Multiple Cytochromes P-450 Are Translated from Multiple Messenger Ribonucleic Acids†

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ABSTRACT: The cytochromes P-450 consist of a family of enzymes that are related structurally and functionally, yet differ in their substrate specificities, in their responses to different inducers, and in their physical and immunological characteristics. We have investigated the molecular basis for the multiplicity of the cytochromes P-450 by characterization of their mRNAs and by immunological analysis of the in vitro translation products of these mRNAs. RNAs isolated from the livers of control, 3-methylcholanthrene-treated, and phenobarbital-treated rats were translated in vitro in a rabbit reticulocyte lysate system, and cytochrome P-450 related peptides were identified by immunoprecipitation with monoclonal and aclonal antibodies followed by sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis. 3-Methylcholanthrene was found to induce translatable mRNAs, coding for three cytochrome P-450 related peptides of 57.9, 55.4, and 45.0 kilodaltons. Phenobarbital induced mRNA that coded for a single cytochrome P-450 related peptide of 51.7 kilodaltons. 3-Methylcholanthrene induced two other mRNAs, coding for peptides of 62.5 and 40.3 kilodaltons. These peptides bound selectively to formalinized *Staphylococcus aureus* but did not interact with P-450-specific antibodies; therefore these peptides were not cytochromes P-450. The induction of mRNAs for these peptides indicates that 3-methylcholanthrene affects the regulation of genes other than those for the cytochromes P-450. Total poly(A^+) RNAs from the livers of 3-methylcholanthrene- and phenobarbital-treated rats were size fractionated under fully denaturing conditions by methyl mercuric hydroxide–agarose gel electrophoresis. The RNA fractions recovered from the gel were translated in vitro. The fractions containing cytochrome P-450 mRNA were identified by immunoprecipitation of the translation products of each fraction with cytochrome P-450 specific antibodies followed by $NaDodSO_4$–polyacrylamide gel electrophoresis. It was found that the cytochromes P-450 from phenobarbital-treated and control rat livers were both translated from mRNAs of about 2.0 kilobases in length. In contrast, the 3-methylcholanthrene-induced cytochromes P-450 were translated from two size classes of mRNA, 2.0 and 2.7 kilobases in length. The larger class probably contains more than one cytochrome P-450 mRNA species, each coding for a peptide of a different molecular weight. These findings, in conjunction with immunological characterization of the peptides translated from these mRNAs using monoclonal and aclonal antibodies specific for cytochrome P-450, suggest that each of the 3-methylcholanthrene-induced cytochromes P-450, as well as the cytochromes P-450 found in control and phenobarbital-treated liver, is derived from separate mRNAs. The molecular weights of the cytochrome P-450 peptides were not always proportional to the sizes of their mRNAs, indicating that these mRNAs have noncoding regions of different lengths. We also present preliminary evidence that the primary translation product of one MC-P-450 mRNA is processed proteolytically in vivo but not during in vitro translation.

The cytochromes P-450 are a multimember family of inducible, mixed function oxidases. These enzymes are responsible for the detoxication of a variety of xenobiotics and drugs, for the activation of carcinogens, and for important reactions in the biosynthesis of steroids and other biological molecules (Gelboin, 1980; Conney, 1967). Control liver contains low levels of at least 11 forms of P-450 (Aoyama et al., 1981). 3-Methylcholanthrene (MC),[1] phenobarbital (PB), and other compounds are known to induce more than one form of P-450 (Guengerich, 1977; Thomas et al., 1981). We are investigating the molecular basis for the multiplicity of the P-450s. This paper investigates the relationships among the different P-450s by characterization of the mRNAs coding for them and by immunological analysis of the P-450 peptides synthesized from these mRNAs in vitro. In this way it is demonstrated that separate inducible mRNAs from MC-treated rats code for at least three peptides that are immunoprecipitated with MC-P-450-specific antibodies. On the other hand, PB-P-450-specific antibodies recognize one major PB-induced peptide. It is also shown that liver from MC-treated rats contains two size classes of MC-P-450 mRNA, 2.0 and 2.7 kilobases in length. The larger of these classes probably contains more than one mRNA species, each coding for a MC-P-450 of a different size. Control P-450 and PB-P-450 are each shown to be translated from a single size of mRNA, 2.0 kilobases.

In contrast to our findings in rat, others (Tukey et al., 1981) have found that the MC-P-450 mRNAs of mouse liver are much larger, about 2.7 and 3.5 kilobases in length, and that both of these mRNAs code for peptides of the same molecular weight (55 000). Thus, although both rat and mouse have two size classes of MC-P-450 mRNA, these classes are distinctly different in size and code for different peptides. Our findings in the rat as well as those for the mouse (Tukey et al., 1981) suggest that the multiplicity of MC-P-450s is not primarily due to peptide modification or processing, although the MC-P-450 peptides may be processed by proteolytic cleavage (Kumar & Padmanabhan, 1980) and by glycosylation (Negishi et al., 1981). Instead, the multiplicity of MC-P-450s is primarily due to the existence of multiple species of MC-P-450 mRNA that are generated either from multiple MC-P-450

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[1] Abbreviations: MC, 3-methylcholanthrene; PB, phenobarbital; MC-P-450, MC-induced cytochrome P-450; PB-P-450, PB-induced cytochrome P-450; poly(A+) RNA, polyadenylated RNA; $NaDodSO_4$, sodium dodecyl sulfate; control-RNA, MC-RNA, and PB-RNA, RNAs isolated from the livers of control and MC- and PB-treated rats; anti-MC-P-450 and anti-PB-P-450, IgG preparations specific for MC- and PB-P-450s, respectively; oligo(dT), oligo(deoxythymidylate); Tris, tris(hydroxymethyl)aminomethane.

genes or from alternative modes of processing of a single MC-P-450 mRNA precursor.

Materials and Methods

RNA Purification. MC (40 mg/kg in corn oil) or PB (80 mg/kg in H_2O) was used for intraperitoneal injections of female Sprague-Dawley rats (220 g). Twenty-four hours later livers were isolated, and RNA was extracted and purified with guanidine hydrochloride (Adams et al., 1977). The resulting RNA preparation contained mRNA and rRNA and was highly active in in vitro translation.

