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Regulation of Cytochrome P-450 Gene Expression

STUDIES WITH A CLONED PROBE*

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A cDNA clone for cytochrome P-450e, a phenobarbitone-inducible species in rat liver, has been isolated and characterized. With the use of this cloned DNA, an attempt has been initiated to elucidate the factors regulating the cytochrome P-450 gene expression. Inhibitors of heme synthesis such as cobalt chloride and 3-amino-1,2,4-triazole block the induction of cytochrome P-450e by phenobarbitone at the level of transcription. This is evident from the decrease in the rate of synthesis of cytochrome P-450e, a decrease in the levels of specific translatable messenger RNA, a decrease in the specific cytoplasmic and nuclear messenger RNA contents, and nuclear transcription of cytochrome P-450e gene, as revealed by hybridization to the cloned probe, under these conditions. It is proposed that heme is a general regulator of cytochrome P-450 gene expression at the level of transcription, whereas the drug or its metabolite would impart the specificity needed for the induction of a particular species.

The cytochrome P-450 group of proteins plays an important role in the metabolism of xenobiotics and is induced by a wide variety of chemicals (1, 2). They exhibit inducer specificity, and prototype chemicals induce different species of cytochrome P-450. For example, phenobarbitone induces cytochrome P-450b and e, whereas 3-methylcholanthrene induces cytochrome P-450c and d (3-5). Pregnenolone-16α-carbonitrile induces yet another form of cytochrome P-450 (6). These are typical examples, and the induction of additional but drug-specific species of cytochrome P-450 are being reported (7, 8). It has been established that the chemicals bring about the *de novo* synthesis of specific cytochrome P-450 species resulting from the induction of specific messenger RNA species (9-12). Recent studies have indicated that the cytochrome P-450 group constitutes a multigene family, and prototype drugs activate specific genes at the transcription level (13-16), although the mechanism of origin of the different messenger RNA species needs to be understood in greater detail.

In an effort to identify the nature and site of action of factors regulating cytochrome P-450 gene expression in rat liver, we have sought to examine the effects of a decrease in the heme pool which is modulated under a variety of conditions (17, 18). Heme, as a prosthetic group, would naturally affect the structural and functional properties of cytochrome P-450. But, we have also shown earlier that chemicals such as cobalt chloride, 3-amino-1,2,4-triazole, and thioacetamide, which inhibit heme synthesis, mixed function oxidase activity, and depress cytochrome P-450 content estimated spectrally, bring about these effects by inhibiting the synthesis of apocytochrome P-450 (19-21).

In the present study, we have constructed a cDNA clone for a cytochrome P-450 species induced by phenobarbitone, and using this probe, we have demonstrated that the inhibitors of heme synthesis block the transcription of the cytochrome P-450 gene.

EXPERIMENTAL PROCEDURES

Treatment of Animals—Male rats of the Institute strain (90-100 g) were given single injections of phenobarbitone (8 mg/100 g, intraperitoneally), cobalt chloride (6 mg/100 g, subcutaneously), and 3-amino-1,2,4-triazole (300 mg/100 g, intraperitoneally), where indicated.

