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Dose Response for Induction of Two Cytochrome P-450 Isozymes and Their mRNAs by 3,4,5,3′4′5′-Hexachlorobiphenyl Indicating Coordinate Regulation in Rat Liver

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SUMMARY

The present study compares the time course and dose-response curves for induction of the two major 3-methylcholanthrene (3-MC)-inducible isozymes of cytochrome P-450 and their mRNAs in livers of male rats after administration of 3,4,5,3′4′5′-hexachlorobiphenyl (HCB). Isozyme concentrations were measured by radioimmunoassay. The corresponding translatable mRNAs were measured by translation of polysomes in a cell-free translational system followed by immunoprecipitation and electrophoretic analysis of the translational products. The time course for induction of the two isozymes by HCB indicated that cytochrome P-448$_{MC}$ (P-450c) peaked sooner than P-448$_{HCB}$ (P450d). However, the time course for induction of the two mRNAs was identical. The dose-response curves for induction of the two isozymes and their mRNAs demonstrated that the ED$_{50}$ for induction of P-448$_{MC}$ was identical to that of P-448$_{HCB}$, suggesting that the two proteins are induced coordinately by this compound in liver. HCB did not induce P-450$_{PB}$ (the major phenobarbital-inducible isozyme) or affect mRNA levels for this isozyme. Although cytochrome P-448$_{HCB}$ is the predominant cytochrome in liver microsomes from HCB-induced rats, the magnitude of the induction of this isozyme (40-fold) is lower than that of P-448$_{MC}$ (600-fold), because cytochrome P-448$_{HCB}$ is present in higher concentrations in livers of untreated rats than P-448$_{MC}$ (90 versus 3 pmol/mg). Polysomes from control rats also contain more translationally active P-448$_{HCB}$ mRNA than P-448$_{MC}$ mRNA (0.009 versus 0.003% of the total translational products). The increase in the translatable mRNAs (12-fold for P-448$_{HCB}$ mRNA and 40-fold for P-448$_{MC}$ mRNA) was less than the increase in the isozymes. The discrepancy between the magnitude of the induction of the isozymes and their respective mRNAs suggests that factors other than an increase in mRNA influence the magnitude of the increase of the isozymes by HCB. However, HCB did not affect translational efficiency of total mRNA as measured *in vitro* in the present study. Differences in half-lives of the proteins or effects of HCB on the stability of the proteins might account for the magnitude of the increase in the isozymes after HCB treatment.

INTRODUCTION

Polychlorinated biphenyls represent major environmental pollutants which alter the level of many enzymes of the mixed function oxidase system (1). The terminal oxygenase of this system, cytochrome P-450, plays a central role in the metabolism of environmental pollutants, carcinogens, and endogenous compounds (2, 3). Cytochrome P-450 exists as a family of isozymes with varying specificities. Therefore, the induction of particular isozymes influences the susceptibility of the animal to the subsequent toxicity and carcinogenicity of other chemicals. Since the expression of these isozymes is regulated by environmental pollutants, as well as diet, age, and genetic makeup, it is important to determine how these factors control expression of the cytochrome P-450 gene family.

The inducers of cytochrome P-450 have been divided into several classes (2). The 3-MC[1] class of inducers has been shown to induce three isozymes of cytochrome P-450 in rat liver (4). One isozyme of 3-MC-inducible rat liver P-450 is known as P-450c (5) or P-448$_{MC}$ (6). However, treatment of rats with 3-MC-type inducers such as 3,4,5,3′,4′,5′-HCB also results in the induction of a second major form of cytochrome P-450 which we

[1] The abbreviations used are: 3-MC, 3-methylcholanthrene; HCB, hexachlorobiphenyl; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; SDS, sodium dodecyl sulfate; RIA, radioimmunoassay, TCA, trichloracetic acid.

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refer to as P-448$_{HCB}$ (6). This form is also induced by isosafrole and appears to be identical to P-450d originally isolated from isosafrole-treated rats, which also has been shown to be induced by 3-MC-type inducers (4, 7).

Antibodies to P-450c (P-448$_{MC}$) and P-450d (P-448$_{HCB}$) are cross-reactive (4, 8), and the proteins share several immunological determinants (9). Despite the structural similarities, the proteins have been shown to be the products of separate mRNAs (10, 11). Induction of aryl hydrocarbon hydroxylase (a cytochrome P-448$_{MC}$-mediated enzyme) and a number of other proteins by 3-MC-type inducers is believed to be the consequence of the binding of these inducers to a cytosolic receptor and a subsequent coordinate increase in the transcription of mRNAs for these proteins (12). Since cytochrome P-448$_{MC}$ and P-448$_{HCB}$ are both induced by the 3-MC class of inducers (4, 13, 14), we were interested in determining whether the two isozymes and their respective mRNAs are induced coordinately by different doses of a 3-MC-type inducer.

