0026-895X/89/050610-07$02.00/0

MOLECULAR PHARMACOLOGY, 35:610-616

Evidence for a PCN-P450 Enzyme in Chickens and Comparison of Its Development with That of Other Phenobarbital-Inducible Forms

NANCY A. LORR, STEPHEN E. BLOOM, SANG SHIN PARK, HARRY V. GELBOIN, HARUKO MILLER, and FRED K. FRIEDMAN

Department of Poultry and Avian Sciences and The Institute of Comparative and Environmental Toxicology, Cornell University, Ithaca, New York 14853 (N.A.L., S.E.B.), and Laboratory of Molecular Carcinogenesis, National Institutes of Health, Bethesda, Maryland 20205 (S.S.P., H.V.G., H.M., F.K.F.)

Received November 17, 1988; Accepted February 28, 1989

SUMMARY

Of four monoclonal antibodies to purified rat liver cytochrome P450s, including those from 3-methylcholanthrene-, phenobarbital-, ethanol-, and pregnenolone-16-α-carbonitrile-treated rats, only the monoclonal antibody against pregnenolone-16-α-carbonitrile-inducible P450 immunodetected proteins in chicken liver microsomes after blotting from sodium dodecyl sulfate-polyacrylamide gels. This protein migrated identically with the pregnenolone-16-α-carbonitrile-inducible P450 detected in microsomes from dexamethasone-treated rats. It was most predominant in liver microsomes from chickens at 1 day posthatching, whereas much lower levels were observed in the embryo and at 36 days posthatch. Phenobarbital and dexamethasone were both effective inducers of this protein. The developmental profile and induction by phenobarbital and dexamethasone of several cytochrome P450-associated catalytic activities were compared with those of the immunodetected protein. Chicken liver microsomal erythromycin demethylase, a characteristic activity of rat pregnenolone-16-α-carbonitrile-inducible P450, was similar in developmental profile and induction to the immunodetected protein, with a high degree of augmentation at 1 day posthatch compared with that in the embryo and at 36 days posthatch; aldrin epoxidase, benzphetamine demethylase, ethylmorphine demethylase, and aminopyrine demethylase were more similar to each other in development and induction and were less well correlated with the immunodetected protein. This evidence suggests the presence in chicken liver of at least two types of P450, one a form related to the pregnenolone-16-α-carbonitrile-inducible P450 family. All of the catalytic activities were induced after pretreatment of chickens with phenobarbital but aldrin epoxidase was most effectively induced. Aldrin epoxidase was also detected in microsomes from untreated embryos as early as 7 days of incubation. Erythromycin demethylase was the only catalytic activity induced by dexamethasone. There was a trend of increased specific activity toward all the substances after hatching, indicating a more efficient P450 system, possibly due to a sharp increase in some isozymes, including the form from the pregnenolone-16-α-carbonitrile-inducible P450 family. This evidence for a pregnenolone-16-α-carbonitrile-inducible P450 in chickens agrees with sequence information that suggests the early evolution of this form and demonstrates the suitability of the chicken for studies of P450 evolution.

The sensitivity of a developing organism to xenobiotics is dependent in part on the state of development of the P450 enzyme system (1–3). This system activates xenobiotics to reactive intermediates as well as detoxifying them to water-soluble products (4). Therefore, it is important to investigate the P450 system during the embryonic and neonatal period, when the organism is developing rapidly and is highly vulnerable to toxic insult. Of particular interest is the determination of the role of particular P450 enzymes in mediating selective tissue damage and transformation during development (5).

This work was supported by National Institute of Environmental Health Sciences Grant ES03499.

The P450s are a diverse group of proteins with distinct but broad and overlapping substrate specificities (6). A recently devised classification system divides the P450s into 10 major gene families (7). The multiple P450 proteins probably evolved from a common ancestor, because there are regions of high sequence similarity in all P450s, including a prokaryotic type (8). The PCN-P450s constitute a multigene family, as shown by Southern blot analysis (9) and the isolation of three forms from rat liver (10). These differ in developmental regulation and inducibility but have quite similar gene sequences (9, 10). The PCN-inducible P450 family (P450III) is estimated to have diverged from preexisting forms approximately 1100 million years ago, whereas the estimate of the time of divergence of the

ABBREVIATIONS: P450, cytochrome P-450; MC, 3-methylcholanthrene; PB, phenobarbital; PCN, pregnenolone-16-α-carbonitrile; TCB, 3,4,3′,4′-tetrachlorobiphenyl; MAb, monoclonal antibody.