Oligo(dT)–Cellulose Chromatography. Polyadenylated RNA was purified from total RNA by two cycles of oligo-(dT)–cellulose (P-L Biochemicals, Inc.) chromatography (Aviv & Leder, 1972), reducing the ribosomal RNA content from 95 to less than 25%.

In Vitro Translation. Total RNA (1 μg) or size fractionated poly(A^+) RNA (0.1 μg) was translated at 37 °C in a rabbit reticulocyte lysate cell-free translation system (New England Nuclear). Translation products were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis (Laemmli, 1970) as modified by Cabral & Schatz (1979) followed by fluorography (Bonner & Laskey, 1974).

Immunoprecipitation of Translation Products. A 24-μL aliquot of the translation reaction was added to 0.5 mL of immunoprecipitation buffer containing 1% Triton X-100, 1% sodium deoxycholate, 0.1% $NaDodSO_4$, 0.5% Trasylol (Sigma), 50 mM Tris-HCl (pH 7.5), and 150 mM NaCl (Richert et al., 1979). Preimmune serum (40.0 μg) was added, and the mixture was incubated on ice for 30 min. Formalinized *Staphylococcus aureus* [120 μL, 10% w/v suspension; Bethesda Research Labs; pretreated according to Richert et al. (1979)] was added and incubated for 30 min on ice. The suspension was centrifuged for 3 min at 4 °C in a microfuge and the pellet discarded. Purified carrier P-450 (0.1 μg; Guengerich & Martin, 1980) and antibody (40 μg) were added to the supernatant and incubated for 30 min on ice. To this mixture was added 100 μL of *Staph. aureus* and incubated for 30 min on ice. After centrifugation the pellets were washed at 4 °C, twice with immunoprecipitation buffer containing 0.5 M KCl, once with immunoprecipitation buffer alone and once with 10 mM Tris-HCl (pH 7.5) and 0.5% Nonidet-P-40 (Sigma). Antigen was eluted from the *Staph. aureus* by boiling in sample buffer containing $NaDodSO_4$ and 2-mercaptoethanol and analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis.

Antibodies Specific for Cytochromes P-450. The preparation and properties of the aclonal antibodies (Guengerich & Mason, 1979; Baron et al., 1978) and the monoclonal antibodies (Park et al., 1982) have been described. Highly purified rat MC-P-450 and PB-P-450 were used as antigens. These antibodies inhibit the aryl hydrocarbon hydroxylase activity of their respective antigens by more than 90% but do not inhibit the aryl hydrocarbon hydroxylase activity of the other antigens significantly (Guengerich & Mason, 1974; Baron et al., 1978; Park et al., 1982). The preimmune serum was taken before immunization from the rabbits in which antibodies to MC- or PB-P-450s were raised.

Recovery of Translatable RNA from Denaturing Agarose Gels. Twenty micrograms of total poly(A^+) RNA, prepared by two cycles of oligo(dT)–cellulose chromatography, was fractionated by methyl mercuric hydroxide–agarose gel electrophoresis (Bailey & Davidson, 1976). The gel contained 0.8% low melt agarose (Bethesda Research Labs) and 6 mM methyl mercuric hydroxide (Alpha Products). Sample buffer containing 20 mM methyl mercuric hydroxide and a 0.6 mm thick slot former were required to obtain satisfactory resolution. By use of ethidium bromide stained molecular weight markers as standards, the appropriate regions of the sample lanes were excised and cut into 1-mm slices. RNA was recovered from each slice by a modification of the procedure of Kuhn et al. (1979). Each slice was homogenized by three passes through a 25-gauge needle with 0.5 mL of 0.5 M ammonium acetate containing 16 μg of calf liver tRNA (Boehringer Mannheim). The solution was cooled to 37 °C and extracted with 0.5 mL of redistilled phenol that had been preequilibrated with 0.5 M ammonium acetate at 37 °C. After centrifugation at room temperature to bring the agarose precipitate to the interface, the aqueous phase was decanted and extracted twice with an equal volume of $CHCl_3$. RNA was ethanol precipitated from the aqueous phase and redissolved in distilled H_2O.

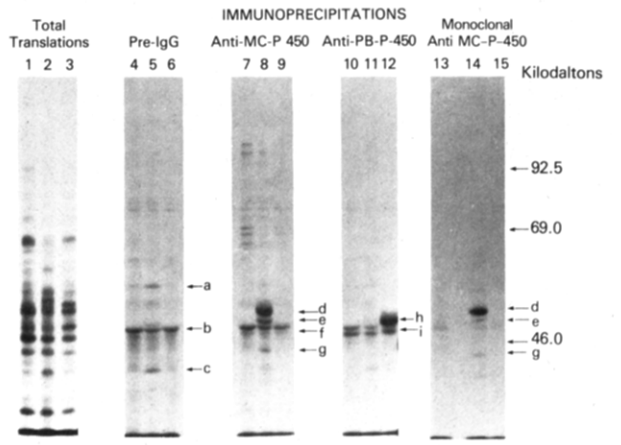

FIGURE 1: In vitro translation products of liver RNA isolated from control rats and rats treated with MC or PB. Total RNA from the livers of untreated, MC-treated, and PB-treated rats was translated in vitro. Translation products were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis directly (lanes 1–3) or immunoprecipitated with preimmune IgG (lanes 4–6), anti-MC-P-450 IgG (lanes 7–9), anti-PB-P-450 IgG (lanes 10–12), or monoclonal anti-MC-P-450 IgG (lanes 13–15). In each set of three lanes, the first contains translation products derived from control RNA and the second and third contain translation products derived from MC-RNA and PB-RNA, respectively. Peptide molecular weights are indicated at the right of the autoradiograms.