The present study examines both the time course and dose response for induction of the two isozymes and their respective mRNAs after administration of HCB. This polychlorinated biphenyl isomer was selected as the inducer because it is one of the more potent 3-MC-type inducers, and it appeared to induce P-448$_{HCB}$ preferentially relative to P-448$_{MC}$ in previous studies (13, 14). The isozymes were measured by a sensitive RIA (13), while the two mRNAs were assessed by electrophoretic analysis of immunoprecipitates of proteins synthesized in a cell-free translational system. This study indicates that P-448$_{HCB}$ and P-448$_{MC}$ are induced coordinately after administration of HCB. The induction of the isozymes was greater than the induction of the mRNAs. In contrast to the induction of these two isozymes, no difference in mRNAs for either albumin or P-450$_{PB}$ (the major phenobarbital-inducible isozyme) was observed after HCB administration, indicating the specificity of this inducer.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats weighing 150–180 g were treated with HCB dissolved in corn oil, starved 24 hr before death, and killed between 9 and 10 a.m.

Purified cytochromes and antibodies. Cytochromes P-448$_{MC}$, P-448$_{HCB}$, and P-450$_{PB}$(b) were purified as previously described (6). Antisera to P-448$_{MC}$, P-448$_{HCB}$, and P-450$_{PB}$ were obtained from rabbits. IgG was prepared by protein A-Sepharose chromatography (15). Cross-reactivity of anti-P-448$_{MC}$ to P-448$_{HCB}$ and that of anti-P-448$_{HCB}$ to P-448$_{MC}$ was removed by repeatedly recycling the IgG over a column of the cross-reacting antigen covalently bound to Sepharose 4B (13). The removal of cross-reactivity from each antibody was verified by a Western blot of microsomes prepared from 3-MC- and HCB-treated rats (8) (Fig. 1) and by a dot-blot procedure (16). Anti-P-448$_{MC}$ contained ≤0.5% cross-reactivity to P-448$_{HCB}$, while anti-P-448$_{HCB}$ contained ≤0.1% cross-reactivity to P-448$_{MC}$. Antiserum to albumin was obtained from Cappel Laboratories.

Preparation of RNA and polysomes. Total liver polysomes were homogenized with vanadyl ribonucleoside complex and isolated as previously described (15) and resuspended at a concentration of 1000–1500 absorbance units/ml at A_{260} in 50 mM Tris (pH 7.5) containing 2 mM EGTA, 5 mM $MgCl_2$, 0.3 M NaCl, 2 mM dithiothreitol, and 2 units/ml of RNasin (Promega Biotec, Madison, WI) and stored at −80°.

