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Identification and Quantitation of a 2.0-Kilobase Messenger Ribonucleic Acid Coding for 3-Methylcholanthrene-Induced Cytochrome P-450 Using Cloned Cytochrome P-450 Complementary Deoxyribonucleic Acid†

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ABSTRACT: We have used a plasmid containing DNA complementary to one of the two size classes of mRNA coding for 3-methylcholanthrene-induced cytochrome P-450 from rat liver to characterize and quantitate that mRNA. The plasmid used was constructed and identified as follows: Total poly(A^+) RNA from 3-methylcholanthrene-induced liver was used as a template for cDNA synthesis. Double-stranded cDNA was inserted into plasmid pBR322 by the G-C tailing procedure. Recombinants were screened by colony hybridization using as probe [^{32}P]cDNA prepared from partially purified cytochrome P-450 mRNA. A differential screening approach was used in which duplicate filters were hybridized with probe from either 3-methylcholanthrene treated or untreated rats. Plasmid p23 was strongly positive by colony hybridization. It was conclusively shown to contain cytochrome P-450 cDNA sequences by demonstrating that the mRNA which specifically hybridized to nitrocellulose-bound plasmid p23 could be translated in vitro into peptides that were immunoprecipitable with monoclonal antibodies specific for 3-methylcholanthrene-induced cytochrome P-450. The size and quantity of the mRNA complementary to plasmid p23 were determined by hybridization of the ^{32}P-labeled plasmid to rat liver RNA that had been fractionated by electrophoresis under fully denaturing conditions and transferred to diazobenzyloxymethyl-paper. Plasmid p23 hybridized strictly to a single size of mRNA that was about 2000 nucleotides in length, the smaller of the two size classes of mRNA coding for 3-methylcholanthrene-induced cytochrome P-450. From this we concluded that, at least within the region of the mRNA contained within the insert of plasmid p23, the two size classes of 3-methylcholanthrene-induced cytochrome P-450 mRNA were very different in sequence. The mRNA complementary to plasmid p23 was increased about 4-fold after in vivo administration of 3-methylcholanthrene under conditions that result in maximal induction of 3-methylcholanthrene-induced cytochrome P-450 enzymatic activity. This increase in cytochrome P-450 mRNA parallels the increase in cytochrome P-450 enzymatic activity observed after 3-methylcholanthrene administration. These data suggest that the regulation of mRNA levels is an important point of control of cytochrome P-450 gene expression.

The cytochromes P-450, the terminal enzymes of microsomal mixed-function oxidases, are central to the metabolism and enzymatic detoxification of a wide variety of endogenous metabolites and xenobiotics that include carcinogens, drugs, and steroids (Conney, 1967). This system is also responsible for the enzymatic activation of many compounds into mutagenic or carcinogenic forms (Gelboin, 1980). It is well established that there are multiple forms of the cytochromes P-450, many of which are regulated by a variety of inducers (Lu & West, 1980; Haugen et al., 1975; Guengerich & Mason, 1979).

Some of the mechanisms of induction of cytochrome P-450 enzymatic activity have been elucidated in cell culture (Nebert & Gelboin, 1968, 1970; Whitlock & Gelboin, 1974). Induction is caused by a variety of treatments including exposure to polycyclic aromatic hydrocarbons such as benzo[*a*]anthracene (Nebert & Gelboin, 1970; Whitlock & Gelboin, 1974) and exposure to cAMP (Yamasaki et al., 1975). Induction also occurs following temporary inhibition of protein synthesis (Whitlock & Gelboin, 1974). Both protein and RNA syntheses are required for induction (Nebert & Gelboin, 1968, 1970; Whitlock & Gelboin, 1970).

The molecular biology of the cytochromes P-450 and their regulation are presently under vigorous investigation. Recombinant DNA techniques are being used to develop the tools needed to examine cytochrome P-450 regulation at the levels of mRNA transcription, processing, and degradation and to elucidate the structure and multiplicity of the cytochrome P-450 genes. To date, cDNAs derived from three different cytochrome P-450 mRNAs have been cloned (Negishi et al., 1981; Fujii-Kuriyama et al., 1981; Bresnick et al., 1981; Adesnik et al., 1981). One cloned cDNA complementary to

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0006-2960/82/0421-6574$01.25/0

rat PB-P-450[1] mRNA was found to be a full length copy of the corresponding mRNA. This cDNA has been completely sequenced (Fujii-Kuriyama et al., 1982), providing a great deal of information about PB-P-450 gene and mRNA structure. The mouse MC-P-450 cDNAs that have been cloned are less than full-length copies of the mRNA (Negishi & Nebert, 1981) but they have been used as a hybridization probes to characterize the corresponding mRNA (Tukey et al., 1981). In this way it has been shown that the mRNA complementary to these cloned cDNAs is about 23 S in size and is induced several fold by MC. A rat MC-P-450 has been cloned (Bresnick et al., 1981), but the properties of the mRNA complementary to this cloned cDNA have not been reported.