MC- (P450I) and PB- (P450II) inducible major families is 800 million years ago (11). Because the bird-mammal divergence was about 300 million years ago (12), the bird genome should contain representatives of all three of these families of P450 genes.

Two chicken P450 genes from the P450II family have been cloned (13). One such gene was sequenced and is about 50% similar to known P450II sequences (14). At least one PB-induced P450 protein and a MC-inducible P450 resembling the rodent forms have been purified from chicken liver (15–17). It is unknown whether proteins homologous to the PCN-induced forms are present in the chicken. Thus, further studies comparing the P450 families in chicken with those in mammals is important in understanding P450 evolution.

Further study of the development of different families of P450 is important in defining the metabolic capabilities of the developing organism and the role of P450 in mutagenicity and teratogenicity. The multiple isozymes of rat hepatic P450 differ in their developmental regulation, with most isozymes being expressed at birth or several weeks later (18, 19). In human fetal liver, however, substantial P450-associated catalytic activities are present as early as the end of the first trimester (20), predominantly one or several PCN-P450s (21).

The early chicken embryo is capable of activating a wide spectrum of xenobiotics to DNA-damaging metabolites (22–24). PB induction of P450 and aminopyrine demethylase is detectable by 7 days of incubation in chicken liver microsomes. Basal and PB-induced aminopyrine demethylase rise steadily throughout the embryonic period, reaching their highest levels at 1 day posthatch (25). The specific activity (per nmol of P450) of aminopyrine demethylase is at least doubled upon hatching, indicating significant changes in the P450 system leading to more efficient metabolism of aminopyrine (25). One possible explanation for the increased specific activity is that the composition of P450 isozymes involved in the metabolism of aminopyrine changes upon hatching.

The PCN-P450s have substrate specificities similar to those of P450II forms and are induced by PB (26–28). The development in chicken liver of a PCN-P450 may contribute to the pattern of embryonic and neonatal development of aminopyrine demethylase observed in previous studies. Because the P450II and P450III families have low amino acid sequence similarity (about 30%) and probably evolved as separate genetic entities for over 1000 million years, we thought it reasonable that an avian PCN-P450 would be present. We, therefore, hypothesized that the change in the chicken P450 system detected neonatally is related to the expression of a PCN-P450 analogous (homologous) to the rat PCN-P450 isozymes.

MAbs toward purified rat liver P450s have been widely used in the immunodetection of antigenic P450s in microsomal preparations (29, 30). Forms of P450 related to rat P450s have been identified in tissues from other species (31–33). Homologous forms in other species are more likely to react with antibodies to a specific form than are P450s from different families within the same species (33). Park and co-workers have prepared panels of MAbs against MC- (34), ethanol- (35), PCN- (36), and PB- (37) inducible rat liver P450s. These MAbs have been used for immunodetection (31, 32), to inhibit enzymatic activity (reaction phenotyping) (34, 35, 38), and for immunopurification (31, 39). The MAbs prepared by Park *et al.* (36) against PCN-inducible rat liver P450, specifically P450 PB-2a/PCN-E (40), include highly specific clones that do not react with P450II forms. One of these highly specific antibodies, MAb 2-13-2, was used in this study to probe for the presence of related forms in chicken liver.

In this study, we give evidence for the presence in chicken liver microsomes of a protein related to the rat PCN-P450s, the level of which dramatically increases after hatching. The development of P450-associated catalytic activities during the embryonic and neonatal period follow two profiles, which may correspond to development of two classes of P450, one related to the rodent P450II forms, another related to the rodent P450III forms.

