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INDUCTION OF CYTOCHROMES P450IA1 AND P450IA2 AS DETERMINED BY SOLUTION HYBRIDIZATION

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Abstract—Two oligodeoxyribonucleotides were synthesized that were specific for the messenger RNAs for the polycyclic hydrocarbon-inducible cytochromes P450IA1 and P450IA2. The solution hybridization technique was modified for the use of these oligodeoxyribonucleotide probes so as to increase the sensitivity and specificity of this method. Using this technique, the steady-state levels of the mRNAs for cytochromes P450IA1 and P450IA2 in control rat liver were determined to be < 3 and 6 molecules/cell, and 1.8 and 4.0 attomol/μg poly $(A)^+$ RNA, respectively. At 15 hr after induction with 3-methylcholanthrene, the steady-state levels of the mRNAs for P450IA1 and P450IA2 were 68 and 200 molecules/cell, and 41.6 and 123 attomol/μg poly $(A)^+$ RNA.

Cytochrome P450-dependent mixed-function oxygenases catalyze the bioactivation of a number of xenobiotics leading to the formation of highly reactive, electrophilic derivatives that often function as mutagens, carcinogens and teratogens [1, 2]. The cytochromes P450 also catalyze critical biotransformation reactions with endogenous substrates such as steroids, fatty acids, and prostaglandins [3]. The regulation of the expression of cytochrome P450 is, consequently, an important determinant of the metabolic fate of a large number of chemicals of different structures; this may dictate the susceptibility of an organism to various toxicologic effects.

A unique feature of the hepatic cytochrome P450 system is the capacity for profound isozyme induction after exposure to certain xenobiotics, e.g. 3-methylcholanthrene (3MC). In the adult rat, 3MC treatment results in an increase in the concentration of two unique members of the P450 family, cytochromes P450IA1 and P450IA2, which is preceded by an elevation in the steady-state levels of P450IA1 and P450IA2 mRNAs (see for example, Ref. 4). Cytochromes P450IA1 and P450IA2 are immunologically comparable [5], encoded by distinct mRNAs [6, 7], and transcribed from two separate genes [8, 9]. Maximum expression of P450IA1 mRNA in rat liver takes place at 15 hr after the administration of 3MC [4].

The ability to measure mRNA levels precisely by hybridization to a specific probe represents an important tool in the analysis of the regulation of gene expression. A number of methods have been developed over the past few years for the quantitation of mRNA. One of the most widely-used methods is laser densitometric analysis of Northern blots or slot-blots that have been hybridized with a gene-specific probe. Another useful method for mRNA quantitation, which has the advantage of sensitivity, is solution hybridization [10, 11]. Recently, solution hybridization analysis has been employed for the detection of small quantities of mRNA in cells [12].

During our attempts to quantitate the mRNAs for cytochromes P450IA1 and P450IA2, we employed a solution hybridization approach [11]. Unfortunately, the sensitivity of this method proved inadequate. The solution hybridization technique was modified so that small quantities of mRNA that would be found in cells or fetal tissue could be quantitated accurately. The induction of cytochromes P450IA1 and P450IA2 in liver after administration of 3MC to rats is reported in this manuscript using this assay.

MATERIALS AND METHODS

Materials. 3-Methylcholanthrene, S1 nuclease, salmon sperm DNA and CsCl were purchased from the Sigma Chemical Co. (St. Louis, MO). T_4 polynucleotide kinase was obtained from Bethesda Research Laboratories (Gaithersburg, MD), and [γ-^{32}P]ATP (3000 Ci/mmol) from Amersham Inc. (Arlington Heights, IL).

Animal treatment. Three- to four-week-old male Sprague–Dawley rats were injected intraperitoneally with a corn oil solution of 3MC at 40 mg/kg body weight or with the vehicle alone. The livers were removed from the rats at 15 hr after administration of the polycyclic hydrocarbon, frozen immediately in dry ice, and stored at −80°.