Here we report the cloning of cDNA derived from rat MC-P-450 mRNA and the use of this cDNA to quantitate and to determine the size of one species of rat MC-P-450 mRNA. Our results are in striking contrast to those reported for mouse MC-P-450 mRNA (Tukey et al., 1981). The size of the mRNA complementary to our cDNA is 2.0 kb, while that isolated from the mouse is 23 S or about 3.5 kb. Furthermore, the mouse mRNA is induced to a much greater extent than that from the rat. In conjunction with data presented elsewhere (J. B. Fagan et al., unpublished experiments), these findings suggest that rat MC-P-450 is translated from two distinct size classes of mRNA, one about 2.0 kb in length and one about 2.8 kb in length.

Materials and Methods

Synthesis of Double-Stranded cDNA. Single-stranded cDNA was synthesized by a modification of published procedures (Myers et al., 1977; Sobel et al., 1978) using total poly(A^+) RNA from MC-induced rat liver as the template. The reaction mixture contained 40 μg/mL $(dT)_{10}$ (P-L Biochemicals, Inc.), 80 μg/ mL mRNA, 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 8 mM $MgCl_2$, 0.4 mM DTT, 1 mM each of dATP, dCTP, dGTP, and TTP, 0.2 mCi/mmol α-^{32}P-labeled dATP, dCTP, and dGTP (Amersham Corp.), 4 mM sodium pyrophosphate (freshly prepared), 50 μg/mL actinomycin D, and 250 units/mL AMV reverse transcriptase (Life Sciences, Inc., St. Petersburg, FL). The mRNA and $(dT)_{10}$ were combined, heated to 70 °C for 1 min, and quick-cooled. The other components were added and incubated for 30 min at 42 °C. The reaction was stopped and incubated with alkali to degrade RNA, phenol extracted, and chromatographed on Sephadex G-50 as described (Sobel et al., 1978). The isolated cDNA was made double stranded with *Escherichia coli* DNA polymerase I (Boehringer Mannheim, Inc.) as described (Efstratiadis et al., 1976) by using [^{3}H]dATP, [^{3}H]dCTP and [^{3}H]dGTP at 0.2 Ci/mmole (New England Nuclear Corp.). Single-stranded ends and loop structures were removed from the double-stranded cDNA by digestion with S1 nuclease (Miles Biochemicals) (Ullrich et al., 1977). Eighty-seven percent of the ^{32}P-labeled deoxynucleotides incorporated into the first strand and 94% of the ^{3}H-labeled deoxynucleotides incorporated into the second strand were resistant to digestion with S1 nuclease. A library of recombinant plasmids containing these cDNA sequences was constructed by the G-C tailing procedure (Bolivar et al., 1977; Roychoudhury et al., 1976; Villa-Komaroff et al., 1978). $(dC)_{15-25}$ was added to the 3′ termini of the S1-treated, double-stranded cDNA by the terminal transferase reaction (Bolivar et al., 1977). The terminal transferase reaction was carried out at 37 °C for 50 min. The reaction components were 2.1 μg of double-stranded, S1 nuclease treated cDNA, 0.7 mM dCTP, 1 μCi of [^{32}P]-dCTP, 50 mM Tes–KOH (pH 7.2), 0.8 mg/mL bovine serum albumin, 40 mM KCl, 2 mM 2-mercaptoethanol, 1 mM $CoCl_2$, and 20 units of terminal transferase (Bethesda Research Laboratories). Recombinant plasmids were assembled by annealing this cDNA to pBR322 DNA that had been linearized with the restriction endonuclease *Pst*I and tailed with $(dG)_{15-25}$ a reaction identical with that for the dC tailing of cDNA, except that 0.7 mM dGTP and 1 μCi of [^{32}P]dGTP were used. The annealing reactions were carried out in sealed capillary tubes that had been treated with dichlorodimethylsilane. The reactions were heated to 60 °C for 2 min and then incubated sequentially at 42 °C for 1 h, 37 °C for 1 h, and 22 °C for 10 min.

Bacterial Transformations and Antibiotic Screening of Transformants. E. coli (strain C600) was transformed with the recombinant plasmid population as described (Enea et al., 1975). Transformants were selected on LB agar plates with 15 μg of tetracycline/mL, and recombinant transformants were distinguished from pBR322 transformants by their inability to grow on LB agar plates with 25 μg of ampicillin/mL. Seven hundred and twenty (15%) of the tetracycline-resistant transformants were ampicillin sensitive.

