Vol. 104, No. 2, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 29, 1982 Pages 641-648

EXPRESSION AND SUBCELLULAR DISTRIBUTION OF MOUSE CYTOCHROME P_1-450 mRNA AS DETERMINED BY MOLECULAR HYBRIDIZATION WITH CLONED P_1-450 DNA

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Received November 5, 1981

SUMMARY: Cytochrome P_1-450 (P_1-450) is defined as that cytochrome most closely associated with 3-methylcholanthrene (MC)-induced aryl hydrocarbon hydroxylase (AHH) activity. Recently a cloned DNA sequence (clone 46) was shown to represent a portion of the P_1-450 structural gene [Negishi et al., Proc. Nat. Acad. Sci. U.S.A. 78: 800-804 (1981)]. Poly(A^+)-enriched RNA was isolated from total liver homogenate, membrane-bound polysomes and from free polysomes at various times after MC treatment of Ah-responsive C57BL/6N (B6) and Ah-nonresponsive DBA/2N (D2) inbred mice. The poly(A^+)-enriched RNA was separated by methylmercury-agarose gel electrophoresis and hybridized to nick-translated [^{32}P]DNA from clone 46. By means of this RNA-DNA hybridization, only 6% of total polysomal P_1-450 mRNA exists in free polysomes after 24 h of MC treatment. The data indicate that the endoplasmic reticulum is the principal site of synthesis for this integral microsomal protein.

Studies of induction kinetics following MC treatment provided the evidence of the rapid increase of total liver and membrane bound P_1-450 mRNA preceding the synthesis of apo-P_1-450 and the increase of AHH activity.

INTRODUCTION

Microsomal cytochrome P-450 is a terminal enzyme of a monooxygenase system by which numerous xenobiotics as well as endogenous substrates such as steroids and fatty acids are metabolized. It is well known that many drugs such as MC and phenobarbital can induce multiple forms of cytochrome P-450 (1-3). Murine Ah locus regulates the induction of these enzymes by polycyclic aromatic compounds (such as MC, β-naphthoflavone and 2,3,7,8-

Abbreviations used are: MC, 3-methylcholanthrene; B6, the C57BL/6N inbred mouse strain; D2, the DBA/2N inbred mouse strain; AHH, aryl hydrocarbon hydroxylase

0006-291X/82/020641-08$01.00/0

tetrachlorodibenzo-p-dioxin) (4). Cytochrome P_1-450, defined as that form of microsomal cytochrome most closely associated with induced AHH activity, is one of those enzymes associated with Ah locus. In order to understand better the complexities of the induction process, we have recently cloned a DNA sequence representing a portion of P_1-450 structure gene (5, 6).

It has been demonstrated by using clone 46 as the hybridization probe that the induction of specific P_1-450 mRNA following MC induction is under transcriptional control (6); however, processes leading to the final increase in AHH activity are undoubtedly complex. Of particular interest is the intracellular site of synthesis for this microsomal integral membrane protein. Phenobarbital induced rat liver P-450 (7, 8), have been reported to be synthesized by membrane-bound polysomes. Adrenal microsomal P-450 (C-21), however, was found not to segregate specifically in either membrane-bound or free polysomes, as determined by an *in vitro* translation assay (9). In this communication, we have taken advantage of clone 46 DNA and have studied the expression and subcellular distribution of mouse P_1-450 mRNA between membrane-bound and free polysomes during MC induction.

MATERIALS AND METHODS

Materials: ABM paper was purchased from Schleicher and Schnell, Inc. (Keene, NH); guanidine-HCl and sucrose from Bethesda Research Laboratories (Rockville, MD); methylmercury (II) hydroxide from Alfa Division, Inc. (Danvers, MA); agarose and MC from Sigma Chemical Company (St. Louis, MO); α-[^{32}P]dCTP (2,000 Ci/mmol) and nick-translation kits from Amersham (Arlington Heights, IL); and XS-5 film from Eastman Kodak Company (Rochester, NY).

Treatment of animals: B6 and D2 mice received intraperitoneally 250 mg/kg of MC and twenty mice were killed at the indicated time points. The twenty livers were combined and minced. The animals were not starved during these experiments.

