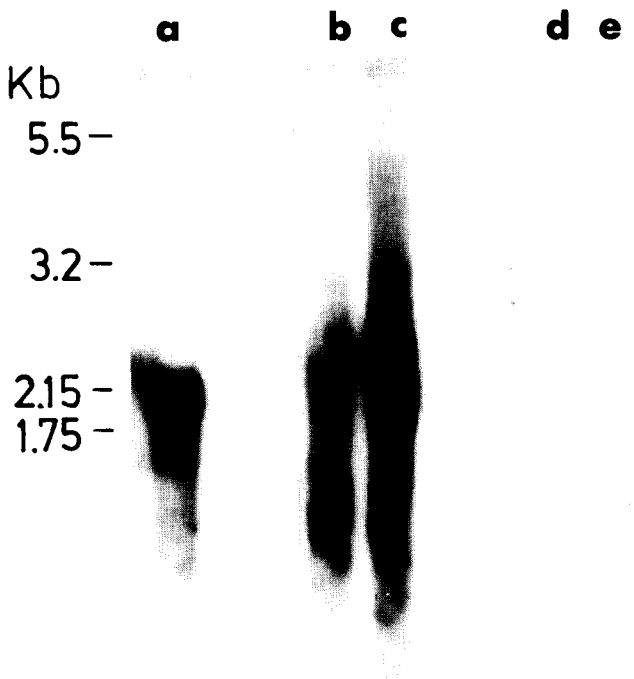

Fig. 1. Northern blot hybridization analysis of PB P-450 mRNA sequences in cytoplasm and nucleus. Cytoplasmic poly(A)$^+$ RNA was isolated from the livers of phenobarbital-treated rats (a). Total nuclear RNA was isolated from livers of phenobarbital-treated (b,c) or untreated rats (d,e). RNA was denatured with glyoxal, elctrophoresed through a 1% agarose gel and blotted to a nitrocellulose filter. The filter was hybridized with [^{32}P]*p*P450(1). Amounts of RNA loaded were 2 μg (a), 10 μg (b), 50 μg (c,d) 100 μg (e). Numbers give the size of RNA standards in kilobases.

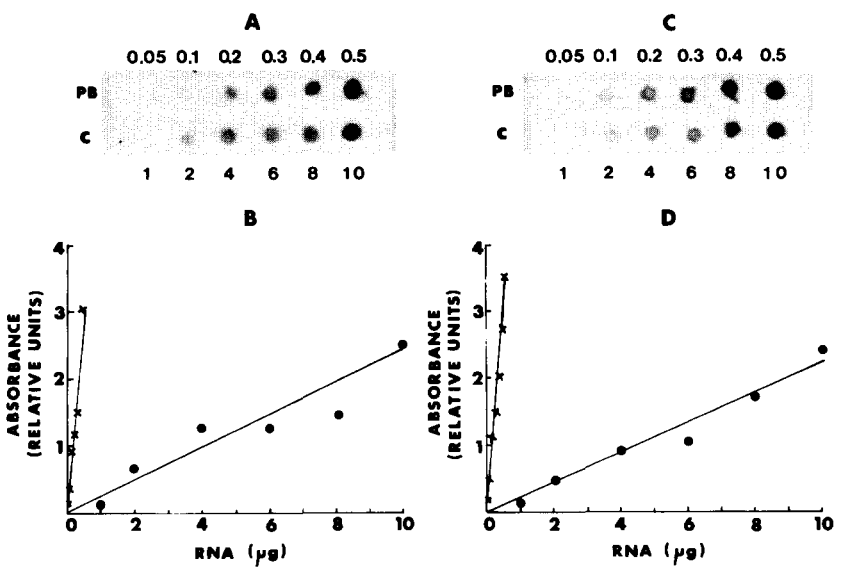

Fig. 2. Induction of PB P-450 mRNA sequences in cytoplasm and nucleus. RNA was extracted from nuclei (A,B) or cytoplasm (C,D) isolated from the livers of phenobarbital-treated (PB) or control (C) rats. Amounts of RNA in μg (as indicated) were applied to nitrocellulose filters and hybridized to [^{32}P] *p*P450(1). A and C represent autoradiograms of the hybridized filters. B and D show plots of the intensity of absorbance (measured by scanning densitometry) of the spots in A and C, respectively, against amount of RNA from phenobarbital-treated (×) or control (●) rats.