Colony Hybridization. Tetracycline-resistant, ampicillin-sensitive transformants were screened by colony hybridization (Grunstein & Hogness, 1975) using a differential screening protocol in which duplicate filters were hybridized with different [^{32}P]cDNA preparations. One [^{32}P]cDNA preparation was derived from partially purified cytochrome P-450 mRNA from MC-induced rat liver and the other was derived from a corresponding mRNA fraction from untreated rats. The preparation of the mRNA fractions used as template for the synthesis of high specific activity ^{32}P-labeled cDNA is described in detail elsewhere (J. B. Fagan et al., unpublished experiments). Briefly, total poly(A^+) RNA prepared by two cycles of oligo(dT)–cellulose chromatography was fractionated by methylmercuric hydroxide–agarose gel electrophoresis (Bailey & Davidson, 1976). RNA eluted from sequential gel slices was translated in vitro. MC-P-450 mRNA activity was located by immunoprecipitation of the translation products with rabbit IgG specific for MC-P-450. The active fraction from MC-induced liver RNA and the corresponding fraction from control RNA were used to generate [^{32}P]cDNA for screening. About 3% of the colonies were found to hybridize to the [^{32}P]cDNA from induced liver and not to that from uninduced liver. These 20 colonies were selected for further analysis.

Hybridization Selected Translation. Plasmid DNA was isolated from chloramphenicol-amplified, mixed cultures containing four to six selected clones by a cleared lysate procedure, and the plasmid DNA was bound to nitrocellulose as described (Parnes et al., 1981). Filter disks, 0.5 cm in diameter, containing about 4 μg of plasmid DNA were hybridized for 4 h at 37 °C to 2 μg of poly(A^+) RNA in 25 μL of 50% formamide (deionized by stirring 1 L with 50 g of Dowex mixed-bed resin for 30 min at room temperature, followed by filtration), 0.4 M NaCl, 10 mM Pipes–NaOH (pH 6.4), 0.1% $NaDodSO_4$, and 1 mM EDTA with 0.5 mg/mL calf liver tRNA carrier. The filters were washed 9 times at 37 °C with 10 mM Pipes–NaOH (pH 6.4), 150 mM NaCl,

[1] Abbreviations: MC, 3-methylcholanthrene; PB, phenobarbital; MC-P-450, MC-induced cytochrome P-450; PB-P-450, PB-induced cytochrome P-450; control-P-450, cytochrome P-450 from untreated rats; DBM-paper, diazobenzyloxymethyl-paper; SSC, 0.15 M NaCl–0.015 M sodium citrate; $NaDodSO_4$, sodium dodecyl sulfate; DTT, dithiothreitol; Pipes, piperazine-*N*,*N*′-bis[2-ethanesulfonic acid]; BSA, bovine serum albumin; Tes, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]-ethanesulfonic acid; kb, kilobase.

1 mM EDTA, and 0.5% $NaDodSO_4$, 4 times at 37 °C with the same buffer without $NaDodSO_4$, and once at room temperature with a 1:10 dilution of this buffer without $NaDodSO_4$. Hybridized mRNA was eluted at 100 °C for 1 min into 300 μL of H_2O containing 16 μg of carrier tRNA, ethanol precipitated, and dissolved in 1.3 μL of H_2O. The eluted RNA was translated in vitro in a reticulocyte lysate translation system (New England Nuclear). Translation products were immunoprecipitated (Richert et al., 1979), with a monoclonal antibody specific for MC-P-450, and along with total translation products, the immunoprecipitates were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis (Laemmli, 1970; Cabral & Schatz, 1979) and fluorography (Bonner & Laskey, 1974).

MC-P-450-Specific Antibodies. IgG specific for rat liver MC-P-450 was prepared in rabbits using highly purified rat MC-P-450 as the antigen. A complete description of this antibody preparation is presented elsewhere (Guengerich & Mason, 1979; Baron et al., 1978). A monoclonal antibody specific for rat liver MC-P-450 was prepared by using highly purified rat liver MC-P-450 as the antigen. This monoclonal antibody inhibits the aryl hydrocarbon hydroxylase activity of purified MC-P-450 by more than 90% but does not inhibit PB-P-450 aryl hydrocarbon hydroxylase activity significantly. Thus, this monoclonal antibody is clearly specific for MC-P-450. The preparation and properties of this monoclonal antibody have been described (Park et al., 1982).

Isolation of p23 Insert DNA, End Labeling, and Restriction Analysis. After *Pst*I digestion of the plasmid, the cDNA insert was isolated by a modification of the method of Kuhn et al. (1979). Briefly, the cDNA was separated from the vector DNA by electrophoresis through low-melt agarose (Bethesda Research Laboratories). The insert band was excised from the gel, melted at 65 °C with 2 volumes of 0.5 M ammonium acetate, and extracted with an equal volume of phenol that was saturated with 0.5 M ammonium acetate. The aqueous layer was extracted twice with $CHCl_3$ and the insert DNA isolated by ethanol precipitation. The labeling of the insert with ^{32}P using polynucleotide kinase and the preparation and electrophoretic isolation of fragments labeled at a single end with ^{32}P were carried out as described (Maxam & Gilbert, 1977).