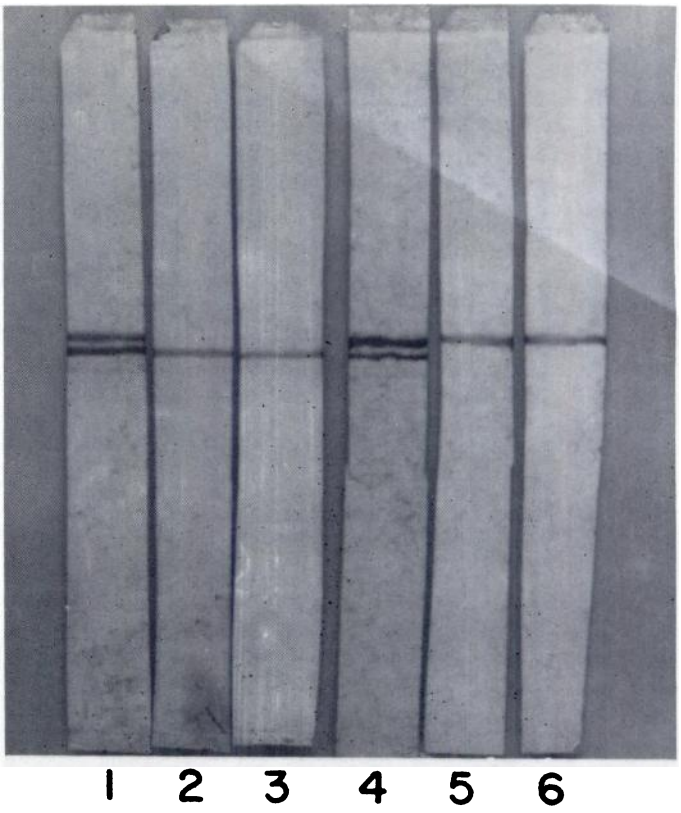

FIG. 1. *Specificity of antibodies to P-448$_{MC}$ and P-448$_{HCB}$*
Microsomes from HCB-treated rats were electrophoresed in SDS-polyacrylamide gels at 10 pmol of P-450/lane. The proteins were then transferred electrophoretically to nitrocellulose sheets and immunostained with antibody as described elsewhere (15). Wells 1 and 4 were incubated with unabsorbed antibody for P-448$_{HCB}$ or P-448$_{MC}$. Lanes 2,3, and 5,6 were incubated with immunoabsorbed anti-P-448$_{HCB}$ and anti-P-448$_{MC}$, respectively.

RNA was isolated from polysomes by extraction with 4 M guanidinium thiocyanate (16). The RNA was washed twice in 3 M sodium acetate (pH 6.0) containing 0.1 mM iodoacetate, twice in 80% ethanol containing 10 mM sodium acetate (pH 6.0), once in absolute ethanol, and then lyophilized. RNA was stored at 1 mg/ml or used for the isolation of poly(A$^+$)-RNA on oligo(dT)-cellulose (T_3) (Collaborative Research, Lexington, MA) (17).

Cell-free translation. A reticulocyte cell-free system was prepared from rabbits made anemic with acetylphenylhydrazine according to the protocol of Pelham and Jackson (18). Either 0.2–10 μg of poly(A$^+$)-RNA, 10 μg of total polysomes (1.0 A_{260} = 20 μg/ml) or 10–15 μg of total RNA was translated for 60 min at 23° in a 25-μl lysate system in the presence of [^{35}S]methionine (800–1000 Ci/mmol, Amersham) at a concentration of 400 μCi/ml. The incubation mixture was then boiled for 5 min with 4% SDS and then diluted with 2.5% (v/v) Triton X-100, containing 90 mM NaCl, 6 mM EDTA, 60 mM Tris (pH 7.5), and 0.1 μg/ml of aprotinin and leupeptin so that the final SDS concentration was 0.2%. An aliquot was used to determine the total TCA-precipitable material (total incorporation). Another aliquot was used for immunoprecipitation of specific cytochrome P-450 isozymes. Two types of blanks were performed in triplicate. The first contained no RNA, and the second contained RNA but used control IgG instead of the specific antibody. Immunoprecipitation was performed in 1% (v/v) Triton X-100 containing 150 mM NaCl, 5 mM EDTA, 0.1% (w/v) SDS, and 0.05 μg/ml leupeptin and aprotinin. Preimmune IgG (20 μg) was added and the mixture was incubated at 4° for 30 min. Heat-killed, formalinized *Staphylococcus aureus* cells (Pansorbin, Calbiochem, La

Jolla, CA) (100 μl of a 10% suspension) were added and the mixture was incubated on ice for 30 min and then centrifuged. Immune IgG (40 μg) was added and incubated overnight at 4°. Prewashed *S. aureus* cells (100 μl of a 10% suspension in immunoprecipitation buffer) were added, incubated at 4° for 1 hr, and then centrifuged. The cells were washed twice with immunoprecipitation buffer containing 0.5 M NaCl, twice with immunoprecipitation buffer, resuspended in 95 μl of 0.125 M Tris (pH 6.8) containing 2% SDS, and boiled for 5 min to release immunoprecipitated products. After centrifugation, mercaptoethanol (5 μl) was added to the supernatant and the sample was heated to 100° for 2 min. An aliquot was used to measure total immunoprecipitable products.

We separated the remainder of the immunoprecipitable products by electrophoresis on a 10% SDS-polyacrylamide gel (19). The gel was fixed and stained. In some cases, the gel was prepared for autoradiography with EN^{3}HANCE (New England Nuclear, Boston, MA). The radioactive band with a mobility identical to that of the pure protein was excised, incubated with 95% H_2O_2, 5% NH_4OH overnight, and counted in Atomlight (New England Nuclear). A duplicate area was excised from samples to which preimmune IgG was added instead of specific antisera. The background radioactivity in these slices was subtracted from that of actual samples.

Other methods. Cytochrome P-450 was determined by the method of Omura and Sato (20). Protein was determined by the Lowry method (21). Cytochrome P-448$_{MC}$ and P-448$_{HCB}$ content of microsomes were assayed by RIA as described previously (13).