Construction of a cDNA Clone for a Species of Cytochrome P-450 Induced by Phenobarbitone—Poly(A)-containing RNA isolated from magnesium-precipitated polyribosomes (22) of phenobarbitone-injected rat livers was fractionated on 5-25% sucrose gradients. The 16-18 S RNA (10 μg), coding for cytochrome P-450b/e as evident from reticulocyte cell-free translation experiments, was used for the preparation of single- and double-stranded cDNAs using reverse transcriptase and DNA polymerase I (Klenow). The double-stranded cDNA was cloned into *Escherichia coli* (C600) using pBR322 as vector and the GC tailing procedure. Tetracycline-resistant, ampicillin-sensitive clones were screened with [^{32}P]single-stranded-cDNA prepared against purified cytochrome P-450b/e messenger RNA. Cytochrome P-450b/e messenger RNA was purified nearly 50-fold by immunobinding of polyribosomes obtained from phenobarbitone-injected rat liver with cytochrome P-450b/e antibodies and isolating the immunobound polyribosomes using protein A-Sepharose chromatography (23), followed by oligo(dT)-cellulose chromatography to isolate poly(A)-containing RNA. In this procedure, 2000 A_{260} units of total polyribosomes yielded 2 μg of RNA, enriched for cytochrome P-450b/e messenger activity as revealed in reticulocyte translation experiments. The screening of a random population of 16 clones yielded two clones showing intense signals (Fig. 1). Clone 91 (marked by *arrow* in Fig. 1) was further analyzed along with a few other clones for fragment size, hybrid-selected messenger RNA translation, restriction mapping, and partial sequencing of a few fragments by the Maxam-Gilbert procedure (24). Digestion of clone 91 DNA with *Pst*I enzyme liberated a fragment, and its mobility in 1.5% agarose gels indicated a size of 1100 base pairs (Fig. 2). Clone 91 DNA or the insert was able to selectively hybrid-select an mRNA, which on translation in the reticulocyte cell-free system followed by immunobinding to cytochrome P-450b/e antibodies and analysis of the immunoprecipitate by SDS[1]-gel analysis and fluorography was found to code for cytochrome P-450b/e species (Fig. 3). Restriction map of the fragment was carried out after end labeling with [γ-^{32}P]ATP and generating partials from unsymmetrically cut halves (Fig. 4). Standard protocols were used in all the cloning manipulations and characterization of the recombinant DNA (25). For sequencing, the fragment was liberated with *Pst*I, and the *Pst*I ends were labeled with [γ-^{32}P]ATP, and then the fragment was cut with *Hin*dIII to generate 460- and 620-base pair fragments. The two fragments were sequenced partially using 40-cm gels. Multiple loadings facilitated the reading

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[1] The abbreviations used are: SDS, sodium dodecyl sulfate; bp, base pairs; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

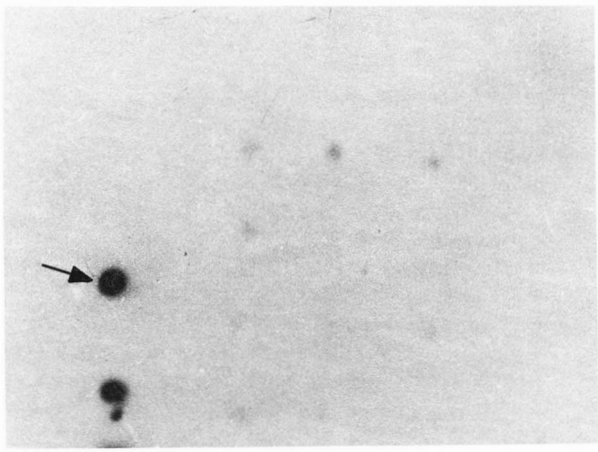

FIG. 1. **Grunstein-Hogness colony hybridization with [^{32}P] cDNA prepared against purified cytochrome P-450b/e mRNA.** Clone 91 indicated by *arrow* was taken for further analysis.