Restriction endonuclease digestions were carried out under the conditions suggested by the manufacturer (New England Biolabs or Bethesda Research Laboratories). Restriction digests were analyzed by electrophoresis on 8% polyacrylamide gels (Peacock & Dingman, 1968). DNA fragments end labeled with ^{32}P were visualized by autoradiography.

RNA Blot Hybridization. Total RNAs, separated by electrophoresis in 1% agarose gels containing 6 mM methylmercuric hydroxide (Bailey & Davidson, 1976), were transferred to DBM-paper and the filters pretreated as described (Alwine et al., 1977). Plasmid DNA was labeled with ^{32}P to a specific activity of (150–300) × 10^6 cpm/μg by nick translation (Maniatis et al., 1975). This probe was hybridized to DBM filters for 24 h at 37 °C in 50% deionized formamide, 5 × concentrated SSC, 1 × Denhardt's solution (without BSA) (Denhardt, 1966), 0.1% (w/v) $NaDodSO_4$, 1% (w/v) glycine, and 1 mM EDTA with 0.15 mg/mL yeast RNA carrier. The filters were washed 4–6 times for 15–30 min at 37 °C with 1 × concentrated SSC, 0.1% $NaDodSO_4$, and 1 mM EDTA, blotted dry, and autoradiographed at −70 °C by using Kodak XAR film and intensifying screens.

Results

In Vitro Translation of Cytochrome P-450 mRNA. Figure 1 compares the translation products derived from mRNA isolated from the livers of untreated rats and rats treated with MC. When the translation products of these two mRNAs were immunoprecipitated with IgG specific for MC-P-450, three peptides with molecular weights of 58 000 (band A), 55 000 (band B), and 45 000 (band C) were found to be markedly increased in the translation products directed by the RNA isolated from the livers of MC-treated rats. The radioactive material appearing at the electrophoretic front probably consists of short peptides resulting from incomplete translation of liver mRNAs. Those in lane 4 appear to have been specifically immunoprecipitated with IgG specific for MC-P-450 and, therefore, were probably derived from MC-P-450 mRNA. The larger two peptides (bands A and B) were present in very low amounts in the translation products of all control RNA preparations. The 45 000-dalton peptide was present in significant amounts in the translation products of some preparations of control RNA but was always much increased by MC treatment. Our objective was to clone cDNAs corresponding to the mRNAs for the MC-specific proteins.

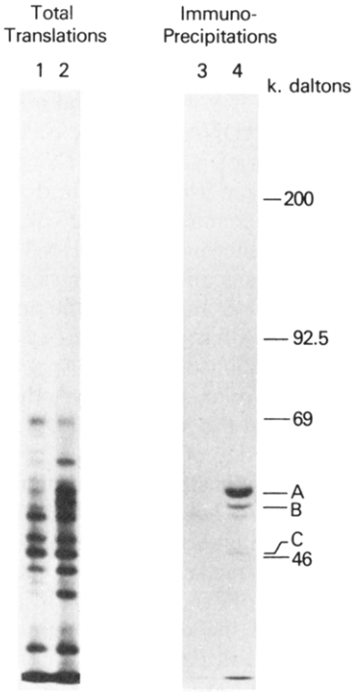

FIGURE 1: In vitro translation products derived from RNA from control rats and from rats treated with MC. Total RNA, isolated from the livers of control rats or of rats injected with 40 mg of MC/kg, ip, 24 h before sacrifice, was translated in vitro. Translation products were immunoprecipitated by using rabbit IgG specific for MC-induced cytochrome P-450 and analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis followed by fluorography. Lanes 1 and 2 are total translation products derived from liver RNA from control and MC-treated rats, respectively. Lanes 3 and 4 are the corresponding immunoprecipitations.

Identification of a Plasmid Containing MC-P-450 cDNA by Hybridization-Selected Translation. We constructed a cDNA library using plasmid pBR322 as the vector. Twenty plasmids containing sequences enriched after MC treatment were identified by colony hybridization. These clones were further characterized by hybridization selected translation. One of these, plasmid p23, contains about 650 nucleotides of inserted cDNA and has sequences complementary to MC-induced cytochrome P-450 mRNA. The data demonstrating this are shown in Figure 2. Lane 3 contains the translation products directed by the mRNA that specifically hybridized