Materials and Methods

Chemicals. PB was purchased from Merck and Co. (Rahway, NJ). The chemicals phenylmethylsulfonyl fluoride, erythromycin, NADP, NADPH, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were all obtained from Sigma Chemical Co. (St. Louis, MO). Dexamethasone sodium phosphate injection solution was obtained through Ascot Pharmaceuticals (Stokie, IL). Ethylmorphine HCl was supplied by Applied Sciences Laboratory (State College, PA), and benzphetamine HCl was the gift of Upjohn Co. (Kalamazoo, MI). Aldrin and dieldrin was furnished by the Shell Development Co. (Modesto, CA). Alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate came from K&P Laboratories. All chemicals and solvents used were of the highest purity commercially available.

Animals, treatments and microsomes. Fertile eggs were obtained from the Cornell K-MFO line of chickens representing a subline of the Cornell K-strain (41). The subline has been selected for uniformity of general egg characteristics and fertility. Additionally, the K-MFO line is highly inducible for PB and MC types of P450 activities (24, 25). Eggs were incubated at 37° and 85% humidity and were rotated once an hour. Experimental eggs were selected for fertility and normal embryonic development by candling. Chick embryos were pretreated by placement of the solution, by pipette, onto the inner shell membrane through a 5-mm diameter hole made in the shell of each egg directly over the air cell (42). The hole was sealed with a piece of adhesive tape and the egg was returned to the incubator. Embryos and hatched chicks were injected intraperitoneally with maximally tolerated doses of PB, as determined in a previous study (25). Embryos at 14 days of incubation received 8.95 mg of PB (5 mmol/kg of body weight); embryos at 18 days of incubation, 11.6 mg (2.5 mmol/kg of body weight); and hatched chickens, 100 mg/kg of body weight (0.4 nmol/kg of body weight). Dexamethasone sodium phosphate injection was administered intraperitoneally at 100 mg/kg of body weight as a 10 mg/ml solution. Twenty-four hours after treatment, embryos or chicks were killed and their livers were removed and rinsed with phosphate-buffered saline. Samples of pooled livers were used; 40 embryos at 14 days incubation, 15 embryos at 18 days of incubation, 10 birds at 1 day posthatch, and 2 or 3 birds at 36 days posthatch. Liver microsomes were prepared by differential centrifugation, as previously described (25). Microsomes for gel electrophoresis and Western blotting were prepared in buffer containing 1 mM phenylmethylsulfonyl fluoride. Pellets from the high speed centrifugation were resuspended in 2 mM Tris, 20% glycerol, 1 mM EDTA, pH 7.4, with or without phenylmethylsulfonyl fluoride at 0.25 ml/g wet weight of liver, divided into 0.5 ml aliquots, and frozen at −80° until use.

Enzyme assays. Demethylase assays were performed as previously described (25), by a modification of the protocol of Nash (43). The final substrate concentrations were erythromycin, 1 mM; benzphetamine, 1 mM; ethylmorphine, 8 mM; and aminopyrine, 5 mM. Incubation times were 15 min for aminopyrine, ethylmorphine, and benzphetamine, and 20 min for erythromycin. The amounts of microsomal protein (0.5 to 1 mg) and incubation times were adjusted so that formaldehyde formation was proportional to these parameters. Catalytic activity is

expressed as nmol of formaldehyde/mg of microsomal protein/min, whereas specific activity is expressed as nmol of product/nmol of P450/min. P450 was measured using a DW-2 Aminco spectrophotometer according to the procedure of Matsubara *et al.* (44), which employs the reduced CO versus oxidized CO difference spectrum.