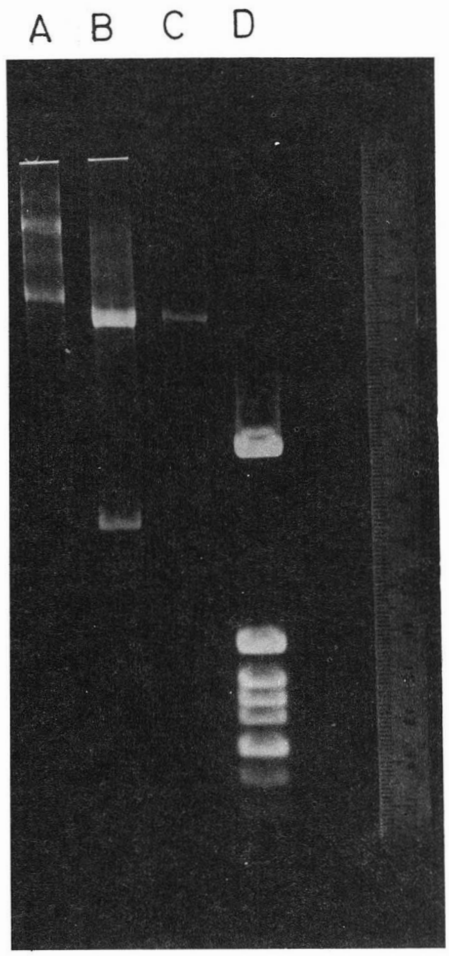

FIG. 2. **Size of the insert in clone 91.** The insert released on *Pst*I digestion of DNA was analyzed on 1.5% agarose gel. *A*, uncut DNA; *B*, *Pst*I-cut DNA; *C*, *Hin*dIII-cut DNA; *D*, size markers obtained with *Hin*f-cut pBR DNA.

of the sequences partially. A stretch of 30 nucleotides in the 460-base pair fragment and a stretch of 175 nucleotides in the 620-base pair fragment (Fig. 5) helped to identify clone 91 as a cytochrome P-450e clone.

Quantification of Messenger RNA Concentration for Cytochrome P-450b/e in the Cytoplasm—Three procedures were employed for this purpose. In the first procedure, microsomes were isolated from the treated animals, and protein synthesis was carried out *in vitro* in the

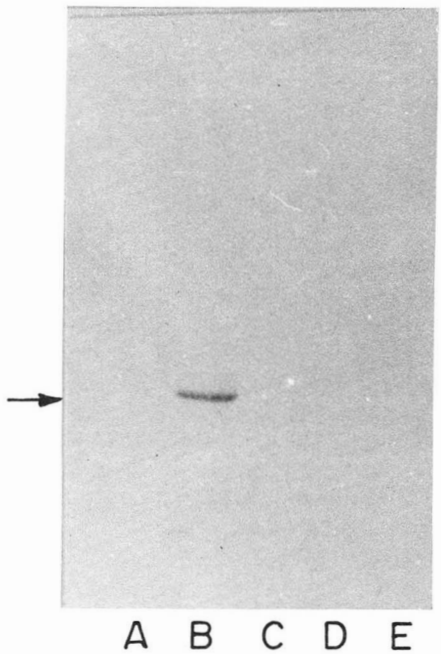

FIG. 3. **Hybrid-selected mRNA translation.** DNA-loaded filters were hybridized to poly(A)-containing RNA isolated from phenobarbitone-treated rat livers. The hybridized RNA was eluted, translated, treated with cytochrome P-450b/e antibody and the immunoprecipitate was analyzed on SDS-polyacrylamide gel electrophoresis (8% gels) and subjected to fluorography. *A*, clone 91 DNA, but translated product immunobound to albumin antibodies; *B*, clone 91 DNA; *C* and *D*, DNAs from clones not positive in Grunstein-Hogness hybridization; *E*, pBR DNA. *Arrow* indicates position of cytochrome P-450b/e.

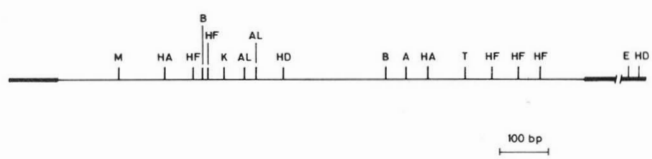

FIG. 4. **Restriction map of clone 91.** The insert was released with *Pst*I and end-labeled with [γ-^{32}P]ATP. The end-labeled fragment was digested with *Hin*dIII and the two labeled fragments, 460 and 620 bp, were used for restriction mapping by the generation of partials. The orientation of the insert with respect to the plasmid DNA was obtained on the basis of the size of the fragments released with *Hin*dIII (Fig. 2). *A*, *Av*II; *AL*, *Alu*I; *B*, *Bgl*II; *HA*, *Hae*III; *E*, *Eco*RI; *HD*, *Hin*dIII; *HF*, *Hin*fI; *K*, *Kpn*I; *M*, *Msp*I; *T*, *Taq*I. *Thick line* indicates pBR322 sequence, not drawn to scale.

presence of ^{14}C-labeled *Chlorella* protein hydrolysate and other components (19). The mixture was incubated at 37 °C for 20 min and then processed by immunoprecipitation and SDS-polyacrylamide gel electrophoresis using 8% gels. The cytochrome P-450b/e band was sliced out, digested with H_2O_2, and radioactivity measured using 0.5% (w/v) in Triton X-100/toluene (1:2) as mixture (19).

