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Biochemistry

Administration of 3-methylcholanthrene to rats increases the specific hybridizable mRNA coding for cytochrome *P*-450c

(polycyclic hydrocarbons/induction of monooxygenases/aryl hydrocarbon hydroxylase/drug metabolism)

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ABSTRACT **Poly(A)$^+$-RNA obtained from the livers of 3-methylcholanthrene (3MC)-treated rats was translated into cytochrome *P*-450c in a cell-free reticulocyte system. In this translational system, no precursor cytochrome *P*-450c was observed. The mRNA responsible for the synthesis of this cytochrome was isolated by immunoprecipitation of liver polyribosomes obtained at 15 hr after 3MC treatment, and a cDNA was constructed by the reverse transcriptase reaction. The cDNA was further purified by hybridizing at a high R_0t (product of RNA concentration and incubation time) to poly(A)$^+$-RNA isolated from control rat liver, and the nonhybridized, single-stranded cDNA was isolated by hydroxylapatite chromatography. This $cDNA_{P\text{-}450c}$ was employed in hybridization reactions with poly(A)$^+$-RNA isolated from the livers of rats treated with 3MC for various times. These studies indicated a maximal induction of $mRNA_{P\text{-}450c}$ at about 15 hr after 3MC injection, although levels of this mRNA were significantly increased by 7 hr. The $mRNA_{P\text{-}450c}$ concentration had diminished by 24 hr but remained higher than control levels for at least 48 hr. These studies establish an effect of 3MC upon the accumulation of $mRNA_{P\text{-}450c}$ in rat liver.**

Cytochrome *P*-450, a class of terminal oxidases of a NADPH-dependent electron transport pathway, participates in the biotransformation of a wide variety of structurally different compounds, including steroids, fatty acids, chemical carcinogens, insecticides, and hydrocarbons (1, 2). The rather broad substrate specificity of this monooxygenase system relates to the presence of multiple forms of cytochrome *P*-450 in tissues (3–7). The existence of individual members of this heterogeneous class of cytochrome *P*-450 has been established through the effects of various "inducers"—e.g., 3-methylcholanthrene (3MC) (8–16). Three highly purified isozymes of cytochrome *P*-450 (*P*-450a, *P*-450b, and *P*-450c) have been isolated from liver microsomes of rats that had been treated with Aroclor 1254, a mixture of polychlorinated biphenyls (17). Cytochrome *P*-450c has also been referred to as cytochrome *P*-448 (18) or P_1-450 (19), and this hemoprotein is the major form of cytochrome *P*-450 induced in rats by 3MC (17, 20).

On the basis of differences in amino acid compositions, partial amino acid sequences, peptide maps of proteolytic digests, and the absence of immunochemical relatedness (17, 20, 21), it has been proposed that cytochromes *P*-450a, *P*-450b, and *P*-450c arise through the transcription of different genes. We have undertaken a detailed investigation of the effects of 3MC administration upon the transcription and translation of cytochrome *P*-450c. In preliminary studies (22, 23), we reported that poly(A)$^+$-RNA from the livers of 3MC-treated rats translated more immunoprecipitable cytochrome *P*-450c in a cell-free reticulocyte assay system than an analogous preparation from control liver. However, because these results could be explained by an increase in "translatability" as well as enhanced synthesis of mRNA, we have studied this problem by using hybridization techniques under conditions of RNA excess. The results reported in this manuscript indicate that the amount of mRNA coding for cytochrome *P*-450c is increased in liver as early as 7 hr after administration of the polycyclic hydrocarbon to rats.

MATERIALS AND METHODS

Materials. Reverse transcriptase (RNA-dependent DNA polymerase) from avian myeloblastosis virus was obtained from J. W. Beard under contract to the National Institutes of Health. [^{3}H]dCTP (19 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) was supplied by Amersham. The *Hin*dIII restriction nuclease fragments of λ phage were obtained from Bethesda Research Laboratories (Rockville, MD). Sprague–Dawley rats were purchased from the Canadian Breeding Labs (St. Constant, Quebec). All other materials were obtained from commercial sources.