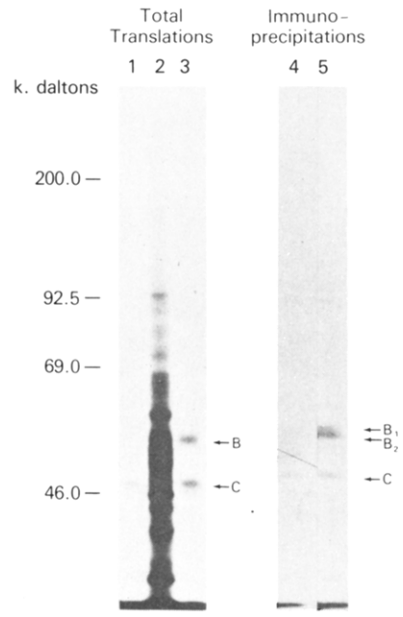

FIGURE 2: Characterization of plasmid p23 by hybridization-selected translation. Poly(A^+) RNA from the livers of rats treated with MC was hybridized for 2 h at 37 °C to nitrocellulose-immobilized plasmid p23. Bound RNA was then eluted and translated in vitro. The translation products and immunoprecipitates were analyzed by Na-DodSO_4–polyacrylamide gel electrophoresis and fluorography. Lane 1 contains translation products of RNA endogenous to the reticulocyte lysate translation system. Lane 2 contains the translation products of total poly(A^+) RNA from MC-treated rats. This RNA was the input RNA for hybridization selection. Lane 3 contains the translation products derived from the RNA that specifically hybridized to nitrocellulose-bound plasmid p23. Lanes 4 and 5 contain immunoprecipitations of the total translation products present in lane 3. The antibody used in lane 4 was a "control" monoclonal antibody produced by a hybridoma formed from the fusion of myeloma cells with spleen cells from nonimmunized mice. The antibody used in lane 5 was a monoclonal antibody specific for rat MC-P-450.

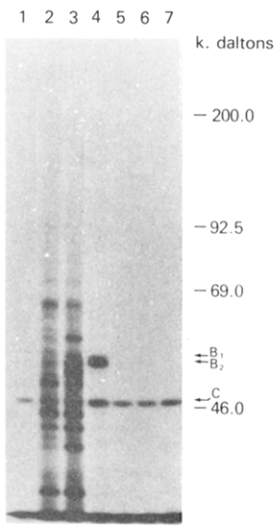

FIGURE 3: Quantitation of MC-P-450 mRNA in control and MC-treated rats by hybridization-selected translation. Liver poly(A^+) RNA from control and MC-treated rats was hybridized to filter-bound plasmid p23 or pBR322. The filters were washed, and specifically hybridizing mRNA was eluted and translated; the translation products were analyzed by Na$DodSO_4$–polyacrylamide gel electrophoresis and fluorography. Lane 1 contains endogenous translation products. Lanes 2 and 3 contain translation products derived from control and MC-induced liver poly(A^+) RNA, respectively. These were the input RNAs for the hybridizations. Lanes 4–7 contain translation products of hybridization selected RNAs. Lanes 4 and 5: MC-induced RNA was hybridized to plasmid p23 (lane 4) or pBR322 (lane 5). Lanes 6 and 7: Control RNA was hybridized to plasmid p23 (lane 6) or pBR322 (lane 7).

to plasmid p23. Two bands can be observed in this lane, bands B and C. Band C is derived from a mRNA which is endogenous to the reticulocyte lysate translation system itself, since it is also present in lane 1 which contains the products of a translation reaction to which no mRNA was added. Band B is the translation product of the mRNA that specifically hybridized to plasmid p23. This peptide corresponds to the 55 000-dalton peptide labeled band B in Figure 1. Lanes 4 and 5 show that the material in band B is immunoprecipitated by monoclonal antibodies specific for MC-P-450 (lane 5) but not by control IgG (lane 4). The endogenous translation product (band C) binds directly to formalinized *Staphylococcus aureus* (data not shown) and, therefore, is present in both immunoprecipitations. Lane 5 also demonstrates that the translation products derived from the mRNA which hybridizes to p23 can be resolved, at least partially, into two bands, labeled B_1 and B_2. This resolution was somewhat variable. Taken together, the data of Figure 2 indicate that plasmid p23 contains MC-P-450 cDNA sequences.

The ability of the inserted DNA of plasmid p23 to select MC-P-450 mRNA by hybridization is also established by the experiment shown in Figure 3. Here we show that only the cDNA insert in plasmid p23 and not the recombinant vector portion of the plasmid is responsible for hybridization selection. This experiment also shows that the mRNAs which are selected by plasmid p23 are present in much larger quantities in MC-treated than in control rats. Liver poly(A^+) RNA from control and from MC-treated rats were hybridized to nitrocellulose-immobilized plasmids p23 and pBR322, and the RNA complementary to these plasmids was eluted and translated in vitro. The second and third lanes contain translation products of total liver poly(A^+) RNA from control and MC-induced rats, respectively. These were the input RNAs for hybridization. The fourth and fifth lanes are translation products of the MC-induced RNA that hybridized specifically to plasmids p23 and pBR322, respectively; the sixth and seventh lanes are translations of the control RNA that hybridized to these plasmids. Bands B_1 and B_2 are present in lane 4 but not in lane 5. This shows that the mRNAs that hybridize specificially to plasmid p23 do not hybridize to plasmid pBR322. Bands B_1 and B_2 are easily detected by in vitro translation of hybridization-purified RNA from MC-induced liver (lane 4). In contrast, these bands are almost undetectable in hybridization-purified RNA from control rat liver (lane 6). Longer exposures do, however, show that the mRNAs coding for these peptides are present in untreated rats. These results indicate that, although the mRNAs for both of these peptides are present at low levels in control liver, these RNAs are highly inducible by MC. As noted above, band C is a translation product derived from a mRNA endogenous to the reticulocyte lysate translation system.