In the second procedure, 8 μg of poly(A)-containing RNA isolated from the livers of rats given the different treatments was translated in the reticulocyte cell-free system (26) in a total volume of 100 μl in presence of [^{35}S]methionine. The translated products were immunobound to cytochrome P-450b/e antibodies. The antigen-antibody complex was isolated using *Staphylococcus aureus* cells and analyzed by SDS-polyacrylamide gel electrophoresis (8% gels) and fluorography.

In the last procedure, 10 μg of poly(A)-containing RNA obtained from rat livers given the different treatments was fractionated on agarose-urea gels (1.5%) and analyzed by Northern blot hybridization with nick-translated [^{32}P]cDNA (10^6 cpm/ml) from clone 91 (27, 28).

Quantification of Messenger RNA Concentration for Cytochrome P-450b/e in the Nucleus—The procedure described by Guertin *et al.* (29) was followed without any modification. Briefly, RNA was first

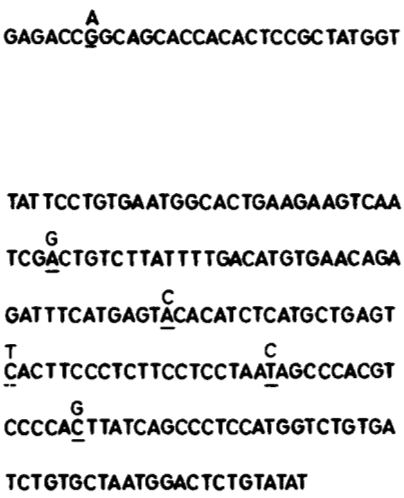

FIG. 5. **Partial sequencing of restriction fragments.** The experimental details are given in text. A, a sequence of 30 nucleotides in the 460-bp fragment. *B*, a sequence of 175 nucleotides in the 620-bp fragment. The *underlined* residues in cytochrome P-450e are replaced by the ones indicated above in cytochrome P-450b.

isolated from the nuclei of control and treated animals. The nuclei were lysed in presence of 2% (w/v) SDS and 7 M urea followed by phenol/chloroform extraction and ethanol precipitation. The pellet was treated with DNase I and proteinase K followed by phenol/chloroform extraction and ethanol precipitation. The final preparation was reprecipitated in presence of 2.5 M LiCl. RNA (10 μg) was loaded onto nitrocellulose filters in 20 × SSC, and the hybridization medium contained nick-translated [^{32}P]cDNA (10^6 cpm/ml) from clone 91.

Cell-free Nuclear Transcription—This was also carried out essentially as described by Guertin *et al.* (29). The reaction mixture in a total volume of 200 μl contained HEPES (pH 7.6), 20 mM; NaCl, 150 mM; magnesium acetate, 5 mM; $MnCl_2$, 1 mM; EDTA, 0.1 mM; dithiothreitol, 2 mM; ATP, GTP, and CTP, 0.4 mM each; [α-^{32}P] UTP, 4 μM; placental RNase inhibitor, 500 units/ml; creatine phosphate, 2 mM; creatine phosphokinase, 3 units/ml; glycerol, 16% (v/v), and nuclei equivalent to 200 μg of DNA. The incubation was at 25 °C for 45 min. Labeled RNA was isolated after DNase I and proteinase K treatments. The RNA preparation was also washed with 5% (w/v) trichloroacetic acid. The final pellet was dissolved in 10 mM Tris, 0.1% SDS (pH 8.0). Clone 91 DNA (40 μg) was loaded onto nitrocellulose discs (30), and hybridization with the RNA transcribed *in vitro* (3.5×10^5 cpm) was carried out as described by Guertin *et al.* (29). After incubation, the filters were washed, dried, and radioactivity directly measured using 0.5% (w/v) 2,5-diphenyloxazole in toluene as mixture.