Treatment of Rats. Male Sprague–Dawley rats, 80–100 g in weight, were injected intraperitoneally with 3MC (in corn oil) at 25 mg/kg of body weight. Control rats were injected with vehicle alone. All rats were killed at 15 hr or as otherwise designated by cervical dislocation and the livers were perfused *in situ* with cold sterile 0.25 M sucrose.

Isolation of RNA Fractions. Poly(A)$^+$-RNA was isolated from the postnuclear supernatant fraction obtained from the livers of control and 3MC-treated rats. Rat liver was homogenized in a heparin-containing buffer (24) and centrifuged at $10,000 \times g$ for 5 min. The supernatant fraction was made 1% with respect to both sodium deoxycholate and Triton X-100 and overlayed on a discontinuous sucrose gradient as outlined by Buehl *et al.* (25). After centrifugation for 4.5 hr at 25,000 rpm in a Beckman SW 27 rotor, the polyribosomes at the interface were removed with a hypodermic needle and syringe and stored frozen at −70°C until used. RNA was extracted from these polyribosomal preparations by the guanidinium chloride method of Chirgwin *et al.* (26). The RNA preparation was heated at 65°C for 5 min, adjusted to 1% $NaDodSO_4$, 0.01 M EDTA, 0.3 M NaCl, and 0.02 M Tris·HCl (pH 7.2), and passed through oligo(dT)-cellulose twice. The poly(A)$^+$-RNA was eluted from the oligo(dT)-cellulose with sterile distilled water and precipitated by the addition of 2 vol of 95% (vol/vol) ethanol. In some instances, RNA was extracted directly from rat liver by the guanidinium thiocyanate method of Chirgwin *et al.* (26) and poly(A)$^+$-RNA was isolated by oligo(dT)-cellulose chromatography after extraction of residual DNA and glycogen from the RNA with 3 M

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Abbreviation: 3MC, 3-methylcholanthrene.

sodium acetate, pH 5. In either case, the poly(A)$^+$-RNA was dissolved in sterile distilled water, frozen in liquid N_2, and stored at −70°C.

The liver poly(A)$^+$-RNA from 3MC-treated rats was subjected to sedimentation analysis in linear 5–20% sucrose gradients containing formamide (27). Each gradient was fractionated into 1-ml samples, 25 μg of rabbit tRNA and NaCl (to final concentration of 0.2 M) were added, and the RNA was precipitated upon the addition of 2 vol of 95% ethanol. After 15 hr at −20°C, the RNA was pelleted by centrifugation, dissolved in water, and assayed for its translational ability in a rabbit reticulocyte system. The translational products were immunoprecipitated either with antiserum against rat albumin (kindly supplied by John Taylor of the Hershey Medical Center) or with monospecific anti-*P*-450c (17, 20).

Immunoprecipitation of Polysomes for the Synthesis of Cytochrome *P*-450c. Liver polyribosomes, prepared as described above, were immunoprecipitated with rabbit anti-*P*-450c, essentially as described for ovalbumin by Shapiro *et al.* (28). In preliminary binding experiments utilizing ^{125}I-labeled anti-*P*-450c prepared with the Bolton–Hunter reagent, we determined that the appropriate ratio of anti-*P*-450c to polyribosomes was approximately 12 μg of protein per A_{260} unit of polysomal RNA. After an overnight incubation at 4°C, the cytochrome *P*-450c-synthesizing polyribosomes were precipitated by using a second antibody, sheep anti-rabbit immunoglobin that was crosslinked by glutaraldehyde (29). After pelleting of the antigen–antibody–second antibody complex and thorough washing of the precipitate as described by Palacios *et al.* (29), the absorbed cytochrome *P*-450c-specific polyribosomes were dissociated with 0.01 M Hepes (pH 7.5)/0.05 M EDTA/heparin (100 μg/ml). Two additional extractions with Hepes/EDTA/heparin were conducted and each time the preparation was centrifuged at 2000 × *g* for 10 min. The supernatant fractions were combined and made 0.2 M with respect to NaCl, and 2 vol of 95% ethanol was added. The cytochrome *P*-450c-specific polyribosomal RNA was precipitated at −20°C overnight. The precipitated RNA was pelleted by centrifugation at 15,000 × *g* for 30 min at 4°C. The mRNA was isolated by two passes through oligo(dT)-cellulose as described above.