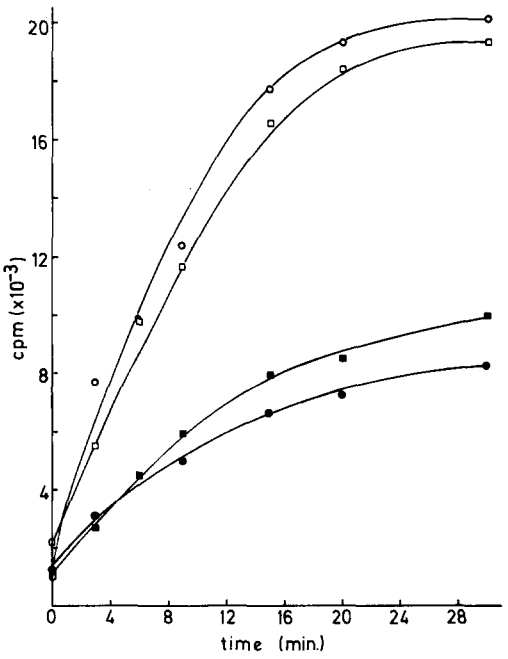

Fig. 3. Time course of transcription of nuclei *in vitro*. Nuclei were isolated from the livers of phenobarbital-treated (■, □) or untreated (●, ○) rats. Nuclei (4×10^7/ml ●, ■; or 10^8/ml, ○, □) were incubated for the times indicated in a buffer containing [^{3}H]UTP. Incorporation of [^{3}H]UTP into trichloroacetic acid insoluble material was measured.

UTP and isolated as described. RNA was hybridized to unlabelled filter bound *p*P450(1) DNA. Nuclei isolated from the livers of phenobarbital treated rats had a 16-fold greater concentration of nascent PB P-450 transcripts than did nuclei from the livers of untreated animals (Table 1). Thus treatment of rats with phenobarbital caused a substantial increase in the transcription of PB P-450 genes. As a control a nitrocellulose filter, to which had been bound *p*9F1 (a plasmid containing a cDNA insert coding for a rat liver protein that is not a cytochrome P-450) was included in the same hybridization tubes. Phenobarbital treatment had no effect on the transcription of genes coding for sequences present in the plasmid *p*9F1 (Table 1). Increasing the amount of radiolabelled RNA in the hybridization reaction resulted in a proportionate increase in the radioactivity bound to the filters (Table 1). This demonstrates that the filter-bound plasmid DNA was in excess and that over the range of nuclear RNA used the radioactivity bound to *p*P450(1) was proportional to the amount of nascent PB P-450 mRNA transcripts. The removal of endogenous nuclear RNA by pre-treatment of nuclei with ribonuclease A eliminated the possibility of competition between pre-existing unlabelled nuclear RNA and radioactively labelled nascent transcripts for hybridization to filter bound DNA. The treatment caused no reduction in the transcriptional activity of the nuclei (data not shown).

Phenobarbital treatment results in no amplification or rearrangement of PB P-450 genes

DNA dot hybridization analysis was used to compare the relative number of PB P-450 genes in nuclei isolated from the livers of phenobarbital treated or untreated rats (Fig. 4A). The results demonstrate that phenobarbital treatment caused no change in the number of genes coding for PB P-450. Analysis by southern blot hybridization showed that the number and pattern of restriction fragments that hybridized to *p*P450(1) was identical for DNA isolated from the livers of phenobarbital-treated or untreated rats (Fig. 4B). Thus phenobarbital treatment resulted in no rearrangement in the organization of PB P-450 genes.

DISCUSSION

In this paper we report the investigation of the molecular mechanism by which phenobarbital induces PB P-450 in rat liver microsomal membranes. The results are summarized in Table 2. PB P-450 mRNA sequences are induced by phenobarbital to the same extent in nuclei as in the cytoplasm. This indicates that the increase in the amount of the mRNA in the cytoplasm involves little or no change in its rate of transport from the nucleus or in its stability in the cytoplasm. The nuclear transcription system we have used does not support re-initiation of transcription *in vitro*. Consequently the amount of radioactive label incorporated into nascent PB P-450 transcripts is a measure of the steady-state number of elongating endogenous RNA polymerase

Table 1. Transcription of PB P-450 genes in rat liver nuclei *in vitro*. Liver nuclei from phenobarbital-treated (PB) or control (C) rats were incubated at a concentration of 3×10^8/ml for 15 min at 26° as outlined in Materials and Methods. [^{32}P]-labelled nascent transcripts were hybridized to filter bound plasmid DNA

Recombinant plasmid	[^{32}P] Nuclear RNA	Input cpm($\times 10^{-6}$)	cpm bound*	Transcription† (ppm)	Fold induction
*p*P450(1)	PB	4.36	1280	294	16
*p*P450(1)	PB	2.18	647	297	16
*p*P450(1)	C	4.25	86	19	
*p*P450(1)	C	2.12	38	18	
*p*9F1	PB	4.36	250	57	–
*p*9F1	PB	2.18	93	43	–
*p*9F1	C	4.25	216	51	
*p*9F1	C	2.12	70	33	

* Corrected for radioactivity that bound to *p*AT153.
† Expressed as ppm of total input cpm that bound to the plasmid DNA.