Preparations of subcellular fractions and of poly(A^+)-enriched RNA: One-third of the minced liver was used for isolation of poly(A+)-enriched RNA from the total liver homogenate. The other two-thirds of the minced liver was homogenized with two volumes of polysome buffer, according to the method described by Steel and Ramsey (10). The homogenate was centrifuged at 740 x g for 2 min; then for 12 min at 131,000 x g. The resulting pellet was used for isolating membrane-bound polysomal mRNA. For the isolation of free polysomes, the supernatant fraction was overlayed on a discontinuous sucrose density gradient, comprised of 1.38 M and 2.0 M sucrose, and centrifuged at 150,000 x g for 25 h.

The yield of membrane-bound and free polysomes was determined by the following method. The pellet derived from the first centrifugation was suspended in a modified polysome buffer containing 1% Triton X-100 and

250 mM KCl. The mixture was then centrifuged at 1,470 x g for 5 min to sediment nuclei. The resulting supernatant fraction was subjected to discontinuous sucrose gradient, as described above for isolation of the free polysomal fraction. After centrifugation, the amounts of polysomes in the 2.0 M sucrose layer and in the pellets were estimated by measurement of optical density at 260 nm.

Total RNA was isolated from the total liver homogenate, membrane-bound polysomal fraction, and free polysomal fraction by the guanidine-HCl method described previously (11). Poly(A^+)-containing RNA was enriched by oligo(dT)-cellulose column chromatography (12).

Isolation and preparation of clone 46 [^{32}P]DNA: Clone 46, which has a portion of the structural gene for cytochrome P_1-450, was isolated by the procedure reported by Negishi et al. (5). The clone 46 DNA was first digested by Pst I. Seven hundred and 400 bp of P_1-450 DNA were separated from the linear plasmid by polyacrylamide gel electrophoresis, followed by electroelution from the gel. The DNA mixture of 700 and 400 bp was then nick-translated with [^{32}P]CTP as the radioactive tracer (13) and used for the probe. Specific radioactivity of the DNA was 1 x 10^8 cpm/μg.

Methylmercury agarose gel electrophoresis and transfer of RNA to DBM paper and [^{32}P]DNA hybridization from clone 46: Five μg of poly(A+)-enriched RNA from the three subcellular fractions at each time point following induction by MC were electrophoresed in 1% agarose gels containing 5 mM methylmercury hydroxide (14). The gel was treated by the method described (15) and blotted on DBM paper. The DBM paper to which RNA was transferred was hybridized with ~1 x 10^7 cpm of nick-translated [^{32}P]DNA from clone 46 and washed under the conditions reported previously (6). The washed paper was then exposed to x-ray film (XS-5). The relative amounts of P_1-450 mRNA were determined by measuring the radioactivity recovered from DBM paper in the area corresponded to the 23S mRNA band. The calibration curve shown in Fig. 1 demonstrates a linear increase of specific P_1-450 mRNA content, up to 5 μg of poly(A^+)-enriched RNA added to an agarose gel.

RESULTS

Specific segregation of P_1-450 mRNA in membrane-bound polysomes

Poly$(A)^+$-enriched RNA was isolated from total homogenates, membrane-bound polysomes, and free polysome fractions of mouse liver at various times following MC induction. P_1-450 mRNA is known to be about 23S in size (6). A time-dependent increase in the specific content of 23S mRNA was seen in all three mRNA fractions from B6 mice (Fig. 2). Control animals receiving corn oil alone had nondetectable levels of P_1-450 mRNA (data not shown). When the amounts of P_1-450 mRNA were compared between membrane-bound and free polysomes, the intensity of the 23S band was much greater in the membrane-bound than in the free polysomal fraction at any time point following MC treatment. The autoradiograph also shows a very low level of P_1-450 mRNA in Ah-nonresponsive D2 mice 24 h after MC induction, and this small amount of P_1-450 mRNA was noted also to segregate with the membrane-bound polysomes.

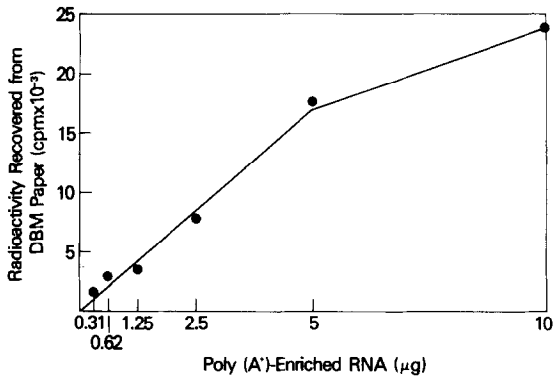

FIG. 1. Radioactivity recovered from DBM paper as a function of the amount of poly(A^+)-enriched RNA added. Ten, 5, 2.5, 1.25, 0.62 and 0.31 μg of poly(A^+)-enriched RNA from MC-treated B6 liver were electrophoresed, transferred to DBM paper, and hybridized to clone 46 [^{32}P]DNA. After blot hybridization, the area of DBM paper corresponded to 23S mRNA for cytochrome P_1-450 was cut and radioactivity measured by liquid scintillation counter.