The assay for aldrin epoxidase was a slight modification of a procedure developed by Krieger and Wilkinson (45), which measures the epoxidation of aldrin to its 6,7-epoxide, dieldrin. Incubations were carried out in open 15-ml glass test tubes in a 37° water bath. Each incubation mixture contained 0.1 to 0.5 mg of microsomal protein, 200 μl of generating system (1.8 mM NADP, 12 mM glucose-6-phosphate, and 2 units of glucose-6-phosphate dehydrogenase) and was brought to a final volume of 1 ml with Tris buffer (5.8 mM Tris-HCl, 14 mM KCl, 1.5 mM EDTA, 3 mM glucose-6-phosphate, pH 7.4). The optimum pH was 7.4, as determined over a range from 6.8 to 7.8. After a few minutes were allowed for temperature equilibration, the reaction was initiated with 5 μl of aldrin solution (4 μg/μl in ethanol) (55 μM final). The reaction was terminated after 10 min with 700 μl of acetone. Blanks were the same as sample tubes except acetone was added before the addition of aldrin. After addition of a small amount of anhydrous sodium sulfate, the contents of each tube were extracted twice with hexane (2 ml/extraction). The organic layers were combined in a ground glass test tube. After a few more crystals of anhydrous sodium sulfate were added to each sample extract, tubes were stoppered, sealed with Parafilm, and refrigerated overnight until gas chromatographic analysis. Dieldrin was assayed using a Perkin-Elmer gas chromatograph (Model 990), equipped with a Ni235 electron capture detector. The column was 3 feet × 2 mm and was packed with 80–100 mesh Chromosorb Q that was coated with QF-1 5% silicon oil. The column temperature was 180° and nitrogen was the carrier gas. Dieldrin concentrations were determined, by the peak height method, from standard curves obtained on the same day samples were assayed; some samples required 10–50-fold dilution to fall within the range of linearity of the standard curve. Each sample was incubated in replicate. Enzyme activities were calculated as nmol of dieldrin/mg of microsomal protein/min.

Preparation of MAbs, gel electrophoresis, and Western blotting. Preparation of MAbs to rat liver P450s induced by MC (34), PB (37), or ethanol (35) and preparation and characterization of MAb 2-13-2 to P450 PB-2a/PCN-E were as described (36). P450 PB-2a/PCN-E (40) is probably identical to one of the three PCN-P450s isolated by Halpert (10), PCNb, which is the predominant PCN-P450 in PB-induced rat liver microsomes.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed using a discontinuous system (46), and electrophoresed proteins were transferred to nitrocellulose (47). The immunoblots were then developed by sequential incubations with MAb 2-13-2 in 0.25 mg/ml ascites fluid, alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (1:1000 dilution), and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate. Immunostained areas were quantitated by densitometry with a Beckman DU-8 spectrophotometer that was equipped with scanning accessory.

Statistical analysis. Means were compared by a two-sample *t* test to determine statistically significant differences.

Results

To screen initially for the immunoreactivity of chicken liver P450s with MAbs against purified rat liver P450s induced by MC, PB, ethanol, and PCN, immunoblotting was performed with chicken liver microsomes from embryos and neonatal chickens, untreated or pretreated with PB or TCB. Only MAb 2-13-2, which specifically recognizes P450 PB-2a/PCN-E (36, 40), detected proteins in the P450 region (Fig. 1). The immunodetectable chicken protein migrated identically with the PCN-P450(s) detected in microsomes from dexamethasone-treated rats (Fig. 1, *lane 1*). This screening study indicated that a P450 form present in untreated and TCB-induced (not shown) neonatal chicken liver and induced by PB at both 14 days of incubation and 1 day posthatch is epitopically related to rat liver PCN-P450(s). To characterize the development of the MAb 2-13-2-reactive protein, additional immunoblot analyses were carried out, with several inducers and at different developmental stages. The protein that was immunodetected with MAb 2-13-2 was most predominant in microsomes from chicken liver at 1 days posthatch (Fig. 1, *lanes 4*, *8*, and *10*), with much lower levels observed in the embryo (Fig. 1, *lanes 2*, *3*, *6*, and *7*) and at 36 days posthatch (Fig. 1, *lanes 5*, *9*, and *11*). PB and dexamethasone were both highly effective inducers of this protein at both 1 and 36 days posthatch, although dexamethasone did not increase the overall level of P450, as determined spectrophotometrically (Table 1). Thus, dexamethasone was a more specific inducer of this protein than PB, based on immunostain intensity/nmol of P450.