Results

Translation and Immunoprecipitation of Total RNAs. Figure 1 presents an analysis of the translation products of RNAs isolated from the livers of control and MC- and PB-treated rats (control-RNA, MC-RNA, and PB-RNA). These RNAs were translated in vitro, and the products of these reactions were immunoprecipitated with monoclonal or aclonal IgG preparations (see Materials and Methods) specific for MC-P-450 (anti-MC-P-450) and for PB-P-450 (anti-PB-P-450). The [^{35}S]methionine labeled translation products and immunoprecipitates were then analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis and fluorography. Lanes 1–3 contained the total translation products directed by control-RNA, MC-RNA, and PB-RNA, respectively. Lanes 4–6 contained the same translation products immunoprecipitated with preimmune IgG and formalinized *Staph. aureus*. Peptides of three molecular weights, 62 000, 50 100, and 40 300, were precipitated under these conditions. Two of these peptides, a and c, were increased in the translation products of MC-RNA. The third peptide, b, was present in the translation products of all three RNAs and was present in slightly lower amounts in the translation products of MC-RNA. The precipitation of these three peptides was not due to interaction with the preimmune IgG but to their direct binding to for-

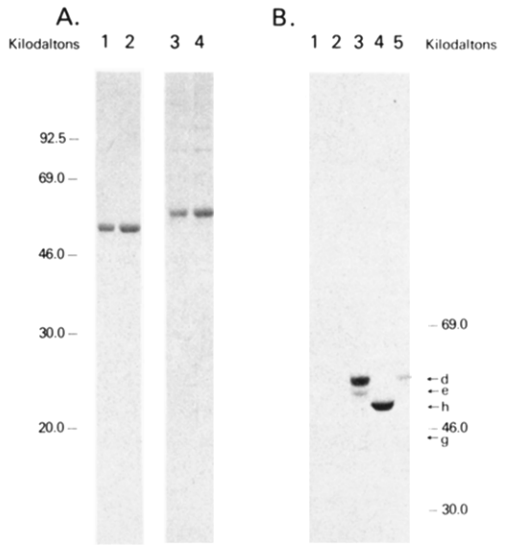

FIGURE 2: Electrophoretic comparison of purified cytochromes P-450 with the translation products immunoprecipitated by antibodies against these proteins. (Panel A) Purified PB-P-450 (lanes 1 and 2) and MC-P-450 (lanes 3 and 4) were electrophoresed through a 10% $NaDodSO_4$–polyacrylamide gel with a 5% stacking gel and the gel stained with Coomassie blue and photographed. Lanes 1 and 3 contained 0.5 μg of protein and lanes 2 and 4 contained 1.0 μg. (Panel B) MC-RNA (lanes 1, 3, and 5) or PB-RNA (lanes 2 and 4) was translated in vitro and immunoprecipitated with preimmune IgG (lanes 1 and 2), MC-P-450-specific IgG (lane 3), PB-P-450-specific IgG (lane 4), or MC-P-450-specific IgG that had been preadsorbed with PB-P-450 linked to Sepharose 4B (lane 5). The immunoprecipitates were electrophoresed through a 7% $NaDodSO_4$–polyacrylamide gel with a 3.5% stacking gel and fluorographed.

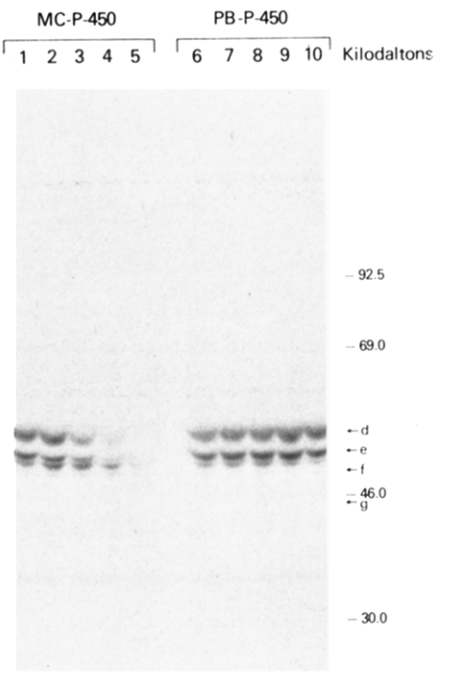

FIGURE 3: Competition of purified MC-P-450 with in vitro translation products of MC-RNA for immunoprecipitation with anti-MC-P-450. The in vitro translation products of MC-RNA were immunoprecipitated as described in Figure 1 except that increasing amounts of purified MC-P-450 (lanes 1–5) or PB-P-450 (lanes 6–10) were present during immunoprecipitation. The immunoprecipitations of lanes 1–5 contained 0.1, 0.5, 1.0, 2.0, and 10.0 μg of MC-P-450, respectively, while those of lanes 6–10 contained 0.5, 1.0, 2.0, 10.0, and 20.0 μg of PB-P-450, respectively.

malinized *Staph. aureus* (data not shown).

Lanes 7–9 and 10–12 contained translation products of control-, MC-, and PB-RNAs immunoprecipitated with anti-MC-P-450 and with anti-PB-P-450, respectively. In addition to peptides a, b, and c, which were precipitated by *Staph. aureus* alone, four other peptides, d, e, f, and g, were present in lanes 7–9. Three peptides, d, e, and g, were immunoprecipitated from the translation products of MC-RNA only. Peptide f was immunoprecipitated from the translation products derived from all three types of RNA both by anti-MC-P-450 and weakly by preimmune serum. As shown in lanes 10–12, anti-PB-P-450 immunoprecipitated two peptides, h and i. Peptide h was immunoprecipitated exclusively from the translation products of PB-RNA. Peptide i, which was of the same apparent molecular weight as peptide f, was precipitated in roughly equal amounts from the translation products of all three RNAs.

Lanes 13, 14, and 15 contained immunoprecipitations of the translation products of control-, MC-, and PB-RNAs using a monoclonal antibody specific for rat MC-P-450 (see Materials and Methods). This antibody specifically immunoprecipitated peptides d, e, and g, although it had a much higher affinity for peptide d than for peptides e and g. The immunoprecipitation of these peptides with this MC-P-450-specific monoclonal antibody indicated that they have antigenic sites in common with rat liver MC-P-450.