Other Procedures—Purification of cytochrome P-450 from phenobarbitone-treated rats, preparation and purification of antibodies conditions for immunoprecipitation, and analysis by SDS-polyacrylamide gel electrophoresis have all been described in previous publications (9, 10, 20, 21).

RESULTS

We have established that clone 91 is for a species of cytochrome P-450 induced by phenobarbitone on the basis of Grunstein-Hogness hybridization (Fig. 1) and hybrid-selected messenger RNA translation (Fig. 3). It is known that cytochrome P-450b and e are highly homologous at the protein and messenger RNA levels (4, 16, 31, 32). The restriction map of the 1100-bp insert in clone 91 agrees very closely with the sequence data provided for R17 which has been characterized to be a cytochrome P-450e clone (31). Some of the specific identifying features for cytochrome P-450e clone obtained in the present study are (a) loss of *Msp*I site at 215 bp, but appearance of a new *Msp*I site at 123 bp. The sequence of 30 nucleotides obtained in this region demonstrates the substitution of an A residue present in cytochrome P-450b by a G residue in cytochrome P-450e such that the *Msp*I site GGCC is created. In addition, a new *Hae*III site at 218 bp resulting in a unique *Hae*III-*Bgl*II fragment of about 100 bp also agrees with that of the sequence reported for the R17 clone. (b) The sequence of 175 nucleotides obtained at the 3′-end for clone 91 is identical to that reported for R17 and shows five single base substitutions compared to cytochrome P-450b. The orientation of the insert in clone 91 with respect to the plasmid sequences is as depicted in Fig. 5.

The ability of microsomes isolated from control and drug-treated animals to synthesize cytochrome P-450b/e *in vitro* as assessed by immunoprecipitation, SDS-gel analysis, and the radioactivity in the protein band is given in Table I. As is now well established (9–12), phenobarbitone treatment enhances cytochrome P-450b/e synthesis. The interesting feature is that $CoCl_2$ given along with phenobarbitone or 90 min after the drug administration is able to inhibit cytochrome P-450b/e synthesis. In the latter case, the inhibitory effect of $CoCl_2$ is less than in the former case. 3-Amino-1,2,4-triazole also inhibits the phenobarbitone-mediated increase in cytochrome P-450b/e synthesis, but only when given in sufficient time before phenobarbitone.

The results obtained in the homologous cell-free system can be confirmed by translating the RNA isolated from the treated livers in the reticulocyte lysate cell-free system (Fig. 6). It is once again clear that $CoCl_2$ and 3-amino-1,2,4-triazole are effective in suppressing the phenobarbitone-mediated increase in the translatable activity of cytochrome P-450b/e messenger RNA. $CoCl_2$ is effective, but predictably to a lesser extent, even when administered 12 h after phenobarbitone treatment. The ability of $CoCl_2$ to block cytochrome P-450 synthesis and the translatability of its messenger RNA even when administered after phenobarbitone treatment, albeit partially, would indicate the rapid turnover of the messenger, requiring continued synthesis to maintain its level.

Northern blot analysis of the polysomal poly(A)-containing RNA isolated from control and drug-treated animals using nick-translated clone 91 is presented in Fig. 7. The content of cytochrome P-450b/e mRNA increases strikingly after phenobarbitone treatment, and $CoCl_2$ treatment effectively blocks this increase. The size of the messenger estimated from the mobility of markers is around 1950 ± 80 nucleotides in all treatment conditions.

The quantitation of cytochrome P-450b/e messenger pre-

TABLE I

Synthesis of cytochrome P-450b/e by microsomes isolated from drug-treated animals

The animals were killed 6 h after phenobarbitone administration, and protein synthesis was carried out with isolated microsomes. After *in vitro* protein synthesis in presence of ^{14}C-labeled *Chlorella* protein hydrolysate, the microsomes were solubilized and treated with cytochrome P-450b/e antibodies. The immunoprecipitates were analyzed on SDS-gels, and the cytochrome P-450 band was cut out, digested with H_2O_2, and radioactivity measured. The amount of total protein radioactivity taken for immunoprecipitation was 1.2×10^5 cpm in each case.