The results from the immunoblots were compared with a series of P450-associated catalytic activities of the same microsomal preparations. Aldrin epoxidase and benzphetamine and erythromycin demethylase were all induced significantly by PB (Table 1) at most of the ages tested. Aldrin epoxidase was particularly responsive to PB induction, with 42-, 18-, 8-, and 7-fold induction at 14 and 18 days of incubation and 1 and 36 days posthatch, respectively. The specific activity of aldrin epoxidase was induced by PB at all ages examined, whereas the other specific activities were not consistently induced by PB (Table 2). Whereas aminopyrine demethylase is undetectable in hepatic microsomes from untreated embryos at 7 days of incubation (33), aldrin epoxidase was detectable in these microsomes at a level of 0.016 nmol/mg of microsomal protein/min and was induced 60-fold by PB. Pentoxyresorufin dealkylase, which is a specific assay for some PB-induced forms in rodents, was not induced by PB in chicken hepatic microsomes, although we observed PB induction in rats.

Dexamethasone induced the amount of MAb 2-13-2-reactive protein 2-fold (Table 1, last column) at 1 day posthatch and 10-fold at 36 days posthatch. Erythromycin demethylase was the only activity significantly induced by dexamethasone (at 36 days posthatch).

The developmental profiles of MAb 2-13-2-reactive protein and the catalytic activities displayed a common pattern, in that they all reach a peak at 1 day posthatch. However, differences were noted in the extent to which the levels of protein or catalytic activities were augmented in the hepatic microsomes of the newborn chick, compared with embryos and older chickens (36 days posthatch) (Fig. 2). The developmental profiles of erythromycin demethylase and the MAb 2-13-2-reactive protein were similar, in that embryonic levels were quite low, a large increase was observed in the neonate, and, by 36 days posthatch, levels dropped substantially. The erythromycin demethylase and MAb 2-13-2-reactive protein profiles showed a good correlation ($r^2 = 0.86$) with each treatment and at all ages. Also, the developmental profiles of aldrin epoxidase, benzphetamine demethylase, ethylmorphine demethylase, and aminopyrine demethylase were positively correlated with each other at levels from $r^2 = 0.92$ to 0.98 but showed much weaker correlations with the MAb 2-13-2-reactive protein ($r^2 = 0.23$ to 0.42).

There was a trend of increased specific activity toward all substrates after hatching (Table 2), indicating that the catalytic

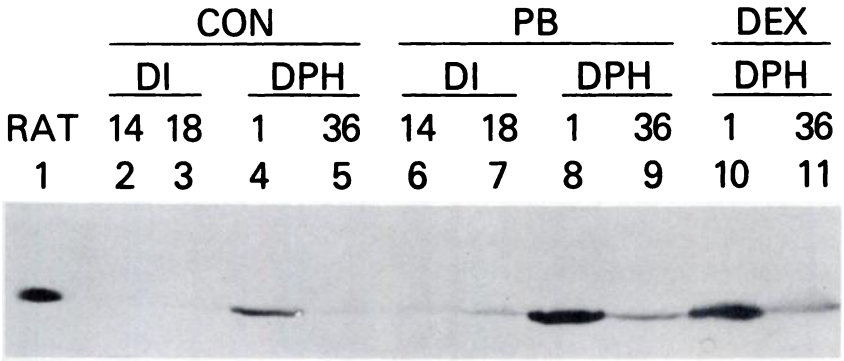

Fig. 1. Immunoblot of embryonic and posthatch chicken liver microsomes using MAb 2-13-2. *Lane 1* contains 2 μg of microsomes from dexamethasone-treated rats. *Lanes 2–5* contain 200 μg of liver microsomes from control chicken embryos at 14 and 18 days of incubation and 1 and 36 days posthatch. *Lanes 6–9* repeat the same sequence for PB-treated chickens. *Lanes 10* and *11* contain microsomes from dexamethasone-treated chickens at 1 and 36 days posthatch. *CON, control; DEX*, dexamethasone; *DI*, days of incubation; and *DPH*, days posthatch.

TABLE 1

P450-associated catalytic activities in chicken hepatic microsomes during embryonic and neonatal development and immunodetection by MAb 2-13-2

P450 is expressed as nmol/mg of microsomal protein. The activities are expressed as nmol of product/mg of microsomal protein/min. Immunostaining was quantitated as described in Materials and Methods. Values are means ± standard deviations; the numbers in parentheses are the numbers of samples.