RESULTS

Because the cytochromes P-448$_{MC}$ and P-448$_{HCB}$ are present at very low levels in microsomes from control rats, we anticipated that the mRNAs for these isozymes would represent low abundance class mRNAs. Initial studies indicated that the addition of poly(A$^+$)-RNA or total RNA to the reticulocyte lysate system increased the incorporation of radioactive methionine into TCA-precipitable products 10- to 20-fold over background. However, polysomes produced a 50–70-fold increased (Fig. 2). By employing polysomes, we increased the sensitivity of the cell-free translational system 3-fold, enabling us to measure the synthesis of proteins translated at a rate of 0.001% of the total translational products. Initial studies indicated that the magnitude of the induction of P-448$_{HCB}$ and P-448$_{MC}$ mRNAs was similar for total RNA, poly(A$^+$)-RNA, or polysomes, indicating that translation of polysomes is a measure of the relative amount of mRNA. Moreover, we observed no difference in the efficiency of incorporation of radioactivity into total TCA-precipitable products, using equivalent amounts of poly(A$^+$)-RNA from livers of control or 24-hr HCB-induced rats (Fig. 2A), indicating that HCB does not affect the translational efficiency of the mRNA. Radioautograms of SDS-polyacrylamide gels of the immunoprecipitable products of polysomes from HCB-induced rats indicated incorporation of radioactivity into discrete polypeptide bands with mobilities identical to those of albumin, P-448$_{MC}$, and P-448$_{HCB}$.

The amount of cytochrome P-448$_{HCB}$ (90 pmol/mg) was considerably higher than the amount of P-448$_{MC}$ (3 pmol/mg) in livers of untreated male rats as noted in our previous study (8). Liver polysomes from untreated rats also translated cytochrome P-448$_{HCB}$ at a higher rate than P-448$_{MC}$ (0.009 versus 0.002% of the total translational products). In contrast, cytochrome P-450$_{PB}$ and albumin accounted for 0.03 and 0.4% of the total translational products.

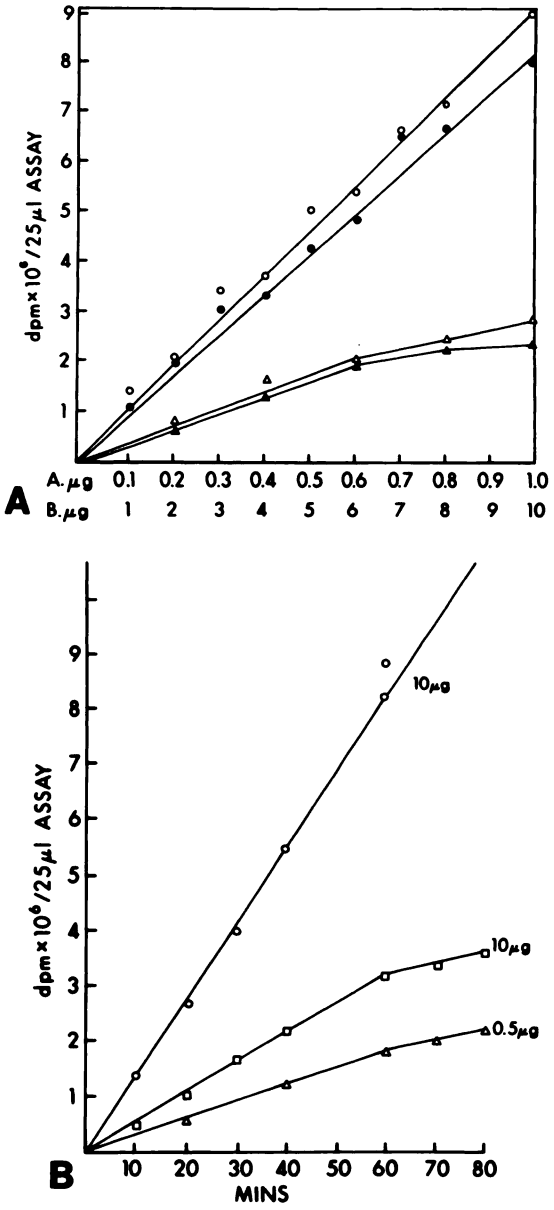

FIG. 2. *Translation of liver polysomes in a cell-free system*

A, comparison of translational efficiency of polysomes or poly(A$^+$)-RNA from livers of control or HCB-treated rats as a function of the concentration of RNA. Increasing amounts of polysomes from livers of control rats (○) or HCB-treated rats (●) or poly(A$^+$)-RNA from control rats (△) or HCB-treated rats (▲) were translated in a cell-free reticulocyte lysate system and incorporation of [^{35}S]methionine into TCA-precipitable material was measured. Row A represents the amount of poly(A$^+$)-RNA (1 A_{260} = 40 μg/ml) and row B is the amount of polysomes (1 A_{260} = 20 μg/ml) used per translation. Background lysate incorporation was 1 × 10^5 dpm. B, linearity of translation by polysomes, total polysomal RNA, and polysomal poly(A$^+$)-RNA with time. Polysomes at 10 μg (○) (1 A_{260} = 20 μg/ml) per 25-μl translation assay, total RNA extracted from polysomes (10 μg) (□), and polysomal poly(A$^+$)-RNA at 0.5 μg (△) were translated in a cell-free reticulocyte lysate system.