Restriction Analysis of Plasmid p23. The restriction map of the cDNA insert of plasmid p23 is presented in Figure 4. The insert is about 650 nucleotides in length. The cleavage sites for all restriction endonucleases known to cut within the insert are indicated. The following restriction endonucleases did not cut within the insert of plasmid p23: *Ava*I, *Ava*II, *Bam*HI, *Bst*NI, *Eco*RI, *Hae*II, *Hin*cII, *Hin*dIII, *Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Pvu*I, *Sal*I, *Sma*I, and *Xor*II.

Quantitation of MC-P-450 mRNA by RNA Blot Hybridization. ^{32}P-Labeled plasmid p23 was used to quantitate the level of corresponding mRNA in MC-treated and untreated rat liver. We used another cloned cDNA, designated p9, as a control. Plasmid p9 was isolated in conjunction with p23 and has not been characterized except to show that the mRNA

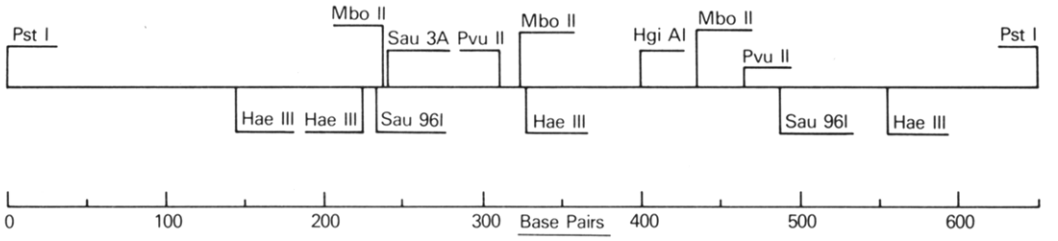

FIGURE 4: Restriction map of the cDNA insert of plasmid p23.

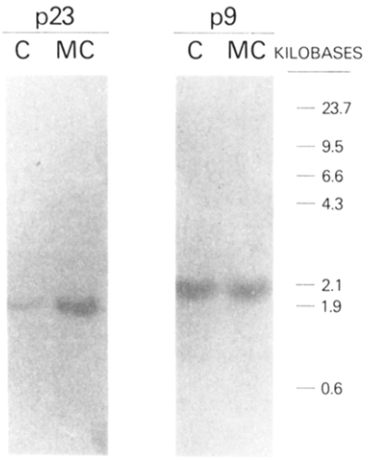

FIGURE 5: RNA blot hybridization of liver RNAs from control and MC-treated rats. Twenty micrograms of total RNA from control rats (C) and rats treated for 24 h with 40 mg of MC/kg (MC) were fractionated by electrophoresis in a methylmercuric hydroxide–agarose gel and transferred to DBM-paper. ^{32}P-Labeled plasmids p23 and p9 were hybridized to duplicate filters and autoradiographed. The position and sizes of DNA marker fragments of phage λ DNA generated by digestion with the restriction endonuclease *Hin*dIII are indicated at the right.

complementary to this plasmid is not induced by MC. Figure 5 presents autoradiograms of transfers of control and MC-induced liver RNA which have been hybridized with ^{32}P-labeled plasmids p23 or p9. Hybridization of plasmid p9 with either control or MC-RNA resulted in single bands of equal intensity. This indicates that control and MC-RNAs were applied uniformly to the gel and transferred uniformly to DBM-paper in this experiment. Hybridization with plasmid p23 also resulted in a single band, with both control and MC-RNAs. However, the band was much more intense in the lane containing MC-RNA than in that containing control RNA. This shows that plasmid p23 hybridizes to mRNA of a discrete size that is markedly increased in MC-induced RNA. This and similar autoradiograms for three preparations of control RNA and three preparations of MC-induced RNA were quantitated by densitometry. By this method MC-P-450 mRNA was found to be 4.1 times more abundant in RNA isolated from MC-induced rats than in RNA isolated from controls. By comparison with molecular weight standards run on the same gel, the MC-P-450 mRNA that is complementary to plasmid p23 is 2000 nucleotides in length. This agrees well with size estimates from independent experiments in which cytochrome P-450 mRNAs were located in denaturing agarose gels by elution and in vitro translation (J. B. Fagan et al., unpublished experiments).