Isolation of RNA from rat liver. Total RNA was isolated from the livers using the method of Iversen *et al.* [13]. Approximately 0.5 g of frozen liver was ground in liquid nitrogen with a precooled pestle, the powder was added to 2.5 mL of 6 M guanidine hydrochloride, and the mixture was homogenized. Solid CsCl was added to a final concentration of 0.5 g/mL and the mixture was centrifuged at 55,000 rpm for 3 hr in a swinging bucket rotor at 20°. The

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RNA was isolated from the bottom of the centrifuge tube, was extracted with phenol–chloroform–isoamyl alcohol (24:24:1, by vol), and was precipitated with 3 vol. of ethanol and 0.1 vol. of 3 M sodium acetate. Poly $(A)^+$ RNA was isolated by means of a Fast-Track kit from Invitrogen (San Diego, CA), using the procedure described by the manufacturer.

Preparation of synthetic oligodeoxyribonucleotides. Two complementary 18 mers and two complementary 26 mers were synthesized for the quantitation of cytochrome P450IA1 and P450IA2 mRNAs, respectively. The sequences of the oligomers employed in these experiments were chosen based on the primary sequence data as reported by our laboratory [8] and Sogawa *et al.* [9], respectively. The DNA sequence from 5097–5114 base pairs (bp) was selected for the measurement of P450IA1 mRNA, whereas 6654–6679 bp was used for P450IA2. No significant homology existed between the sequences of these oligomers and any other genes that are listed in Genbank. The 18 mer probe had the sequence 5′-CTGAGGACCAGAAGACCG-3′; the sequence of the 26 mer probe was 5′-TTCACTTGGAGAAGCGTGGCCAGGCC - 3 ′. The complements of these sequences were also synthesized.

The oligomers were purified by elution of appropriate bands after electrophoresis on 20% polyacrylamide gels. The concentrations of the oligodeoxyribonucleotides were determined spectrophotometrically at 260 nm, pH 7, and 25° using a molar absorptivity of 2.07×10^5 and 1.705×10^5 $M^{-1} cm^{-1}$ for the two 18 mers and 2.95×10^5 and 2.89×10^5 $M^{-1} cm^{-1}$ for the two 26 mers. These molar absorptivities were calculated as $\Sigma_i n_i A_i$, where i designates a constituent nucleotide containing either adenine, guanine, cytosine, or thymine; n_i is the mol of nucleotide i per mol of oligonucleotide; A_i is the molar absorptivity of the corresponding 5′-nucleotide at 260 nm and pH 7. The values of A_i for AMP, CMP, GMP [14], and TMP [15], respectively, were 1.54×10^4, 7.30×10^3, 1.17×10^4, and 8.80×10^3 $M^{-1} cm^{-1}$. The calculated molar absorptivities for the oligonucleotides represent maximal estimations since they did not account for hypochromic effects due to base stacking.

Northern blot and slot-blot analyses of RNA. The induction of the cytochrome P450IA1 and P450IA2 genes by 3MC was measured by Northern blot analysis of total RNA using these synthetic, specific oligonucleotides. For Northern blot analysis, 20 μg of total RNA was electrophoresed on 1% agarose–formaldehyde gels under denaturing conditions, and then the RNA was blotted onto nylon membranes, as described [16], and baked at 80° for 2 hr. For slot-blot analysis, 2 and 4 μg of poly $(A)^+$ RNA were dotted onto nylon filters using a Minifold 11 Slot Blotter (Schleicher & Schuell, Keene, NH) as described in the guidelines of the manufacturer.

Radioactive labeling of the DNA. Albumin and actin probes were ^{32}P-labeled using a Prime Time labeling kit (International Biotechnologies, New Haven, CT) as described by the manufacturer. The synthetic oligomers were 5′-end labeled with T_4 polynucleotide kinase and [γ-^{32}P]ATP as described by Richardson [17]. The reactions were incubated for 60 min at 37°, and additional polynucleotide kinase was added. After a further 60 min incubation, the reactions were stopped by the addition of EDTA to 20 mM. The labeled oligomers were purified by Nensorb-20 spin columns (NEN Research Products, Boston, MA). The resulting specific radioactivities obtained for the oligomers were typically 8–9×10^6 cpm/pmol.