In order to achieve maximal recoveries of ^{35}S-labeled peptides in the experiment of Figure 1, large amounts (40 μg) of antibody were used in each immunoprecipitation. These levels of antibody distorted the electrophoretic migration of peptides d and e, making it impossible to accurately determine their molecular weights from this experiment. Figure 2B presents immunoprecipitations carried out with much lower levels of antibodies, allowing the accurate determination of the molecular weights of the P-450 peptides. Figure 2A presents an electrophoretic analysis of the purified MC-P-450 and PB-P-450 that were used as antigens in the preparation of both the aclonal and monoclonal antibodies used in the experiments reported here. Comparison of electrophoretic mobilities shows that the antigen used to generate anti-PB-P-450 antibodies (lanes 1 and 2, Figure 2A) and the major in vitro translated peptide recognized by this antibody (band h, lane 4, Figure 2B) have the same apparent molecular weight, about 52 000. The minor peptide recognized by anti-PB-P-450, peptide i, was not visible in this autoradiogram. For the MC-P-450s the situation was more complex. The purified protein ran as a single band of M_r 55 500 (lanes 3 and 4, Figure 2A), while both the antibodies that were developed against this antigen and that inhibit MC-P-450 enzymatic activity immunoprecipitated translation products of four distinct molecular weights, 57 900, 55 400, 49 000, and 45 000 (bands d, e, f, and g, respectively, of lane 3, Figure 2B). Bands f and g were only faintly visible in this autoradiogram. Lane 5 of Figure 2B presents the translation products recognized by anti-MC-P-450 that had been preadsorbed with purified PB-P-450-linked to Sepharose 4B to remove IgG species recognizing epitopes common to both MC-P-450 and PB-P-450. Only band d was precipitated by this antibody.

Figure 3 shows that the purified MC-P-450 that migrated as a single band in Figure 2A, lanes 3 and 4, competed with all four in vitro translated peptides for binding to anti-MC-P-450 IgG. In this experiment the competition between purified MC-P-450 and ^{35}S-labeled, in vitro translated peptides for binding and immunoprecipitation with anti-MC-P-450 IgG was measured. The translation products of MC-RNA were immunoprecipitated under standard conditions, except that increasing amounts of purified MC-P-450 were added in lanes 1–5 and, as a control, increasing amounts of PB-P-450 were

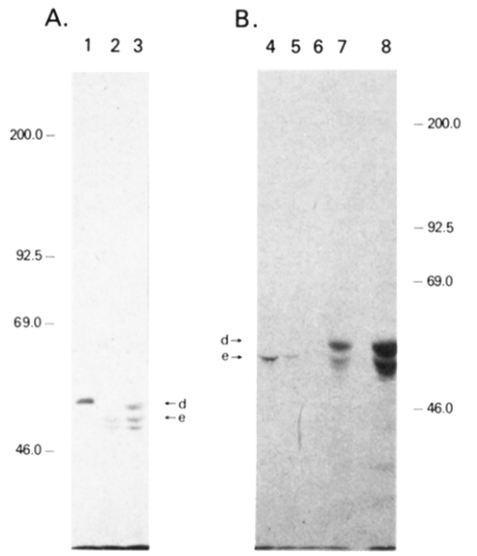

FIGURE 4: Sequential immunoprecipitations of the translation products of MC-RNA using monoclonal and aclonal antibodies to MC-P-450. (Panel A) An in vitro translation reaction of MC-RNA was first immunoprecipitated with MC-P-450-specific monoclonal antibody MA-A (lane 1). The material that was not precipitated with MA-A was then immunoprecipitated with aclonal anti-MC-P-450 IgG (lane 2). A parallel reaction was immunoprecipitated only with aclonal anti-MC-P-450 IgG (lane 3). (Panel B) A single in vitro translation reaction of MC-RNA was immunoprecipitated sequentially first with MC-P-450-specific monoclonal antibody MA-B (lane 4), a second time with MA-B (lane 5), third with *Staph. aureus* alone (lane 6), and, finally, with aclonal anti-MC-P-450 IgG (lane 7). A parallel translation reaction was immunoprecipitated only with aclonal anti-MC-P-450 IgG (lane 8).

added in lanes 6–10. PB-P-450 had no effect on the efficiency with which ^{35}S-labeled peptides were immunoprecipitated with anti-MC-P-450. Increasing amounts of purified MC-P-450 caused a progressive decrease in the quantities of ^{35}S-labeled peptides that were immunoprecipitated. The extent of this competition did, however, vary with different ^{35}S-labeled peptides. Immunoprecipitation of peptide d was partially blocked by 1 μg of purified MC-P-450 and completely blocked by 10 μg. The immunoprecipitation of peptides e and f was only partially blocked, even by 10 μg of purified MC-P-450. In longer exposures, this experiment showed that competition with band g followed a pattern similar to band d. Even though the unlabeled, purified antigen had a molecular weight of 55 500, it competed more strongly with the M_r 57 900 than the M_r 55 400 in vitro translated peptide.

Figure 4 further clarifies the relationships among the peptides recognized by MC-P-450-specific antibodies. In this experiment monoclonal and aclonal antibodies were used to demonstrate that, although the MC-inducible peptides d, e, and g are antigenically related, they also have antigenic determinants that are different from each other. In each part of this experiment, a single translation reaction was immunoprecipitated sequentially with two different antibodies to assess the antigenic relatedness of the peptides recognized by these two antibodies. All of the antibodies used in this experiment strongly inhibit MC-P-450 enzymatic activity; therefore the peptides recognized by them are certainly antigenically related to MC-P-450 and may be MC-P-450 peptides themselves. In the first panel of Figure 4 the translation products of MC-RNA were first immunoprecipitated by monoclonal antibody A (MA-A), resulting in the electrophoretic pattern shown in lane 1 which is similar to that found in lane 14 of Figure 1. The material that was not immunoprecipitated with MA-A was then incubated with aclonal anti-MC-P-450 IgG, resulting in the electrophoretic pattern shown in lane 2. When MC-RNA translation products were immunoprecipitated with aclonal anti-MC-P-450-IgG without previous treatment with another antibody, the pattern of peptides shown in lane 3, similar to that of lane 8 of Figure 1, resulted. These results suggest that the major peptide recognized by MA-A is identical with peptide d, recognized by aclonal anti-MC-P-450 IgG, since the immunoprecipitated translation products contained in lane 3 included peptide d, while those of lane 2, which had been first immunoprecipitated with MA-A before immunoprecipitation with aclonal anti-MC-P-450 IgG, lacked this peptide.