Time course. The time course for the induction of P-448$_{MC}$ and P-448$_{HCB}$ by HCB indicated that P-448$_{HCB}$ was induced somewhat more slowly than P-448$_{MC}$ (Fig. 3A). In contrast, the mRNAs for the two isozymes peaked

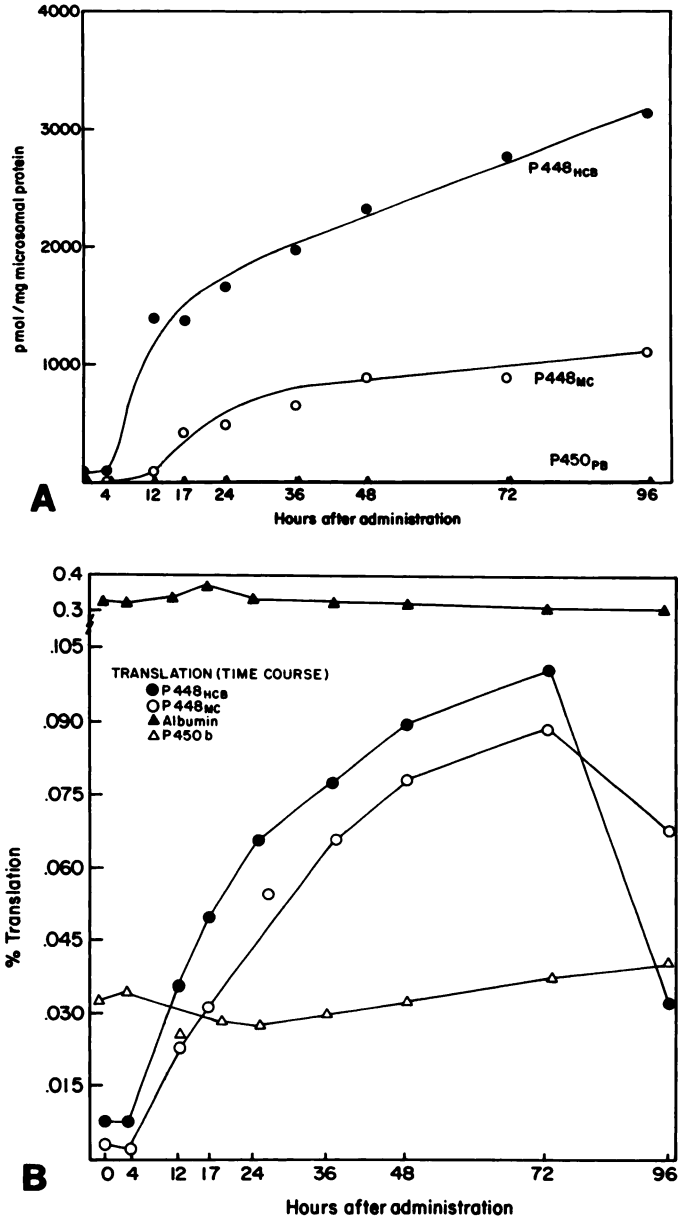

FIG. 3. *Time course for induction of cytochrome P-450 isozymes (A) and their respective translatable mRNAs (B) after administration of HCB*

Microsomes or polysomes from three control or induced rats were pooled at each time point. Isozymes were measured by RIA. Polysomes were translated in a cell-free reticulocyte lysate system using 8 μg of RNA/25 μl of lysate (18). 2–20 × 10^6 cpm were used for immunoprecipitation with specific antibodies. Immunoprecipitated proteins were electrophoresed, and incorporation of radioactivity into a polypeptide band with a molecular weight equal to that of the pure protein was determined. Translation is expressed as the counts in the specific polypeptide band divided by total TCA-precipitable counts × 100 (% translation).

at the same time, 72 hr after administration (Fig. 3B). There was a difference in the magnitude of the induction of the isozymes and their respective mRNAs. Cytochrome P-448_{MC} and P-448_{HCB} were increased 600- and 40-fold, respectively. In contrast, P-448_{MC} mRNA was increased only 40-fold, while P-448_{HCB} mRNA was induced only 12-fold over controls. However, the amounts of P-448_{HCB} and its mRNA were actually higher than that of P-448_{MC} or its mRNA in livers from both control and HCB-treated rats. In contrast to the induction of the mRNAs for the two cytochrome P-450 isozymes, the mRNAs for albumin or cytochrome P-450_{PB} mRNA were not induced by HCB administration.