Translational Assay. The ability of the RNA fractions to stimulate protein synthesis was tested by using a cell-free rabbit reticulocyte translational kit provided by New England Nuclear. The radioactive amino acid was either L-[^{3}H]leucine or [^{35}S]methionine; the K^+ and Mg^{2+} concentrations were 80 mM and 6 mM, respectively. After incubation at 37°C for 60 min, an aliquot of the translation assay was removed for trichloroacetic acid precipitation, and the remainder was generally used for the immunoprecipitation reaction—e.g., with anti-*P*-450c. For the latter, 5 μg of cytochrome *P*-450c and an excess of antibody were added. Pansorbin A was added to ensure precipitation of the antigen–antibody complex, and after 2 hr at 4°C, the aggregate was pelleted, washed, and redissolved in electrophoresis buffer containing 0.4% Emulgen 911. Aliquots were subjected to electrophoresis on 7.5% polyacrylamide gels containing $NaDodSO_4$ (30).

Synthesis of cDNA to the mRNA for Cytochrome *P*-450c ($cDNA_{P\text{-}450c}$). Poly(A)$^+$-RNA from the immunoprecipitated polyribosomes of the livers of 3MC-treated rats (at 15 hr) was used as template for cDNA production in the avian myeloblastosis virus reverse transcriptase reaction under the conditions described by Myers *et al.* (31) with [^{3}H]dCTP as the labeled precursor. The cDNA mixture was treated with alkali to hydrolyze the poly(A)$^+$-RNA, chromatographed on Sephadex G-50 to eliminate the small molecular weight contaminants, and precipitated in the presence of 0.2 M NaCl and 2 vol of 95% ethanol. The cDNA was dissolved and hybridized at a R_0t (product of RNA concentration and incubation time) of 10^3 mol·s·liter^{-1} to poly(A)$^+$-RNA obtained from the livers of control rats. The conditions were: 20 mM Hepes buffer (pH 7.7), 0.5 M NaCl, 2 mM EDTA, various amounts of RNA, and 0.2% $NaDodSO_4$. After incubation at 68°C for 24 hr, the mixture was diluted 1:20 in 0.12 M sodium phosphate buffer (pH 6.8) and applied to a column of hydroxylapatite that was maintained at 60°C. The single-stranded $cDNA_{P\text{-}450c}$ was eluted with the 0.12 M phosphate buffer, and the hybridized material was removed with 0.5 M phosphate buffer (pH 6.8) at 60°C. The $cDNA_{P\text{-}450c}$ was desalted on Sephadex G-50 and precipitated overnight at −20°C in the presence of 0.2 M NaCl, 50 μg of rabbit tRNA, and 2 vol of 95% ethanol.

The hybridization of the cDNA to poly(A)$^+$-RNA obtained from control or 3MC-treated rats was conducted under conditions of RNA excess in 50% (vol/vol) formamide at 42°C as described by Chiu *et al.* (32).