A similar sequence of immunoprecipitations in the second panel of Figure 4 demonstrates, first, that band e of Figure 1 consists of at least two components and, second, that another monoclonal antibody preparation, MA-B, has affinities for the P-450 peptides that are different from those of MA-A, even though MA-B is known to inhibit enzymatic activity similarly to MA-A. In this experiment a single MC-RNA-directed translation reaction was immunoprecipitated sequentially first with MA-B (lane 4), then a second time with MA-B (lane 5), then with *Staph. aureus* alone (lane 6), and, finally, with aclonal anti-MC-P-450 (lane 7). A second MC-RNA-directed translation reaction was immunoprecipitated only with aclonal anti-MC-P-450 (lane 8). Lane 4 demonstrates that MA-B recognizes a single peptide. Lanes 5 and 6 demonstrate that essentially all of this peptide and all of MA-B were removed before addition of aclonal anti-MC-P-450. Densitometric quantitation of a lighter exposure of lanes 7 and 8 demonstrates that, relative to band d, band e is less in lane 7 than in lane 8. Thus, essentially complete removal of the antigen recognized by MA-B results in a 24% decrease in the intensity of band e, precipitated with aclonal anti-MC-P-450. This suggests that band e consists of more than one species and that at least one of these is recognized by both MA-B and aclonal anti-MC-P-450 while the other species found within band e are recognized only by the aclonal antibody.

Size Fractionation of MC-RNA. The upper panel of Figure 5 presents immunoprecipitations of the translation products of size-fractionated MC-RNA. Total translation products are presented for comparison in the lower panel. Poly(A+) MC-RNA was fractionated by size by methyl mercuric hydroxide–agarose gel electrophoresis. The gel was sliced and the RNA from each slice eluted and translated in vitro. The translation products were immunoprecipitated with anti-MC-P-450 and analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis and fluorography. Figure 5 shows that this antibody recognized three MC-inducible peptides, d, e, and g. These were translated from mRNAs of two distinct sizes. Peptides d and g were derived from mRNAs that were about 2700 nucleotides in length, while peptide e was translated from a mRNA of about 2000 nucleotides. The mRNA for the MC-inducible, *Staph. aureus* binding peptide, a, also had a molecular size of about 2000 nucleotides. The uninduced *Staph. aureus* binding peptide, b, was derived from a mRNA that was slightly smaller than those coding for peptides a and e, about 1930 nucleotides in length. Smaller yet was the mRNA coding for peptide f, which is only faintly visible in Figure 2. Its mRNA was about 1900 nucleotides in length. Peptide c of Figure 1 was also only faintly visible in Figure 5; its mRNA was about 1400 nucleotides in length. Two other ^{35}S-labeled peptides, having molecular weights of 50 100 and 49 000 were immunoprecipitated with anti-MC-P-450 (unlabeled arrows, Figure 5). They were translated from mRNAs that were 2600 and 2500 nucleotides long, respectively. Since

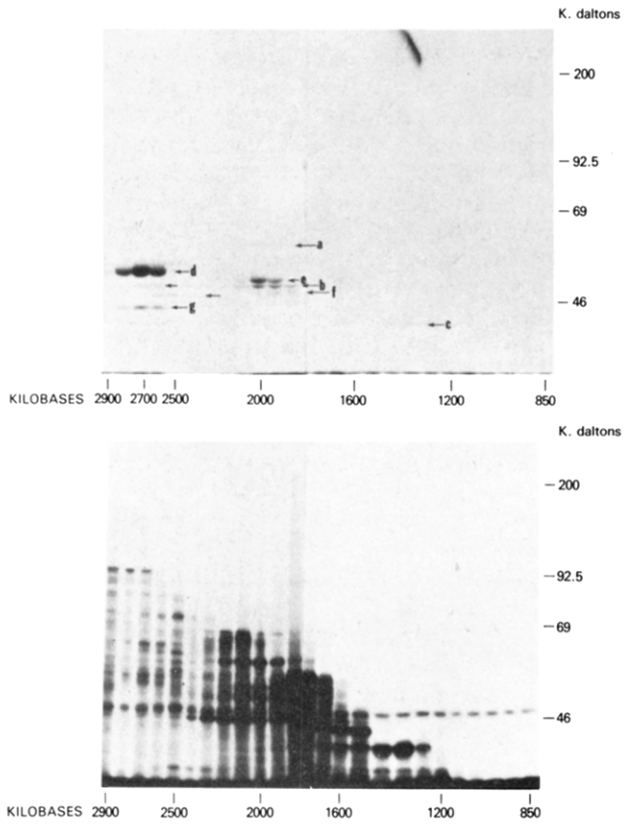

FIGURE 5: In vitro translation products of size-fractionated liver RNA from MC-treated rats. Poly(A+) liver RNA from MC-treated rats was fractionated by methyl mercuric hydroxide–agarose gel electrophoresis. RNA was eluted from sequential gel slices and translated in vitro, and the translation products were immunoprecipitated with rabbit IgG specific for rat liver MC-P-450. Total translation products (lower panel) and immunoprecipitates (upper panel) were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis and fluorography. The molecular size of the RNA in each fraction is indicated below the autoradiogram. Peptide molecular weights are indicated at the right of the autoradiogram.

these peptides were recognized by anti-MC-P-450, they may be minor P-450s.

The sizes of the mRNAs contained in each gel slice of Figures 2 and 5 were initially estimated by comparing the measured electrophoretic mobility of each slice with that of ethidium bromide stained molecular weight markers that were run in an adjacent lane of the same gel. This estimate was later confirmed by rerunning aliquots of selected fractions on an analytical methyl mercuric hydroxide–agarose gel (Figure 6). It is clear that the RNA extracted from each gel slice ran as a discrete size fraction. The spread of molecular weights present in each fraction varied from 300 bases for larger mRNA species to 170 bases for smaller species. The range of molecular weights of the RNAs contained in each fraction were calculated by using *Hin*dIII restriction fragments of phage λ DNA and rat 18S and 28S rRNAs as molecular weight standards.