Hybridization of RNA with genomic probes and synthetic oligonucleotides. In preparation for use of the albumin or actin probes, the RNA blots were prehybridized for 4 hr at 42° in 50% formamide, 5 × SSC (SSC = 0.15 M NaCl–0.015 M sodium citrate, pH 7.0), 100 μg/mL salmon sperm DNA, 0.2% sodium dodecyl sulfate (SDS) and 5× Denhardt's solution (1 × Denhardt's = 0.02% Ficoll, polyvinylpyrrolidone 360, and bovine serum albumin). Prehybridization prior to use of the synthetic oligonucleotides was conducted under slightly different conditions, i.e. incubation at 48° for 3 hr in 20% formamide, 5 × SSC, 0.2% SDS, 5× Denhardt's solution, and 10 μg/mL of denatured and sheared salmon sperm DNA. Hybridization of the filters was carried out for 18–24 hr at 42° in 50% formamide, 5 × SSC, salmon sperm DNA (100 μg/mL with the genomic probes; 10 μg/mL of denatured and sheared salmon sperm DNA with the oligonucleotide probes), 0.2% SDS, Denhardt's solution (1× with the genomic probes; 5× with the oligonucleotides), and 10^6 cpm/mL of ^{32}P-labeled albumin (*rsa*57) or actin probes or oligonucleotides. At the end of the hybridization, the membrane was washed twice with 2 × SSC–0.1% SDS for 15 min at room temperature, and once for 20 min at 65° with 0.2 × SSC–0.1% SDS in the case of the genomic probes, or once at 45° for 10 min after hybridization with the oligomeric probe. The membranes were exposed at −70° to Kodak XAR-5 film with a DuPont Lightning Plus intensifying screen. Size markers consisted of ^{32}P-labeled DNA fragments of bacteriophage λ, generated by HindIII digestion.

The relative quantity of mRNA present in each lane was normalized by hybridizing with the albumin probe. The intensity of each signal on the autoradiograph was measured by densitometric analysis.

Solution hybridization. The solution hybridization experiments were performed in duplicate at 48° for 18 hr in a total volume of 10 μL containing: 0.75 M NaCl; 0.2% SDS; 4 mM EDTA; 20 mM Tris–Cl, pH 7.5; 1.0 fmol of ^{32}P-labeled oligonucleotide; and various amounts of either total or poly $(A)^+$ RNA. In each experiment, a synthetic oligomer which was complementary to the labeled oligomer was used as a standard for hybridization. In the latter experiments, the concentration of complementary oligomer varied from 0 to 4 fmol, in place of the total or poly $(A)^+$ RNA. At completion of the hybridization reaction, the samples were digested with S_1 nuclease, 8 units/mL, for 90 min at 37° in 1 mL of a buffered solution which contained: 100 μg/mL of salmon sperm DNA; 0.75 M NaCl; 2.8 mM $ZnSO_4$; and 0.07 M sodium acetate, pH 4.5. The nucleic acids were precipitated at 4° for 60 min by the addition of 0.2 mL of 6 M trichloroacetic acid. The precipitates were collected by filtration through