RESULTS

Poly(A)$^+$-RNA from livers of 3MC-treated rats proved more efficacious than that from control rats in the translational assay (Table 1). At 15 hr after injection of the polycyclic hydrocarbon, a 2-fold enhancement in total translational activity was observed. The increased efficiency in translation of the poly(A)$^+$-RNA from 3MC-treated rats was not caused by a greater contamination of control systems with ribosomal RNA, because gel electrophoretograms of both poly(A)$^+$-RNA preparations were similar. The increased translatability of the poly(A)$^+$-RNA from 3MC-treated rats may result from an enhanced stability of existing mRNA populations as well as from an increase in the total number of unique mRNA molecules. In other experiments the translational products were immunoprecipitated with anti-*P*-450c and Pansorbin A and the washed immunoprecipitate was subjected to $NaDodSO_4$ gel electrophoresis. Only when poly(A)$^+$-RNA was isolated from livers of 3MC-treated rats was any cytochrome *P*-450c observed. The translated cytochrome *P*-450c had an electrophoretic mobility that was identical to the purified hemoprotein (M_r 56,000). Furthermore, when poly(A)$^+$-RNA that had been obtained from the livers of phenobarbital-treated rats (80 mg/kg, 15 hr earlier) was employed in the translational assay, no cytochrome *P*-450c synthesis was demonstrable either by comparison of electrophoretic mobilities of products or by immunoprecipitation (data not shown). These results are consistent with the very low levels of cytochrome *P*-450c in livers of control and phenobarbital-treated rats (20).

Table 1. Translational activity of liver poly(A)$^+$-RNA from control and 3MC-treated rats

RNA added	[^{35}S]Methionine incorporation, cpm × 10^{-5} per assay
Control, 30 μg	6.0
60 μg	13.0
3MC-treated, 30 μg	12.5
60 μg	22.6
Globin mRNA, 1 μg	41.0

Poly(A)$^+$-RNA was isolated from the livers of control and 3MC-treated rats, 15 hr after injection of the polycyclic hydrocarbon. The purified RNA was added to a [^{35}S]methionine-dependent (50 μCi added per assay), nuclease-treated reticulocyte lysate and translation was allowed to continue at 37°C for 1 hr. The globin mRNA was added as a positive control. Radioactivity was quantified from trichloroacetic acid precipitates of the translation system.

The sedimentation characteristics of the mRNA responsible for the synthesis of cytochrome *P*-450c were compared with those of the mRNA for albumin synthesis. For this purpose, liver poly(A)$^+$-RNA from 3MC-treated rats was subjected to sucrose density gradient sedimentation and the RNA in each fraction was tested by immunoprecipitation techniques for its ability to direct the synthesis of either of these two proteins. The mRNA activity for the translation of cytochrome *P*-450c sedimented with almost the same speed as that of rat albumin (data not shown)—i.e., 17 S (33).

The mRNA specific for cytochrome *P*-450c was purified and cDNA to this hemoprotein was subsequently prepared as follows. Approximately 10,000 A_{260} units of liver polyribosomes from 3MC-treated rats (15 hr after injection) was immunoprecipitated with rabbit anti-cytochrome *P*-450c. We obtained approximately 12 μg of specific poly(A)$^+$-RNA. A portion of this $mRNA_{P\text{-}450c}$ was translated in the reticulocyte lysate system. The efficacy of this mRNA is indicated in Table 2, and the $NaDodSO_4$ gel electrophoretic profile of the translational products is given in Fig. 1. The data of Table 2 indicate that all of the translational products could be immunoprecipitated by antibody to cytochrome *P*-450c. In contrast, less than 15% of the products translated in the presence of the positive control adenovirus mRNA were precipitated by this antibody. These results are confirmed by analysis of the electrophoretic profile of the translational product (Fig. 1). The major product was cytochrome *P*-450c as judged from comparison with the electrophoretic mobility of authentic cytochrome *P*-450c (17).

A portion of the $mRNA_{P\text{-}450c}$ was employed in the reverse transcriptase reaction to obtain cDNA. The cDNA was pelleted and further purified by hybridization to poly(A)$^+$-RNA obtained from control rat liver (34). After hybridization at 68°C for 24 hr, the single-stranded cDNA was obtained by elution from a hydroxylapatite column with 0.12 M sodium phosphate buffer, pH 6.8. Approximately 60% of the starting radioactivity was recovered in this fraction, which contained the $cDNA_{P\text{-}450c}$ used in subsequent studies.