Discussion

We have constructed a library of recombinant plasmids containing cDNAs complementary to the total mRNA population isolated from the livers of MC-treated rats. From this library we have isolated recombinants containing cDNA inserts complementary to the 2000 nucleotide mRNA for MC-induced cytochrome P-450. In this paper one MC-P-450 cDNA containing plasmid, designated p23, that has an insert of about 650 nucleotides, has been used to characterize one size class of MC-P-450 mRNA. The primary evidence that plasmid p23 contains MC-P-450 cDNA is that it hybridizes to mRNA that codes for peptides that are immunoprecipitable with a monoclonal antibody specific for MC-P-450. The specificity of this antibody is well established; as well as reacting positively with MC-P-450 by radioimmune assay, it inhibits MC-P-450 aryl hydrocarbon hydroxylase activity by 80–90% (Park et al., 1982). The peptides coded by the p23-specific mRNA have molecular weights in the range expected for MC-inducible cytochromes P-450. The size (2000 bases) and inducibility (4-fold) of the mRNA complementary to plasmid p23 are also consistent with the conclusion that this mRNA codes for an MC-inducible cytochrome P-450.

We have found translation and immunoprecipitation evidence that there are at least two size classes of mRNAs that code for MC-P-450 peptides (J. B. Fagan et al., unpublished experiments). One class of MC-P-450 mRNAs is about 2.0 kb in length and the other is about 2.8 kb. Analogous experiments in mouse have demonstrated the existence of two size classes of MC-P-450 mRNA in that species, having sizes of 2.7 and 3.5 kb (Negishi et al., 1981). Thus, it appears that for each species there are two size classes of mRNA that code for MC-P-450 but that the actual size of the mRNAs are different for rat and mouse. Plasmid p23 hybridizes very specifically to the 2.0-kb rat MC-P-450 mRNA and not to the 2.8-kb size class. This indicates that, at least within the region of the mRNA complementary to the cloned cDNA, the two size classes of MC-P-450 mRNA are very different in sequence. The mouse MC-P-450 cDNA that has been cloned is complementary to the larger 3.5-kb mouse mRNA that codes for a 55 000-dalton peptide (Tukey et al., 1981). Thus, to date, cDNAs derived from the smaller class of rat and the larger class of mouse MC-P-450 mRNAs have been cloned. Tukey et al. (1981) have found that the mRNA complementary to their MC-P-450 cDNA clone is induced much more than the 4-fold induction that we observe. The differences in the extents of induction of these two mRNAs may be due to differences in the inducibility of the different size classes of P-450 mRNA, although viable alternative explanations also exist. Quantitation of both message classes in both species should provide an explanation for this observation. The size of the mRNA complementary to the rat MC-P-450 cDNA cloned by Bresnick et al. (1981) was not reported, nor did these workers quantitate the levels of MC-P-450 mRNA using their MC-P-450 cDNA clone. However, they do present a restriction map that can be compared to that of the insert of plasmid p23. The cDNAs cloned by both groups were made by using oligo(dT) as the primer and therefore should correspond in sequence to the 3′ end of the mRNA. Furthermore, the insert of the plasmid described by Bresnick et al. (1981) is 1000 while that of plasmid p23 is 650 bases long. Therefore, if these clones were derived from the same mRNA, it would

be expected that they would overlap and that a major portion of the restriction maps of these two inserts would be similar. This is not the case. The insert of the plasmid described by Bresnick et al. (1981) contains three *Hae*III sites that are oriented quite differently from the four *Hae*III sites in our insert. Their insert is cut by *Taq*I and by *Ava*II while ours is not. From these data, it appears that the two groups have cloned sequences from different mRNAs.

Lane 5 of Figure 2 indicates that the translation products derived from the mRNA complementary to plasmid p23 consists of two species that are partially resolvable by Na-DodSO_4–polyacrylamide gel electrophoresis. Two possible explanations for this observation are, first, that two mRNAs that code for P-450s of slightly different molecular weights might be homologous in sequence to plasmid p23 and, second, that the smaller peptide might be a proteolytic digestion product of the larger. These alternatives are presently under investigation.