Size Fractionation of PB-RNA. Figure 7 presents translations and immunoprecipitations of size-fractionated PB-RNA. The design of this experiment was the same as that of Figure 5, except that poly(A+) PB-RNA and anti-PB-P-450 were used. This figure shows that the PB-inducible mRNA coding for the 51.7 kilodalton peptide h is about 2000 nucleotides in length. Close comparison of Figures 5 and 7 reveals that the mRNA for peptide h is slightly larger than the MC-induced mRNA that codes for peptide e. The autoradiogram presented in Figure 7 was exposed only briefly;

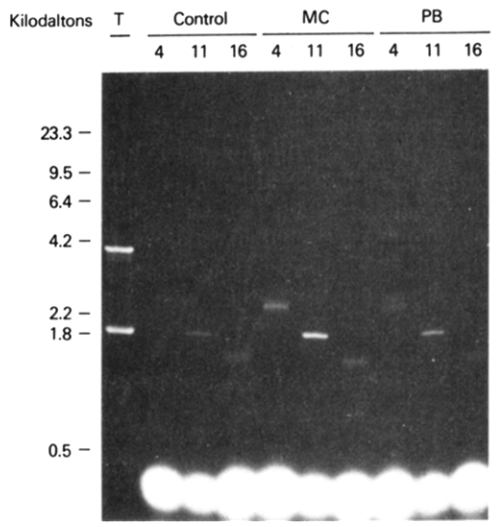

FIGURE 6: Analytical methyl mercuric hydroxide–agarose gel electrophoresis of RNA fractions recovered from a preparative methyl mercuric hydroxide–agarose gel. Aliquots of RNA fractions 4, 11, and 16, whose translation products are among those presented in Figures 2 and 5, were subjected to methyl mercuric hydroxide–agarose gel electrophoresis. The gel was stained with ethidium bromide and photographed. Phage λ DNA, digested with the restriction endonuclease *Hin*dIII (marker bars), and rat ribosomal RNA (lane T) were used as molecular weight standards. The low molecular weight material staining with ethidium bromide, present in the liver RNA fractions, was carrier tRNA. The numbers above the lanes are fraction numbers.

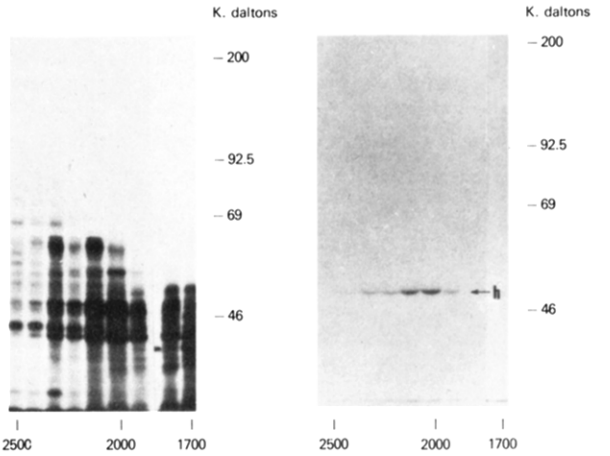

FIGURE 7: In vitro translation products of size-fractionated liver RNA from PB-treated rats. Poly(A+) liver RNA from PB-treated rats was size fractionated, eluted, and translated, and the translation products were immunoprecipitated as described in Figure 2, except that anti-PB-P-450 was used in place of anti-MC-P-450. (Left panel) Total translations; (right panel) immunoprecipitations.

thus only peptide h is visible. After longer exposure, peptide i, the peptide recognized by anti-PB-P-450 and present in the translation products of all three mRNAs, is also visible. Similar in size to peptide f, the mRNA coding for peptide i is about 1900 nucleotides in length. Control-RNA was fractionated and analyzed in the same manner that MC-RNA and PB-RNA were analyzed in Figures 5 and 7, using both anti-MC-P-450 and anti-PB-P-450 (data not shown). These results confirmed those presented in Figures 5 and 7, indicating that the mRNAs for peptides f, i, and b were, 1900, 1900, and 2000 nucleotides in length, respectively.

Discussion

The existence of multiple cytochromes P-450 is well established (Guengerich, 1977; Thomas et al., 1981). We have examined the relationships between these proteins by char-

Table I: P-450 mRNA and Peptide Molecular Weights

band designation	inducer	mRNA size (nucleotides)	peptide size (kilodaltons)	antibody recognition	comments
d	MC	2700 ± 150	57.9	anti-MC	MC specific
g	MC	2700 ± 150	45.0	anti-MC	in all RNAs but induced by MC
e	MC	2000 ± 110	55.4	anti-MC	MC specific
a	MC	2000 ± 110	62.2	*Staph. aureus* alone	in all RNAs but induced by MC
c	MC	1400 ± 85	40.3	*Staph. aureus* alone	in all RNAs but induced by MC
h	PB	2000 ± 110	51.7	anti-PB	PB specific
f, i	none	1900 ± 110	49.0	anti-MC, anti-PB	present in all RNAs; not induced
b	none	2000 ± 110	50.1	*Staph. aureus* alone	present in all RNAs; not induced

acterization of the mRNAs that code for them and by immunological analysis of the peptides synthesized from these mRNAs in vitro.