Table 2. Synthesis of cytochrome *P*-450c as directed by $mRNA_{P\text{-}450c}$

Conditions	[^{35}S]Methionine incorporation, total cpm
Adenovirus mRNA, 1 μg	156,000
$mRNA_{P\text{-}450c}$, 0.8 μg	180,000
$mRNA_{P\text{-}450c}$, immunoprecipitated translational products for *P*-450c	176,000
Adenovirus mRNA, immunoprecipitated translational products for *P*-450c	20,000

mRNA specific for cytochrome *P*-450c was obtained from liver polyribosomes (15 hr after treatment of the rats with 3MC) by the immunoprecipitation procedure described in *Materials and Methods*. The $mRNA_{P\text{-}450c}$ (0.8 μg) was translated in the reticulocyte lysate system with [^{35}S]methionine. Adenovirus mRNA was used as the positive control. The total cpm incorporated into protein was determined by spotting an aliquot of the translational products on filter paper, precipitating the products with 5% trichloroacetic acid, treating with alkali to disrupt aminoacyl-tRNA, washing four times with 5% trichloroacetic acid, and finally rinsing the filters in 95% ethanol. After drying, the filters were placed in glass scintillation vials, NCS solubilizer (Amersham) was added, and, after 1 hr, the radioactivity was determined by liquid scintillation counting techniques. An additional aliquot was employed in the immunoprecipitation reaction with antibody to cytochrome *P*-450c. Radioactivity obtained in the absence of any RNA has been subtracted from the above figures. The counting efficiencies were similar in both sets of experiments.

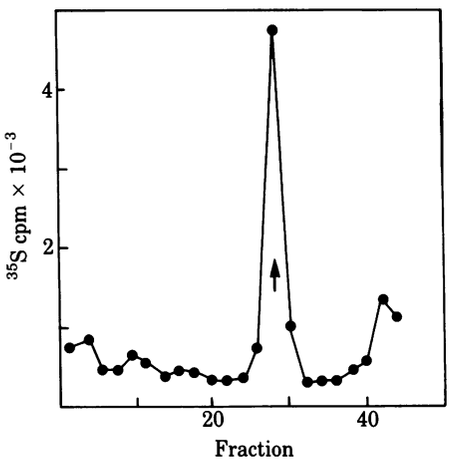

FIG. 1. Translation of poly(A)$^+$-RNA purified from immunoprecipitated liver polyribosomes. Poly(A)$^+$-RNA was obtained from immunoprecipitated liver polyribosomes from 15-hr 3MC-pretreated rats. This RNA, 0.8 μg, was tested in the translational assay with [^{35}S]methionine as precursor. The product was subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis and the gel was cut into 2-mm fragments, which were solubilized in NCS and their radioactivity was quantified. The direction of electrophoresis is from right to left. The position of authentic standard cytochrome *P*-450c was at fraction 28 (arrow). The standards, bovine serum albumin (M_r 68,000) and ovalbumin (M_r 43,000), would be found in fractions 32 and 22, respectively. A background of approximately 35 cpm was found across the gels when RNA was not added to the translational system.

An aliquot of the ^{3}H-labeled $cDNA_{P\text{-}450c}$ was subjected to sucrose density gradient analysis to obtain an estimate of the number of its bases. These data are presented in Fig. 2. The sedimentation was compared with that of the *Hin*dIII restriction nuclease fragments of λ phage. From a relationship of the logarithm of the number of bases as a function of the fraction in which the nuclease fragment was present, it was calculated that the peak in Fig. 2 corresponds to 630 nucleotides. The maximum number—i.e., fraction 10—corresponded to 1300 nucleotides. One would estimate that mRNA for cytochrome *P*-450c (and therefore its cDNA), a protein with a molecular