The relative amounts of P_1-450 mRNA were shown in Table I. The ratio of mouse liver membrane-bound to free polysomes in our preparation ranged between 1.6 and 2.0. At 24 h following MC treatment, approximately 94% of P_1-450 mRNA in total liver polysomes is associated with the membrane-bound fraction, whereas at 3 h and 6 h after MC the values are 86% and 89%, respectively.

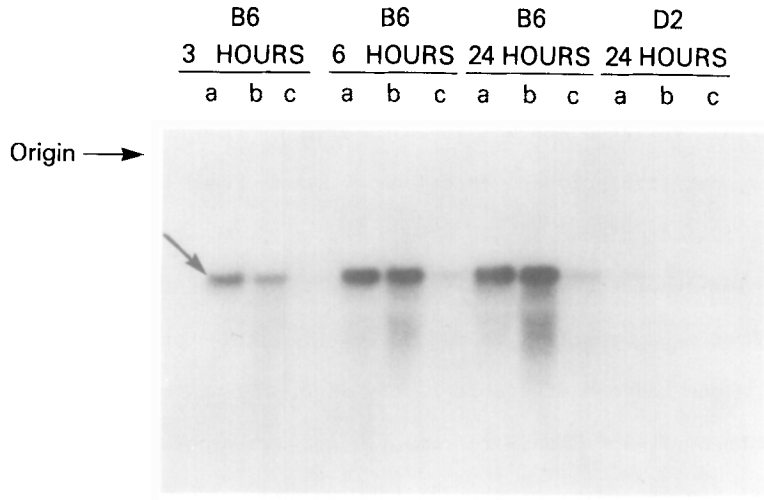

FIG. 2. Blot hybridization analysis of poly(A^+)-enriched RNA with clone 46 [^{32}P]DNA, separated by methylmercury-agarose gel. Following a single dose of MC in Ah-responsive B6 and Ah-nonresponsive D2 mice, mRNA was isolated at the indicated times. Five μg of poly(A^+)-enriched RNA from (a) total liver homogenate, (b) membrane-bound polysomes, and (c) free polysomal fractions were electrophoresed on methylmercury-agarose gels, transferred to DBM paper, and hybridized with [^{32}P]DNA from clone 46. The bands marked with the arrow correspond to the RNA size of 23S.

TABLE I

DISTRIBUTION OF P_1-450 mRNA IN MOUSE LIVER MEMBRANE-BOUND AND FREE POLYSOMES

	Hours after MC treatment						Control	
	3 Hours		6 Hours		24 Hours			
	Bound	Free	Bound	Free	Bound	Free	Bound	Free
1. Distribution of polysomal RNA[a] (ratio)	1.6	1.0	1.7	1.0	2.0	1.0	1.8	1.0
2. P1-450 mRNA content (cpm)[b]	3,240	850	10,920	2,310	15,470	1,830	N.D.	N.D.
3. Relative amount of P1-450 mRNA (line 1 x line 2)	5,180	850	18,560	2,310	30,940	1,830	-	-
4. Relative amount of P1-450 mRNA as % of P1-450 mRNA in total polysomes	86%	14%	89%	11%	94%	6%	-	-

N.D. = *nondetectable.*

[a]The distribution of polysomal RNA in membrane-bound and free polysomal fractions was estimated by A_{260} after purification of the polysomal fraction through a discontinuous sucrose gradient, as described under "Materials and Methods."

[b]P_1-450 mRNA was determined by measurement of radioactivity recovered from DBM paper at the 23S region after blot hybridization.