Treatment	Radioactivity in cytochrome P-450
	cpm
Control	138
Phenobarbitone	708
Phenobarbitone + $CoCl_2$[a]	152
Phenobarbitone + $CoCl_2$[b]	258
Phenobarbitone + 3-amino-1,2,4-triazole	145

[a] $CoCl_2$ administered at the same time as phenobarbitone.
[b] $CoCl_2$ administered 90 min after phenobarbitone.

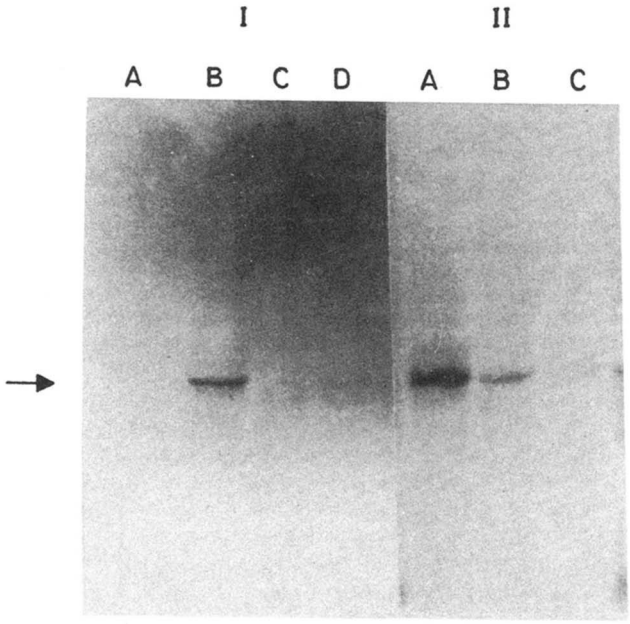

FIG. 6. **Effect of $CoCl_2$ on phenobarbitone-mediated increase in the translatable levels of cytochrome P-450b/e mRNA.** *I: A*, control; *B*, phenobarbitone treatment (6 h); *C*, phenobarbitone + $CoCl_2$ treatment (6 h); *D*, phenobarbitone (6 h) + 3-amino-1,2,4-triazole (10 h) treatment. *II: A*, phenobarbitone treatment (12 h); *B*, phenobarbitone (12 h) + $CoCl_2$ (2 h) treatments; *C*, control. *Arrow* indicates the position of cytochrome P-450b/e.

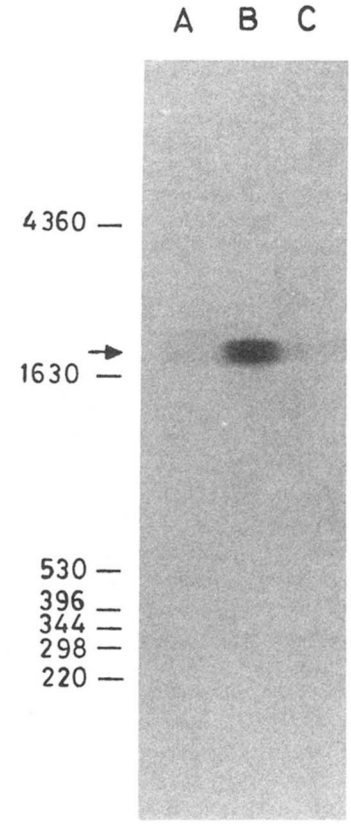

FIG. 7. **Northern blot analysis of cytochrome P-450e mRNA under conditions of phenobarbitone and phenobarbitone + $CoCl_2$ treatments.** Poly(A)-containing RNA was isolated from rat livers given the drug treatment for 6 h. 10 μg of RNA was analyzed on urea-agarose gels (1.5%), blotted into nitrocellulose filters, and then hybridized to nick-translated clone 91 DNA insert. *A*, control; *B*, phenobarbitone treatment; *C*, phenobarbitone + $CoCl_2$ treatment. The position of size markers and cytochrome P-450b/e is indicated by *arrows*.

cursor in the nucleus by dot-blot hybridization is presented in Fig. 8. Discernible hybridization can only be seen after phenobarbitone treatment, indicating an increase in the content of rapidly turning over pools of the cytochrome P-450b/e messenger precursors under these conditions. Once again, $CoCl_2$ and 3-amino-1,2,4-triazole treatments effectively block the phenobarbitone effect.