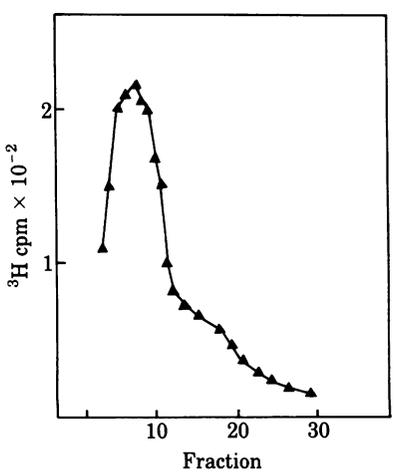

FIG. 2. Sucrose density gradient analysis of $cDNA_{P\text{-}450c}$. A sample of ^{3}H-labeled $cDNA_{P\text{-}450c}$ was overlaid on a linear 5–25% alkaline sucrose density gradient that contained 0.1 M NaOH, 0.9 M NaCl, and 2 mM EDTA. The tubes were centrifuged in a Beckman SW 41 rotor at 39,000 rpm for 24 hr, the product was fractionated, and the radioactivity of each fraction was determined. In addition, *Hin*dIII digests of nonradioactive λ phage DNA were added and similarly centrifuged. The 600-, 1960-, 4260-, 6670-, and 9460-base fragments migrated to fractions 5, 13, 19, 23, and 26, respectively.

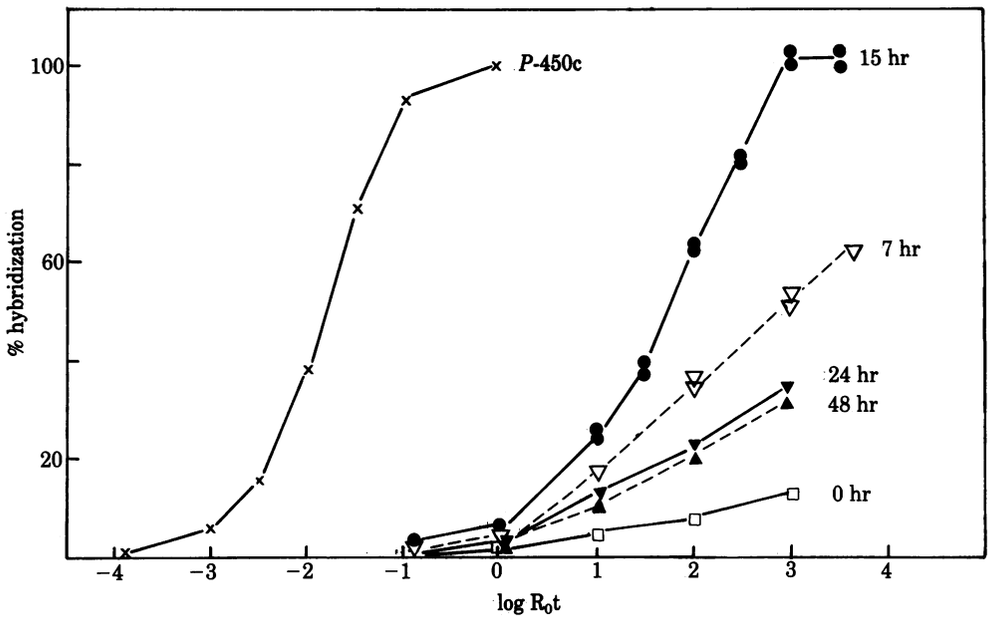

FIG. 3. Hybridization of $cDNA_{P\text{-}450c}$ to liver $mRNA_{P\text{-}450c}$. $cDNA_{P\text{-}450c}$ was hybridized to liver poly(A)$^+$-RNA obtained from control rats and rats treated with 3MC 7, 15, 24, and 48 hr before sacrifice. The hybridization was conducted at 42°C in 50% formamide for 72 hr. The hybridized material represents S1 nuclease-resistant macromolecules. R_0t is defined as moles of nucleotide per liter × seconds.

weight of 56,000 (17) and consisting of 500 amino acids (21), should contain a minimum of 1500 nucleotides.