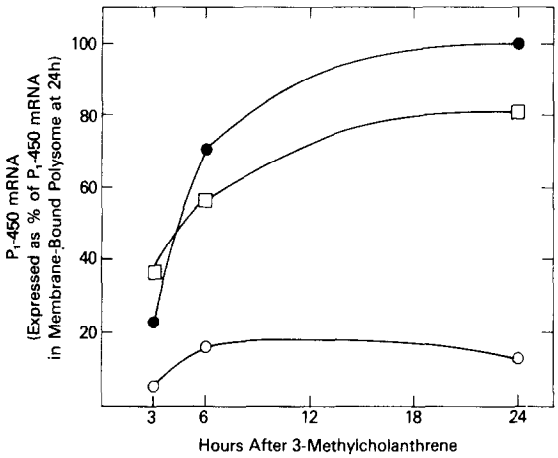

FIG. 3. Kinetics of induction of P_1-450 mRNA in total liver homogenates (□—□), membrane-bound polysomes (●—●), and free polysomes (○—○). B6 mice were given a single injection of MC for 3, 6, or 24 h. After blot hybridization, radioactivity recovered from DBM paper in the area corresponding to the 23S band was determined. Further experimental details are described in the legends to Fig. 2 and under "Material and Methods."

Induction kinetics of P_1-450 mRNA in MC-treated B6 mice

Although the amount of P_1-450 mRNA is increased in total homogenates and membrane-bound fraction as a function of time following MC treatment, the kinetics of the increase was found to be somewhat different in these two fractions (Fig. 3). During the first 3 h after MC, the increase in P_1-450 mRNA content appeared to be much faster in the total homogenate than in membrane-bound polysomes: the mRNA level extracted from total liver homogenate had already reached 45% of its 24-h level, whereas the level of membrane-bound P_1-450 mRNA was only 21%. At 6 h and 24 h after MC treatment, however, the concentration of P_1-450 mRNA was greater in membrane-bound polysomes than in the total homogenate. One possible interpretation is that accumulation of non-ribosomal P_1-450 mRNA occur in the very early stage of induction by MC.

DISCUSSION

Previous studies on the subcellular distributions of mRNAs for microsomal cytochrome P-450 have been based primarily on measurement of (i) binding of ^{125}I-labeled specific antibodies to ribosomes *in vitro* (8), (ii) immunoprecipitation of ^{3}H-puromycin-nascent peptides (8), and (iii) *in vitro*

translation programmed by polysomes or polysomal mRNA followed by immunoprecipitation with specific antibodies (9). These techniques, however, provide indirect evidence for the subcellular distribution of a specific mRNA. In addition, various factors and conditions such as (i) specificity of so-called "monospecific antibodies," (ii) purity and recovery of an immunoprecipitate, and (iii) different efficiencies of *in vitro* translation of free versus membrane-bound mRNA (16) may influence the final conclusions.

To eliminate these facts, we used in this study RNA-DNA hybridization to examine the exact subcellular localization of P_1-450 mRNA. We have extracted poly(A^+)-enriched RNA from membrane-bound and free polysomal fractions of MC-treated B6 mice and performed blot hybridization to nick-translated [^{32}P]DNA from clone 46, which contains a portion of the P_1-450 structural gene.

During the early phase of induction by MC, ~14% (at 3 h) and ~10% (at 6 h) of P_1-450 mRNA are associated with free polysomes. At 24 h this value decreases to 6%. Using soluble hybridization of RNA to DNA, Adesnik and Maschio (17) found that 3% to 8% of liver mRNA isolated from tightly-bound polysomes on endoplasmic reticulum is in the free polysomal fraction. Yap *et al*. (18) reported that 2% of albumin mRNA is found in free polysomes. Our data indicates that P_1-450 mRNA segregates into ribosomes bound on the endoplasmic reticulum, in a manner similar to that for albumin mRNA. We thus have provided the first evidence, obtained by DNA-RNA molecular hybrization, indicating that specific mRNA of eukarytotic membrane protein segregates predominantly with membrane-bound polysomes.

It is interesting to note that during the first 6 h of induction, the specific content of P_1-450 mRNA already reaches 70% of its maximal induction level (Fig. 3). However, the levels of the induced AHH activity and apoprotein of P_1-450 determined by immunological methods are only ~5% of their maximal levels at 6 h following MC treatment (19). This kind of time lag between the binding of P_1-450 mRNA-containing polysomes to the endoplasmic reticulum and the accumulation of immunoprecipitable translated product (P_1-450) in the endoplasmic reticulum was not observed in the case of rat

liver microsomal NADPH-cytochrome P-450 reductase and epoxide hydrolase during their induction by phenobarbital (20). The exact mechanisms of the early stage of this induction process remain to be elucidated.

ACKNOWLEDGMENTS

We thank Dr. Daniel W. Nebert for valuable discussions of this work and critical review of this manuscript. Thanks is also due to Dr. Michitoshi Nakamura. The expert secretarial assistance of Ms. Ingrid E. Jordan is also greatly appreciated.

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