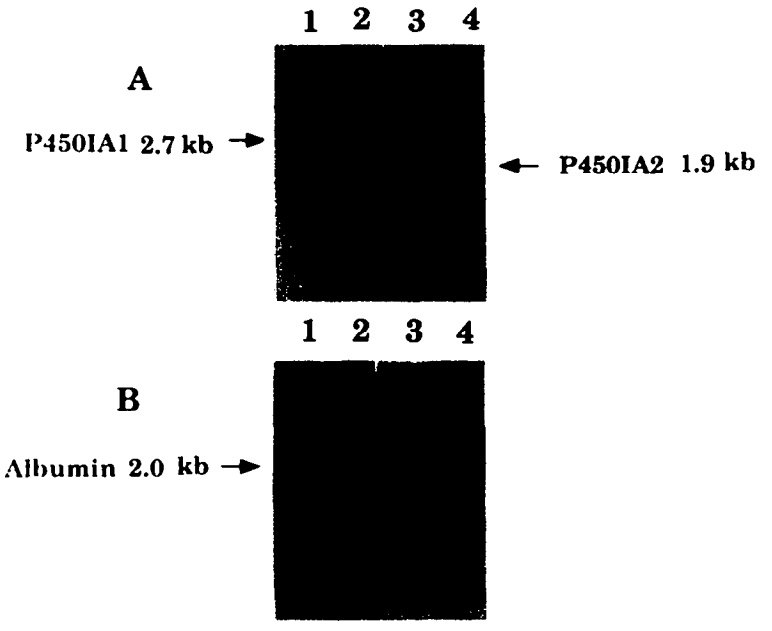

Fig. 1. Hybridization of RNA immobilized on nitrocellulose with oligodeoxyribonucleotide probes. Twenty micrograms of liver total RNA from control and 3MC-treated rats were electrophoresed on formaldehyde-denaturing gels, transferred to nitrocellulose, and hybridized with ^{32}P-labeled P450IA1 or P450IA2 oligonucleotide probes (specific activity = 8×10^6 dpm/pmol). Panel A: Oligonucleotides for P450IA1 (lanes 1 and 2) or P450IA2 (lanes 3 and 4) were used. Liver RNA from control (lanes 1 and 3) and 3MC-treated rats (lanes 2 and 4) was electrophoresed. The positions for mRNA of P450IA1 and P450IA2, i.e. 2.7 and 1.9 kb, respectively, are indicated. Panel B: The membrane of panel A was stripped of probe and rehybridized with an albumin gene. The position of authentic albumin mRNA, i.e. 2 kb, is indicated.

glass fiber filters, and the latter were washed and counted as described by Durnam and Palmiter [10].

RESULTS

The specificities of hybridization of the synthetic oligonucleotides to cytochrome P450IA1 and P450IA2 mRNAs are presented in Fig. 1. The P450IA1-specific oligonucleotide hybridized to only one RNA species, at approximately 3 kilobases (kb), which is the position of authentic mRNA. The P450IA2-specific oligonucleotide hybridized to an RNA species at approximately 2 kb, which represents the position of authentic P450IA2 mRNA. It is recognized that a positive signal at one position of the Northern hybridization analysis does not necessarily mean that only a single mRNA is present at this position.

Treatment of 4-week-old rats with 3MC resulted in an increase in the steady-state levels of cytochrome P450IA1 and P450IA2 mRNAs in liver as shown

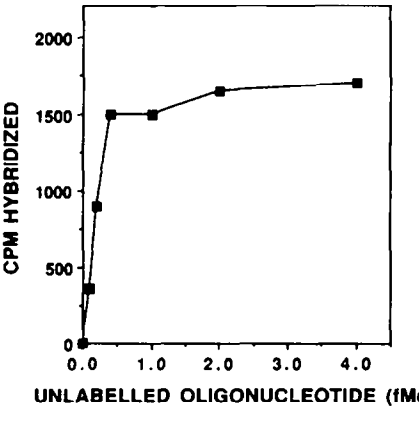

Fig. 2. Hybridization of cytochrome P450IA1 as a function of oligonucleotide concentration. Solution hybridization was conducted as described in the text. Exactly the same hybridization profile was obtained with the P450IA2 oligonucleotide probe.

Table 1. Quantitation of cytochrome P450IA1 and P450IA2 mRNAs by solution hybridization in control and induced rat liver

Probe	Pretreatment	fmol mRNA/μg poly $(A)^+$	pg mRNA*/μg poly $(A)^+$	Molecules/cell†
P450IA1	Control	$<1.8 \times 10^{-3}$	ND‡	<3
	3MC	41.6×10^{-3}	37.1	68
P450IA2	Control	4.0×10^{-3}	2.5	6
	3MC	123.1×10^{-3}	77.2	200

Rats were injected with either corn oil (control) or 3MC in corn oil, 40 mg/kg, i.p., and were killed 15 hr later. The details are presented in Materials and Methods.

* pg mRNA/μg poly $(A)^+$ = [(fmol mRNA/μg poly $(A)^+$) · (330 g/mol of nucleotide) · (number of nucleotides/molecule)]/1000.

† Number of molecules/cell = (mol of mRNA hybridized/μg poly $(A)^+$) · (2.71 pg poly $(A)^+$/cell) · (6×10^{23} molecules/mol). The amount of poly $(A)^-$ RNA/cell was taken from Ref. 18.

‡ Not done.

in Fig. 1A. Cytochrome P450IA1 mRNA was not detectable in control rat liver. On the other hand, cytochrome P450IA2 mRNA was observed in control rat liver albeit at a low level. Although the Northern analysis of Fig. 1 appears "smeary", this was caused by overexposure of the autoradiograph in order to see if any signal corresponding to P450IA1 and P450IA2 mRNAs was present in control liver.