Finally, data on *in vitro* transcription with nuclei isolated from control and drug-treated animals are presented in Table II. It is clear that cytochrome P-450b/e transcripts can be detected in significant quantity soon after phenobarbitone treatment. $CoCl_2$ and 3-amino-1,2,4-triazole treatments are at least 65% effective in blocking the action of phenobarbitone, in this runoff transcription assay where the chains initiated *in vivo* are completed. This is probably a minimal estimate since phenobarbitone induces cytochrome P-450b/e gene expression very rapidly, and the depletion of the heme pool *in vivo* at the time period examined may not have been adequate to completely block initiation of transcription. Alternatively, inhibitors of heme synthesis may also have an additional effect on cytochrome P-450 synthesis at post-transcriptional sites. It may be pointed out that none of the treatments led to a significant change in the total transcription rates measured with isolated nuclei.

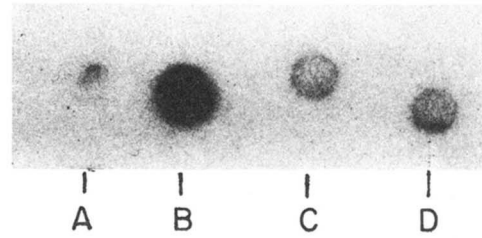

FIG. 8. **Dot-blot hybridization of nuclear RNA.** Nuclear RNA was isolated 4 h after the drug treatments and 10 μg was used for dot-blot hybridization with nick-translated clone 91 DNA insert. *A*, control; *B*, phenobarbitone treatment; *C*, phenobarbitone + $CoCl_2$ treatment; *D*, phenobarbitone + 3-amino-1,2-4-triazole treatment.

DISCUSSION

Heme is implicated in regulating the levels of hemoproteins and enzymes involved in heme metabolism and influences a wide variety of molecular processes (33–35). Earlier studies in this laboratory (19–21) as well as the results obtained in this study clearly establish that inhibitors of heme synthesis modulate the expression of the cytochrome P-450 gene at the level of transcription. This we have established on the basis of the quantitation of the cytochrome P-450b/e species by SDS-gel electrophoresis and radial immunodiffusion, measurements of the rate of cytochrome P-450b/e synthesis in homologous cell-free systems, and the translatability of the messenger RNA in heterologous cell-free systems, quantitation of cytochrome P-450b/e messenger RNA in the cytoplasm and the transcripts in the nucleus, and finally measurement of the rate of cytochrome P-450b/e gene transcription with isolated nuclei *in vitro*. It has not, however, been possible to counteract the effect of the inhibitors of heme synthesis by the administration of exogenous hematin. We feel that this is only a case of the accessibility of the regulatory site to exogenous heme, in view of its known nonspecific interaction with proteins and complications arising out of the time-dependent regulation of the endogenous heme pool by exogenous heme. The mechanism of translocation of a regulatory pool of heme

TABLE II

Transcription with nuclei isolated from drug-treated animals

The animals were killed 4 h after phenobarbitone administration and nuclei isolated. Labeled RNA (3.5×10^5 cpm) transcribed *in vitro* was hybridized to filters containing clone 91 DNA insert, and the radioactivity of the hybridized RNA was measured.

Treatment	RNA hybridized
	cpm
Control	15
Phenobarbitone	291
Phenobarbitone + $CoCl_2$	85
Phenobarbitone + 3-amino-1,2,4-triazole	96

into the nucleus remains to be worked out. We would like to propose that the expression of cytochrome P-450 genes is governed at the level of transcription by at least two parameters. One is the inducing chemical or its metabolite and the other one is heme. Heme may function as a general regulatory molecule governing the synthesis of all cytochrome P-450 species, whereas the drug may impart the specificity needed for the induction of a particular species. It would be interesting to examine the sites of interaction of the drug and heme at the level of chromatin in this regard.

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