Dose-response studies. We examined the dose response at two time points (24 and 72 hr) (Figs. 4 and 5). It is apparent that both P-448_{HCB} and P-448_{MC} and their respective mRNAs are induced coordinately by various doses of HCB at both time points. At 72 hr, the ED_{50} for induction of P-448_{MC} (2.6 mg/kg) by HCB was similar to that of P-448_{MC} (2.3 mg/kg). The ED_{50} values for induction of the two RNAs were more difficult to estimate but appeared to be fairly similar (0.8 mg/kg for P-448_{HCB} mRNA versus 1.7 mg/kg for P-448_{MC} at 72 hr). In contrast, P-450_{PB} (the major phenobarbital-inducible isozyme) and its mRNA were not induced by any dose of HCB.

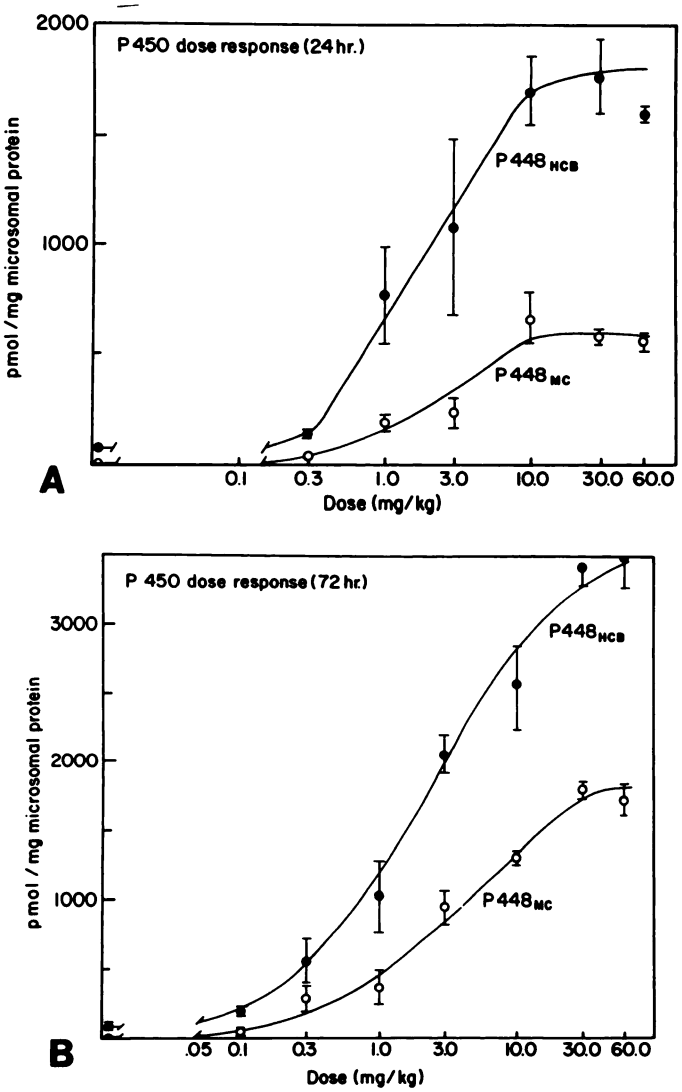

FIG. 4. *Dose response for induction of hepatic P-448_{MC} (○) and P-448_{HCB} (●) after administration of varying doses of HCB at 24 (A) and 72 (B) hr*

Values represent the means ± standard error for three animals per dose.