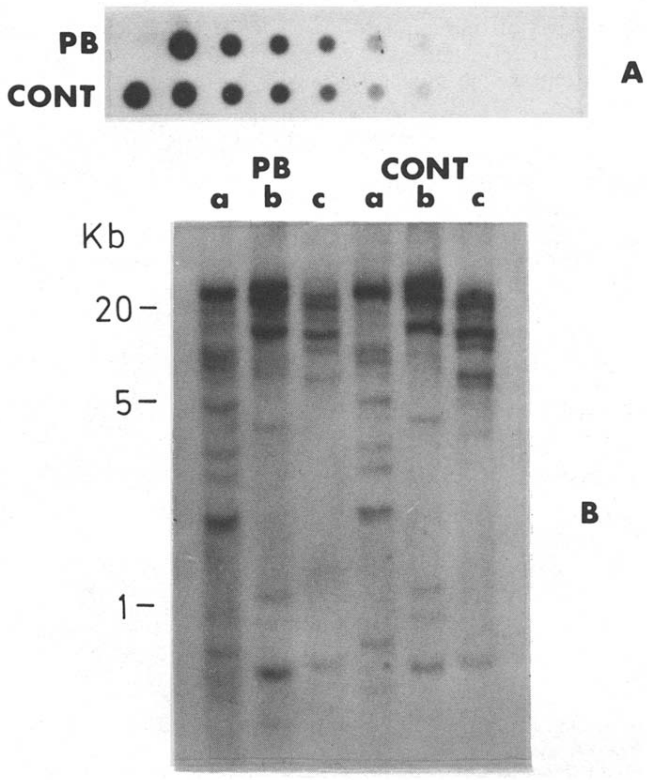

Fig. 4. Effect of phenobarbital on amplification or rearrangement of PB P-450 genes. DNA was isolated from the livers of phenobarbital-treated (PB) or control (CONT) rats and analysed by DNA dot (A) or Southern blot (B) hybridization to [^{32}P]*p*P450(1). (A) DNA was serially diluted (1 : 1) from 4 μg down (CONT) or 2 μg down (PB) and applied to the filter. (B) DNA (10 μg) was digested with *EcoRI* (a), *BamHI* (b) or *Hind*111 (c) and electrophoresed through a 0.8% agarose gel. DNA was blotted to a nitrocellulose filter. Filter was hybridized to [^{32}P]*p*P450(1) and autoradiographed.

II molecules on the PB P-450 gene(s) at the time at which the nuclei were isolated. Our results indicate that phenobarbital treatment caused a 16-fold increase in the number of RNA polymerase molecules engaged in the transcription of PB P-450 genes. Similar results have been obtained using a cytochrome P-450 cDNA clone whose sequence was not reported [25]. Assuming that there is no change in the rate of elongation *in vitro*, this suggests that the increase in the amount of PB P-450 mRNA in the cytoplasm is mediated by a 16-fold increase in the rate of initiation of transcription of PB P-450 genes *in vivo*. Thus to induce PB P-450, phenobarbital acts, either directly or indirectly, at the level of the genome and there is little or no effect on the rate of processing, transport, or stability of PB P-450 mRNA. Phenobarbital treatment had no effect on the overall transcriptional activity of rat liver nuclei, or on the transcription of a gene (*p*9F1) coding for a protein other than PB P-450. Thus the xenobiotic has no effect on transcription rates in general. As PB P-450 is encoded by a multigene family [26–28], the increase in production of PB P-450 transcripts could be due to an increase in the rate of transcription of gene(s) that were already active, or to the 'switching on' of additional genes that were previously inactive. The induction in cell culture of mouse P-450 by TCDD has also been

Table 2. Mechanism of induction of PB P-450 by phenobarbital

	Method of measurement	Fold induction by PB	Reference
Total cytochromes P-450	CO difference spectra	2–3	[7]
PB P-450	Radioimmunoassay	43	[7]
PB P-450 mRNA (translatable)	Immunoprecipitation of Translation products	22	[8]
PB P-450 mRNA (cytoplasmic)	Solution hybridization	20	[9]
PB P-450 mRNA (cytoplasmic)	RNA dot hybridization	20	This study
PB P-450 mRNA (nuclear)	RNA dot hybridization	20	This study
Transcription of PB P-450 gene(s)	Filter hybridization of *in vitro* nuclear transcripts	16	This study

shown to be mediated at the level of transcription [29]. It is clear that the action of TCDD is mediated through a receptor [30]. In contrast, no receptor for phenobarbital has been identified [31] and the method by which phenobarbital triggers an increase in transcription of PB P-450 genes is not known. The possibility has been suggested [32] that the induction of cytochromes P-450 by xenobiotics may involve a rearrangement of their genes. Our results indicate that the induction of PB P-450 by phenobarbital involves no amplification or rearrangement of PB P-450 genes.

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