It has been previously shown that cytochrome P-450 mRNA translational activity is elevated in the livers of rats treated with MC or PB (Kumar & Padmanabhan, 1980; Bar-Nun et al., 1980; Dubois & Waterman, 1979; Lechner et al., 1979; Colbert & Bresnick, 1979). The results presented in Figure 1 confirm this finding and those of Figure 3 demonstrate that this is the case for the MC-P-450 mRNA that is complementary to plasmid p23. Since in vitro translation measures the biochemical activity of a mRNA species, it was not clear from these data whether the increase in translational activity reflected an alteration in the number, or in the translatability, of the cytochrome P-450 mRNA molecules. The RNA blot hybridization experiment of Figure 5 was carried out to determine the size and to measure directly the levels of the cytochrome P-450 mRNA complementary to plasmid p23 in RNA from control and MC-induced rat liver. The dose of MC (40 mg/kg) and the period of induction (24 h) used in this experiment give the maximal induction of MC-P-450 enzymatic activity obtainable with a single injection (Conney et al., 1956, 1957). Detailed kinetics of the induction process will be dealt with elsewhere. This experiment demonstrates that, under the induction conditions used, the levels of this MC-P-450 mRNA are increased about 4-fold. Thus, the increase in translational activity is probably the consequence of an increase in the number of cytochrome P-450 mRNA molecules per cell and not the consequence of some biochemical alteration of the cytochrome P-450 mRNA molecule resulting in an increase in its translational activity. Other investigators (Negishi et al., 1981; Tukey et al., 1981) have reached this same conclusion for the induction of P-450 by MC in mouse liver. The correlation between cytochrome P-450 induction and increased cytochrome P-450 mRNA levels suggests that the regulation of mRNA levels is an important point of control of cytochrome P-450 gene expression. This has also been found with other eukaryotic genes, such as those for fibronection (Fagan et al., 1981) and hemoglobin (Wood et al., 1978). Whether this regulation occurs in the P-450 system at the level of transcription, as has been suggested (Tukey et al., 1981), or at the level of mRNA degradation cannot be determined from the data presented here or from that presently available in the literature. These data provide information only on the total quantity of a particular mRNA species present at a given point in time. That quantity is the result of the dynamic balance between mRNA transcription and degradation. Experiments in which P-450 mRNA is pulse–chase labeled and quantitated by hybridization to cloned P-450 cDNA are required to gain information concerning the rates of MC-P-450 mRNA transcription and degradation.

The cytochrome P-450 system is quite complex. It is thought to consist of a family of structurally related enzymes that have distinct, yet overlapping substrate specificities and that are regulated by a variety of inducers. Using recombinant DNA techniques, it should be possible to develop highly specific reagents for the genes and mRNAs that code for each member of this family of enzymes, making it possible to study their properties and behavior individually. Plasmid p23 is one such reagent. As the collection of recombinant plasmids expands to include specific reagents for each member of the family of cytochromes P-450, increasingly more specific questions will be approachable experimentally. These plasmids will be used to study the regulation of the cytochrome P-450 system at the levels of mRNA transcription, processing, and degradation. They will also serve as tools for studying the molecular genetics of the cytochromes P-450 and the structure and mutliplicity of the cytochrome P-450 genes. Not only will this contribute to our basic understanding of gene structure and gene regulation but also this will give us practical insight into the role of the cytochromes P-450, and of their regulation, in carcinogenesis and in drug and xenobiotic metabolism.

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Configurations of Glycosidic Phosphates of Lipopolysaccharide from *Salmonella minnesota* R595†

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ABSTRACT: The anomeric configurations of the reducing terminal glucosamine and 4-amino-4-deoxy-L-arabinose phosphates in lipopolysaccharide from *Salmonella minnesota* R595 have been determined by nuclear magnetic resonance. Chemical shifts for the anomeric protons were obtained by selective decoupling of the phosphorus spectrum and proton–proton coupling constants by polarization transfer from protons to phosphorus. In both cases, the phosphate is attached to the sugar in an axial orientation.

Considerable progress has been made in the determination of the chemical nature of LPS[1] from Gram-negative bacteria (Galanos et al., 1977). The lipidic subunit of the molecule, known as lipid A, is similar in both *Escherichia coli* K-12 (Rosner et al., 1979a,b,c) and *Salmonella minnesota* (Gmeiner et al., 1969; Mühlradt et al., 1977), but some aspects of the structure remain undetermined. In particular, the anomeric configuration is known for only one of the three sugars in lipid A. These configurations will play an important part in determining the shape of the molecule and its ability to pack into bilayer membranes. The information is also of importance to workers attempting to synthesize the minimal active subunit of the endotoxin (Inaga et al., 1981; Nashed & Anderson, 1981).

Strain R595 of *S. minnesota* is a deep rough mutant (Gmeiner et al., 1969), and LPS from it has a side chain consisting only of a branched trimer of 3-deoxy-D-*manno*-octulosonic acid attached at the 3′ position of lipid A (Figure 1). The lipid A itself consists of two β1→6-linked D-glucosamine residues, with approximately five ester-linked and two amide-linked fatty acids. The 1 and 4′ positions of the disaccharide are phosphorylated. The 1 substituent is mostly

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[1] Abbreviations: LPS, lipopolysaccharide; NMR, nuclear magnetic resonance; EDTA, disodium ethylenediaminetetraacetate; KDO, 3-deoxy-D-*manno*-octulosonic acid.

0006-2960/82/0421-6580$01.25/0