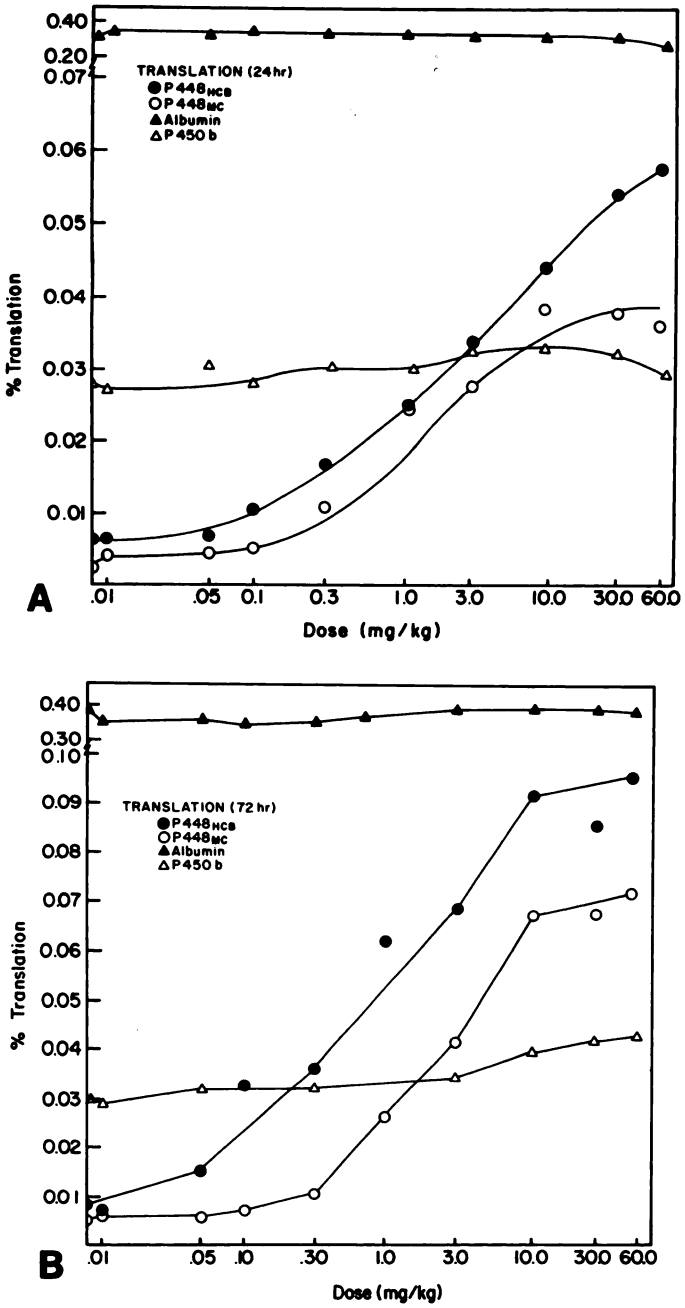

FIG. 5. *Dose response for induction of mRNAs by HCB at 24 (A) and 72 (B) hr*

Polysomes from three rats at each dose level were pooled for analysis. Values represent means of duplicate analyses of at least two separate experiments.

DISCUSSION

This study examines whether equal doses of a potent polychlorinated biphenyl isomer (3,4,5,3′4′5′-HCB) are required to induce the two major 3-MC-inducible isozymes of cytochrome P-450 in the rat. Since differences in isozyme content may reflect changes in the stability of the protein as well as changes in gene expression, we also examined the dose-response for increases in translationally active mRNAs. A number of earlier studies have examined translation of P-450c and P-450d using mRNA or total RNA from 3-MC-treated animals (10, 22). However, these studies did not utilize the data to estimate the increase in mRNA, presumably because the mRNAs for these isozymes are very low in uninduced rats. In the present study, we optimized the translational system to allow us to detect incorporation of as little as 0.002% of the total translational products into specific polypeptides. Improvements included the use of polysomes prepared with vanadyl ribonucleoside complex and RNase inhibitor. Gaetani *et al.* (23) have utilized a similar cell-free system for translation of polysomes *in vitro*. Polysomes prepared in this manner had a translational activity 50–100 times greater than background, while poly (A^+)-RNA or total RNA stimulated translation only 10–20-fold. Moreover, the rate of translation by polysomes was linear for a longer period of time (2 hr) than total RNA or poly(A^+)-RNA (30–60 min). Although differences in efficiency of translation of the different RNA preparations could complicate results, initial studies indicated that the magnitude of the increase produced by HCB was similar for different RNA preparations.

The similarities between the ED_{50} values for induction of P-448_{MC} and P-448_{HCB} and their respective mRNAs by HCB in the present study suggest that the two isozymes are induced coordinately by HCB. These data are consistent with the hypothesis that HCB induces the two isozymes via interaction with a common receptor. Since the completion of the present work, Kawajiri *et al.* (24) have reported that the ratio of induction of the mRNAs for P-450c and P-450d was similar for 3-MC, β-naphthoflavone, and a commercial PCB mixture. Our translational data are consistent with their results. In contrast, they found that isosafrole appeared to differ from the other three compounds by producing a preferential induction of P-450d mRNA compared to P-450c mRNA. Moreover, Ikeda *et al.* (25) have also reported that isosafrole induced the mRNA for P_2-450 in the nonresponsive mouse while it had little or no effect on the mRNA for P_1-450. Conceivably, isosafrole may induce P-450d by a mechanism other than binding to the *Ah* receptor as was suggested by that laboratory (25).