After treatment with 3MC, a significant induction in the steady-state level of both the mRNAs was apparent; a greater amount of P450IA2 mRNA was noted. Densitometric analysis of these autoradiographs which were normalized by albumin mRNA expression (Fig. 1B) revealed an 18-fold increase in the steady-state level of P450IA2 mRNA in liver at 15 hr after 3MC administration. At this time, approximately five times more P450IA2 mRNA compared to P450IA1 mRNA was observed.

The amounts of mRNA were quantitated more exactly by solution by hybridization. For these experiments, the amount of mRNA to be added in each assay was quantitated by slot-blot analysis with an actin gene as probe. Poly $(A)^+$ RNA, 2 and 4 μg, was slot-blotted on nitrocellulose and hybridized to the actin probe as described in Materials and Methods. The relative amount of mRNA present in each sample was quantitated by laser densitometry. It was important to quantitate the mRNA in this fashion for consistent results. It was also important to accurately quantitate the amount of unlabeled complementary oligonucleotide.

The extent of hybridization to labeled oligonucleotide as a function of the concentration of the oligonucleotide is presented in Fig. 2. This standard curve was used in the calculation of fmol of P450IA1 or P450IA2 mRNA per μg of either total or poly $(A)^+$ RNA in liver. It is germane to mention the reason for the achievement of the maximum of hybridization at less than the stoichiometric value. In RNA–DNA hybridization experiments, the concentration of labeled oligonucleotide is at least 1000 times more than the specific RNA that is hybridized. Under these circumstances, the hybridization reaction exhibits first order kinetics and the concentration of the labeled oligonucleotide remains virtually constant. Therefore, complete hybridization of the RNA can occur. The linear portion of the plot is used for the calculation of the hybridized RNA.

The concentrations of mRNA per μg of poly $(A)^+$ RNA before and after administration of 3MC as determined by solution hybridization are shown in Table 1. As observed in the Northern analysis of Fig. 1, the level of cytochrome P450IA1 mRNA in control liver was undetectable; this would correspond to <3 molecules/cell or <1.8 attomol mRNA/μg poly $(A)^+$ RNA. In contrast, the amount of cytochrome P450IA2 mRNA in control rat liver was 6 molecules/cell and 4.0 attomol mRNA/μg poly $(A)^+$ RNA. After induction by 3MC, the concentrations of P450IA1 and P450IA2 mRNAs were 41.6 and 123 attomol/μg poly $(A)^+$ RNA, respectively, corresponding to 68 and 200 molecules/cell. It is recognized that the linearity and sensitivity of this assay could have been more convincing were several doses of 3-methylcholanthrene chosen for administration to the rats. However, in previous studies from our laboratory (not presented), maximum induction of P450IA1 mRNA *in the rat* occurred after administration of 3-methylcholanthrene at 20 mg/kg body wt. Furthermore, the amount of mRNA has been altered in our assay system at least ten times, and a simulated dose–response relationship has been obtained in this fashion.

DISCUSSION

Solution hybridization with oligonucleotides has several advantages over the other available methods. It is simple and the hybridization requires no special apparatus. High stringency conditions for the hybridization, however, are required in order to lower the "noise" level. The resultant calculations of hybridized RNA are simple, and the specific activity of the labeled oligonucleotide and the efficiency of filtration do not interfere with the calculations, thus increasing the sensitivity of the method [11]. The increased sensitivity allows the quantitation of mRNA species that are present in only small amounts in the cell. The sensitivity can be customized to the relative abundance of the mRNA by using either larger or smaller amounts of the complementary oligonucleotide in construction of the standard curve.

Specifically in regard to cytochrome P450IA1 and P450IA2 mRNA, the latter was present in detectable amounts in control rat liver whereas the former existed at <3 molecules/cell. Consequently, any level in rat liver above this value more probably reflects prior exposure of the animal to a pollutant of the polycyclic hydrocarbon type or may be due to genetic variation. Fifteen hours after its administration in rats, 3MC profoundly increased both mRNAs, i.e. from 6 to 200 and from <3 to 68 molecules of P450IA2 and P450IA1 mRNA, respectively. Exposure of the animal to 3MC also increased the relative abundance in rat liver of the P450IA2 and P450IA1 mRNAs by approximately 30-fold, and by >24-fold, respectively.

In summary, a modified solution hybridization analysis has been used to improve sensitivity and specificity for the detection of the steady-state levels of the low-abundancy cytochrome P450IA1 and P450IA2 mRNAs under basal and induced conditions with synthetic oligonucleotides as probes.

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