Table I summarizes the sizes of the P-450 mRNAs, the sizes of peptides for which they code, and the immunological characteristics of these peptides. The most striking observations presented here are that MC induces translatable mRNAs of two distinct size classes and that these mRNAs code for at least three distinct peptides. These peptides are antigenically related to MC-P-450 and are possibly P-450s themselves, since they are recognized not only by aclonal anti-MC-P-450 IgG but also by two different MC-P-450-specific monoclonal antibodies. These monoclonal antibodies have been shown to interact specifically with rat liver microsomal MC-P-450 both by their ability to bind to highly purified MC-P-450 in a radioimmune assay and by their ability to strongly inhibit the enzymatic activity of purified MC-P-450 (Park et al., 1982). A monoclonal antibody is a highly specific probe for a single antigenic site and will bind to any protein containing that specific site. The number of proteins to which a monoclonal antibody will bind is not a reflection of how specific the antibody is but of how many different proteins contain the antigenic determinant that it recognizes. Previous examples of a single monoclonal antibody binding to two or more distinct proteins that have an antigenic feature in common have been reported (Pruss et al., 1981; Lane & Hoeffler, 1980; Crawford et al., 1982). Although the sharing of an antigenic determinant does not necessarily demonstrate that two proteins are functionally related, it is suggestive of such a relationship (Lane & Hoeffler, 1980; Crawford et al., 1982). Our finding that monoclonal antibody MA-A recognizes peptides d, e, and g is clearly another occurrence of the phenomenon of a single monoclonal antibody recognizing more than one peptide. This indicates that these peptides are antigenically related and suggests that they may be functionally related as well. The P-450s have many structural and functional characteristics in common, including their substrate specificities, their heme binding sites, and their binding sites for NADPH–cytochrome P-450 reductase. Therefore it is not surprising that if peptides d, e, and g are MC-P-450s, they would have some antigenic features in common, even though they are distinct peptides derived from different mRNAs.

We have presented evidence that the MC-P-450-related peptides have unique antigenic features as well as common ones. Figure 4 shows that monoclonal antibody MA-B binds to only a single 55 400-dalton peptide, without detectable interactions with peptide d or g. Thus, this 55 400-dalton peptide has antigenic sites different from those on peptides d and g. Further evidence for the presence of unique antigenic features is presented in Figure 2B, lane 5, where it is shown that preadsorption of aclonal anti-MC-P-450 with PB-P-450 removes all IgG species except a population of IgGs that binds to peptide d. Thus, peptide d contains some antigenic sites different from those found on e and g. Other investigators have obtained similar findings with aclonal MC-P-450 antibodies preadsorbed with PB-P-450 (Colbert et al., 1979). Preadsorption in this way is useful for the identification of P-450 species that have one or more antigenic features that are distinct from those found on other classes of P-450s. However, as can be seen from the findings presented here, such antibody preparations give a deceptively low estimate of the number of related P-450 species. The finding that preadsorption of aclonal anti-MC-P-450 antibodies with PB-P-450 reduces the number of immunoprecipitated peptides from three to one suggests that the preparation of MC-P-450 used as antigen might have contained low levels of other forms of P-450 and thus contained antibodies to these forms as well. One alternative to this explanation is that these peptides could be MC-P-450s that have antigenic features that are shared by both MC-P-450s and PB-P-450s. This possibility must be considered seriously in light of the finding that peptides e and g, which are not recognized by the preadsorbed antibody, are recognized by MA-A, a monoclonal antibody that is known to interact directly with MC-P-450 by the fact that it inhibits MC-P-450 enzymatic activity, and in light of the observation that the mRNAs for both of these peptides are induced by MC.

Another possible explanation for the immunoprecipitation of multiple peptides of different molecular weights by a single antibody might be that the smaller peptides are proteolytic breakdown products of the larger. This may be the case for some minor peptides. However, we have shown clearly that peptides d and e are translated from separate mRNAs and have evidence suggesting that g is derived from a separate mRNA, as well (see discussion below). This eliminates proteolytic degradation as a possible explanation for the immunoprecipitation of these peptides by a single antibody.

The experiment presented in Figure 5 demonstrates that the mRNAs coding for the MC-P-450-related peptides are of two size classes, 2.0 and 2.7 kilobases. The 2.7-kilobase mRNA size class seems to contain multiple mRNAs for P-450-related peptides. The experiment presented in Figure 5 partially resolves the mRNAs for the two MC-P-450-related peptides having molecular weights of 50 100 and 49 000 from each other and from the mRNAs for peptides d and g. In some experiments it was possible to partially resolve by size the mRNAs coding for peptides d and g, as well (data not shown); however, in other experiments this separation was not observed, possibly due to variation in slicing of the gel. From these findings it appears likely that each MC-P-450-related peptide is translated from a distinct mRNA. It is interesting that the largest and smallest MC-P-450-related peptides (57.9 and 45 kilodaltons) are translated from 2.7-kilobase mRNAs, while that of intermediate size (55.4 kilodaltons) is translated from the smaller, 2.0-kilobase mRNA. This probably reflects differences in the sizes of the 3′- and 5′-noncoding regions of these mRNAs.

Findings in the mouse (Tukey et al., 1981) contrast with

our present findings in rat. Although there are two sizes of murine MC-P-450 mRNA, these mRNAs are much larger than those in the rat, about 2.7 and 3.5 kilobases in length. Furthermore, instead of coding for peptides of different sizes, both murine mRNAs code for peptides of about 55.0 kilodaltons. The murine mRNAs, especially the 3.5-kilobase species, are considerably larger than is required to code for a 55.0-kilodalton peptide. While the apparent differences between the sizes of rat and mouse P-450 mRNAs may be due to species variation, they might also be attributed to differences in the techniques used for the estimation of molecular weights. We have used both ribosomal RNAs and *Hin*dIII fragments of phage λ DNA as standards for estimating the molecular sizes of mRNAs. We find that our measurements are self-consistent and that our estimates of the molecular sizes of the 18S and 28S ribosomal RNAs agree well with published values (Weinberg & Penman, 1970; Loening, 1968).

Evidence is presented in Figures 2 and 3 that proteolytic processing of MC-P-450 peptides may occur in vivo. It is shown that the purified MC-P-450 used as antigen for preparation of both monoclonal and aclonal MC-P-450 specific antibodies had a molecular weight of 55 500 whereas the in vivo translated peptide recognized most strongly by these antibodies has a molecular weight of 57 900. One possible explanation for this finding is that the biogenesis of the 55 500-dalton purified MC-P-450 occurs by the proteolytic processing of a larger, 57 900-dalton peptide identical with peptide d. An alternate explanation is that the 57 900-dalton peptide is distinct from, yet antigenically related to, the 55 500-dalton purified MC-P-450 and is therefore immunoprecipitated by antibodies raised against this protein. Other investigators have reported evidence that both supports (Kumar & Padmanabhan, 1980) and denies (Bresnick et al., 1981) the existence of MC-P-450 precursors.