In the present study, both P-450_{HCB} and its translationally active mRNA were higher than P-450_{MC} or its mRNA in livers of HCB-induced male rats. However, the magnitude of the induction was less for P-450_{HCB}, since both the isozyme and its translationally active mRNA were higher in livers of uninduced animals. Kawajiri *et al.* (24) also found the mRNA for P-450d to be higher in livers of 3-MC-induced animals than the mRNA for P-450c. They did not measure isozyme levels in their study, but noted a discrepancy between their findings and isozyme determinations from another laboratory which indicated that P-450c was three times higher than P-450d in 3-MC- or β-naphthoflavone-treated rats (4). They suggested that the discrepancy could result from differences in efficiencies of translation for the two mRNAs, different turnover numbers for the proteins, or experimental difficulties in estimating such immunochemically similar proteins. We do not find such a discrepancy, since our RIA data indicate that P-450c and P-450d are present in similar concentrations in livers of 3-MC-treated Sprague-Dawley rats (13). The differ-

ence in the relative amounts of the two isozymes reported in livers of 3-MC-treated rats in various laboratories (4, 13) could reflect differences in methodology. The similarities between the increases in the translationally active mRNAs for P-448$_{MC}$ and P-448$_{HCB}$ (40- and 10-fold) in the present study and the increases in actual mRNA observed by Kawajiri *et al.* (24) for a maximally inducing dose of 3-MC (50- and 10-fold) suggest that there is no major difference in the translational efficiency of the two mRNAs in HCB-induced animals. Moreover, HCB did not affect the total translational efficiency of liver polysomes, poly (A$^+$)-RNA, or total RNA in the present study. In contrast, phenobarbital has been shown to increase translational efficiency of total poly(A$^+$)-RNA in rats (26, 27). However, an effect of 3-MC-type inducers on translational efficiency of specific mRNAs cannot be ruled out until recombinant probes are used to determine differences in efficiencies of translation of mRNAs for specific P-450 isozymes and does not rule out the possibility of an effect on the translational efficiency *in vivo*.

In the present study, the magnitude of the increase in the two isozymes (40-fold for P-450d and 600-fold for P-450c) was much greater than the magnitude of the increase in their respective translationally active mRNAs (12- and 40-fold). Israel and Whitlock (28) found equivalent increases in P$_1$-450 and its mRNA in Hepa 1c1c7 cells (25–50-fold). However, Berlin and Shimke (29) have reported that the magnitude of induction of a protein is dependent not only on the rate of synthesis but also on the rate of turnover of the protein. Certainly, the similarities in the shape and maxima of the dose-response curves for the P-450 mRNAs and the isozymes suggest that mRNA is the rate-limiting factor in induction of the enzymes. Conceivably, differences in the initial rates of synthesis of the two isozymes and in their half-lives may explain some of the differences in the magnitude of the increases observed after various inducers (13, 14). The induction of P-448$_{HCB}$ appeared to be slower than the induction of P-448$_{MC}$ in the present study, suggesting that the half-life of P-448$_{HCB}$ is greater than that of P-448$_{MC}$. Steward *et al.* (30) have recently reported isosafrole (a compound which binds to P-450d) increased the amount of P-450d in hepatocytes without affecting synthesis, and that this compound changed the stability of the protein. Conceivably, formation of a cytochrome P-450 inducer complex might also make the isozyme resistant to degradation and increase the half-life of the protein.

The time course for induction of the two mRNAs by HCB indicated that the maximum increase in the present study occurred 72 hr after administration. Previous studies have indicated that the maximum increase occurs 17 (mRNA) to 24 hr (translatable mRNA) after 3-MC administration (22, 24). The more sustained induction of mRNA administration after the HCB administration probably relates to the relatively long half-life of the polychlorinated biphenyl isomer compared to 3-MC. Gozukara *et al.* (22) have reported that 3-MC decreases translation of albumin. HCB had no effect on translation of albumin or P-450b in the present study, suggesting that neither of these proteins is regulated by the *Ah* receptor. The absence of an increase in P-450b mRNA is in agreement with previous reports that the isozyme is not induced by 3-MC or HCB (1, 13, 14).

From the time course and dose-response data presented in this study, we conclude that HCB induces two major 3-MC-inducible isozymes of cytochrome P-450 and their mRNAs in a coordinate manner, probably via a common mechanism. Our data are thus consistent with the hypothesis that both genes are probably regulated by a single receptor in the rat. The magnitude of the increase in the isozymes was greater than the increase in the amount of translationally active mRNA in polysomes, suggesting that, in addition to increases in transcription of mRNA, other factors may influence the relative induction of these P-450 isozymes.

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