We then assayed the poly(A)$^+$-RNA obtained from the livers of rats that had been treated with 3MC for various times for $mRNA_{P\text{-}450c}$ activity by employing the RNA-excess hybridization technique. In addition, the purified $mRNA_{P\text{-}450c}$ was hybridized to the $cDNA_{P\text{-}450c}$. These data are summarized in Fig. 3. Maximal hybridization occurred with the 15-hr sample; this yielded a R_0t at which 50% hybridization occurred (i.e., $R_0t_{1/2}$) of 60 mol·s·liter^{-1}. Although we cannot compute a $R_0t_{1/2}$ for the 7-hr time period, it is apparent that $mRNA_{P\text{-}450c}$ was substantially increased over that of control. By 24 and 48 hr, the percent hybridization was reduced substantially but was still higher than that observed with poly(A)$^+$-RNA from control rat liver. The $R_0t_{1/2}$ for the hybridization of $mRNA_{P\text{-}450c}$ to its cDNA was 1.5×10^{-2} mol·s·liter^{-1}. This $R_0t_{1/2}$ for $mRNA_{P\text{-}450c}$ is compatible with that of a pure mRNA of 1500 bases.

DISCUSSION

Several studies have recently been published in which the *in vitro* synthesis of microsomal cytochrome *P*-450 was investigated (35–38). Dubois and Waterman (35) and Bhat and Padmanaban (36) presented evidence that cytochrome *P*-450b synthesis as judged by a mRNA-dependent assay system was maximal by 12 hr after administration of phenobarbital. In the studies from our laboratory (37), we were able to demonstrate an increased mRNA activity for cytochrome *P*-450b synthesis as early as 6 hr after administration of this barbiturate with a maximum increase at 12–18 hr. Bar-Nun *et al.* (38), using mRNA from the livers of rats treated with phenobarbital, translated and subsequently determined the sequence of the NH_2-terminal portion of cytochrome *P*-450. The sequence obtained was identical to the NH_2-terminal region of the hemoprotein purified from phenobarbital-treated rats.

The biosynthesis of proteins ultimately destined for export from the cell or for incorporation into the membranous protein of cellular organelles—e.g., endoplasmic reticulum membranes—has been postulated to involve a precursor, the "prepiece" (39), which bears at its NH_2 terminus an additional peptide of 10–15 amino acids. This mechanism, which is referred to as the signal hypothesis, also implies the existence of a hydrophobic peptide immediately following the pre-piece. The mature protein may or may not retain the pre-piece. Evidence has been obtained indicating the lack of a precursor segment in cytochrome *P*-450b (35–38). In contrast, Kumar and Padmanaban (40) have reported that cytochrome *P*-450c is synthesized with a molecular weight 6000 greater than the mature protein. In the studies described, we have been unable to confirm the existence of this precursor segment of cytochrome *P*-450c. In our translational reactions, only a 56,000 molecular weight protein is synthesized, which comigrates in $NaDodSO_4$ with the protein purified from 3MC-treated rats.

We have, in addition, demonstrated in this report that the number of $mRNA_{P\text{-}450c}$ molecules increases in liver after the administration of 3MC. Although the results of inhibitor studies *in vivo* (reviewed in ref. 41) implied this, the action of the polycyclic hydrocarbon upon the accumulation of $mRNA_{P\text{-}450c}$ in rat liver had not been unequivocally demonstrated previously. The time for maximal induction appears to be about 15 hr after injection of the polycyclic hydrocarbon, although by 7 hr a substantial increase is already noted. The $mRNA_{P\text{-}450c}$ concentration, although significantly higher than in control liver, is markedly diminished by 24 and 48 hr. This $cDNA_{P\text{-}450c}$ now makes possible a detailed study of the organization of the cytochrome *P*-450c gene.

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