From calculations based on the molecular weights of the P-450-related peptides and of the mRNAs coding for them, it appears that, while the noncoding regions of the 2.0-kilobase MC-P-450 mRNA and of the PB- and control-P-450 mRNAs are typical in size (20%), those of the mRNAs coding for peptides d and g are uncommonly large (35 and 45%, respectively). Although most mRNAs have a much smaller proportion of noncoding sequences (20–30%) (McReynolds et al., 1978; Heindell et al., 1978; Efstratiadis et al., 1977; Fagan et al., 1979), there have been reports of other mRNAs having large noncoding regions; for example, the mRNA for preparathyroid hormone is 50% noncoding (Stolarsky & Kemper, 1978). One possible explanation for this finding is that the P-450 mRNAs that seem to have extensive noncoding regions might actually code for longer peptides that are proteolytically cleaved, giving rise to the peptides observed by electrophoresis. Although, as mentioned above, we and others have found evidence for the possible proteolytic processing of P-450s in vivo, in order to account for the observed discrepancy between peptide and mRNA sizes by this mechanism, it would be necessary for the pre-P-450 peptides to be quantitatively cleaved by a site-specific protease (as opposed to nonspecific proteolysis) in vitro in the rabbit reticulocyte lysate translation system itself. This is unlikely.

The results presented here also provide further evidence that proteins other than the cytochromes P-450 are induced by 3-methylcholanthrene. The ability of MC to alter the levels of translatable mRNA for glutathione *S*-transferase and albumin has recently been reported (Pickett et al., 1982). We have detected two mRNAs that are induced by MC but that code for peptides that are not P-450s (Figure 1, peptides a and c). The identity of these peptides is unknown. However, their existence indicates that MC affects the regulation of genes for proteins other than the cytochromes P-450.

Registry No. Cytochrome P-450, 9035-51-2; 3-methylcholanthrene, 56-49-5; phenobarbital, 50-06-6.

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Molecular Weights of Mitochondrial and Cytoplasmic Aminoacyl-tRNA Synthetases of Beef Liver and Their Complexes†

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ABSTRACT: In eukaryotes, multienzyme complexes containing five to nine aminoacyl-tRNA synthetase activities have frequently been reported. In this study, we report the existence, in bovine liver cytoplasm, of a multienzyme complex containing at least 16 activities which can be disrupted by homogenization to give rise to smaller complexes and noncomplexed synthetases. Determination of the size and component activity of these complexes and of the molecular weights of all 20 free synthetases suggests that the smaller complexes and free activities normally identified arise from the larger complex by well-defined stages during homogenization. We also show that similar, though not identical, complexes are found in bovine liver mitochondria and give the molecular weights of 16 mitochondrial synthetases.

The aminoacyl-tRNA synthetases, a class of enzymes playing a vital role in protein synthesis, have been extensively studied in recent years. Synthetases from many prokaryotic sources have been well characterized and appear to occur as independently active, nonassociated enzymes. For eukaryotes, on the other hand, Dang et al. (1982) have recently compiled over 70 references which suggest that the synthetases occur in one or more multienzyme complexes. All 20 synthetase activities may occur in a single supramolecular complex (Bandyopadhyay & Deutscher, 1971). Such a complex has been reported to contain tRNA, elongation factors, tRNA-modifying enzymes, and lipid (Soll & Schimmel, 1974). However, the existence, in vivo, of a complex containing all 20 synthetase activities is not universally accepted. Aggregation of its components following disruption of the cell (Irvin & Hardesty, 1972) or, alternatively, the presence of several homotypic complexes of similar type and behavior (Dickman & Boll, 1977) cannot be completely discounted.

If a supramolecular complex does exist, it is extremely fragile and apparently breaks down readily during purification to give rise to a fairly stable "core complex" containing seven to nine of the synthetase activities. The composition of this core complex is similar in a large range of tissues examined in many laboratories, but its size and composition do vary a little with the method of preparation. Again, there is not complete agreement that the core material is a heterocomplex. Dickman & Boll (1977), for example, claim that purification methods "mild enough not to break the types of bonds expected" alter the ratio of the components and hence suggest a mixture of homocomplexes. There have been a number of reports of the existence of homocomplexes containing multiple copies of a single synthetase (Pan et al., 1976; Dietrich et al., 1978; Zaccai et al., 1979) which have sometimes been quoted as evidence for the existence of a set of inseparable homocomplexes with similar properties rather than a heterocomplex. The homocomplex of cysteine studied by Pan et al. (1976), however, is much smaller than the heterocomplexes normally observed. The valyl-tRNA synthetase complex studied by the other workers appears to involve an electrostatic interaction between tRNA and enzyme and is readily disrupted by quite low ionic strengths while the core complex normally observed is stable to much higher ionic strengths. In fact, while it is impossible to disprove the existence of a set of homotypic complexes with very similar properties, there is an increasing weight of evidence in favor of the existence of a heterocomplex of defined structural composition and organization. Dang & Yang (1982) claim that the heterotypic multienzyme complex model is strongly favored over the homotypic complex model on the basis of cosedimentation and coelution of different synthetases from gel filtration, hydroxyapatite, and affinity resins with immobilized tRNA or amino acid. They write that "the sequential disassembly of the complex indicates specific association between synthetases", and the purification of a heterotypic core complex by Kellerman et al. (1979) indicates a fairly well-defined composition. The present study which presents evidence for complexes larger than the stable core complex usually observed, which also show correlation between the size and number of components and which also display sequential disassembly, provides further support for the heterotypic model.

There is, at present, little evidence whether, or by what intermediate stages, the stable core complex might arise from a supramolecular complex. Some results documenting such a process are provided by Dang & Yang (1979) in the form of a histogram tabulating the frequency of reports of the occurrence of a particular activity within a complex and thereby identifying "core" and "peripheral" activities.

Studies of the effects of methods of homogenization on the size and composition of the complex have produced contradictory results. Deutscher (1974) claims that the complex is very unstable to homogenization, though he suggests that homogenization may disrupt the lysosomes and increase proteolysis, rather than disrupting the complex directly. In contrast, Roberts & Olsen (1976) find that the ionic strength,

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