Age	Treatment	P450	Aldrin epoxidase	Benzphetamine demethylases	Erythromycin demethylase	Relative area of staining with MAb 2-13-2
		nmol/mg		*nmol/mg/min*		
14 DI[a]	Control	0.14 ± 0.01 (4)	0.06 ± 0.01 (3)	0.34 ± 0.09 (2)	0.08 ± 0.02 (2)	0.00
	PB	0.35 ± 0.14 (4)[b]	2.50 ± 0.32 (3)[b]	1.56 ± 0.77 (2)	0.27 ± 0.14 (2)	0.03
18 DI	Control	0.24 ± 0.05 (4)	0.18 ± 0.04 (3)	0.86 ± 0.03 (3)	0.16 ± 0.04 (3)	0.02
	PB	0.81 ± 0.12 (4)[b]	3.31 ± 0.72 (3)[b]	3.95 ± 0.89 (3)[b]	0.54 ± 0.19 (3)[b]	0.07
1 DPH[c]	Control	0.30 ± 0.11 (4)	0.97 ± 0.28 (4)	2.56 ± 0.17 (3)	0.67 ± 0.03 (2)	0.22
	PB	1.02 ± 0.16 (4)[b]	7.93 ± 1.20 (4)[b]	9.82 ± 2.79 (3)[b]	2.37 ± 0.47 (3)[b]	0.62
	Dexamethasone	0.28 ± 0.04 (3)	1.37 ± 0.18 (3)	2.69 ± 0.10 (2)	1.22 ± 0.29 (2)	0.42
36 DPH	Control	0.28 ± 0.03 (3)	1.00 ± 0.04 (2)	4.28 ± 1.81 (3)	0.28 ± 0.06 (3)	0.02
	PB	0.64 ± 0.09 (4)[b]	7.14 ± 1.55 (3)[b]	9.91 ± 1.61 (3)[b]	1.02 ± 0.45 (3)[b]	0.16
	Dexamethasone	0.22 ± 0.03 (3)	1.95 ± 1.01 (2)	3.68 ± 0.94 (3)	0.59 ± 0.12 (3)[b]	0.18

[a] DI, days of incubation.
[b] Significantly different from control ($p < 0.05$).
[c] DPH, days posthatch.

TABLE 2

Specific activities of chicken hepatic microsomes during embryonic and neonatal development

Values are expressed as nmol of product/nmol of P450/min. Values for aldrin epoxidase and benzphetamine and erythromycin demethylase are the mean of 2–4 replicates, whereas those for ethylmorphine and aminopyrine are from single samples.

Age	Treatment	Aldrin epoxidase	Benzphetamine demethylase	Ethylmorphine demethylase	Aminopyrine demethyl-ase	Erythromycin demethylase
				nmol/nmol/min		
14 DI[a]	Control	0.4	2.4	ND[b]	ND	0.6
	PB	6.8[c]	3.8	ND	ND	0.7
18 DI	Control	0.8	3.4	2.5	6.1	0.6
	PB	4.3[c]	4.9[c]	7.9	8.1	0.7
1 DPH[d]	Control	3.0	6.8	6.1	17.3	2.3
	PB	8.0[c]	11.1[c]	14.9	17.0	3.0
	Dexamethasone	5.0	9.1	12.0	24.5	4.0
36 DPH	Control	5.0	14.6	8.9	16.5	1.0
	PB	11.7[c]	14.6	21.6	28.5	1.5
	Dexamethasone	8.8	18.4	21.5	36.4	3.0[c]

[a] DI, days of incubation.
[b] ND, not determined.
[c] Significantly different from control ($p < 0.05$).
[d] DPH, days posthatch.

activities examined increased more than the P450. This result suggests a P450 system more efficient in the catalysis of the whole series of substrates.

Discussion

Immunoblot analysis of chicken hepatic microsomes by MAb 2-13-2 against rat PCN-P450 indicates the presence of a related P450 in chicken liver. Supporting evidence for the presence of PCN-P450 in chicken liver is the induction of erythromycin demethylase by dexamethasone, as has been reported in rodents (28), and the correlation of erythromycin demethylase with the amount of immunodetected protein, with the different treatments and age groups. The presence of P450s other than the known PB-induced forms in chicken (13–17) is suggested by a difference in developmental profiles for the catalytic assays;

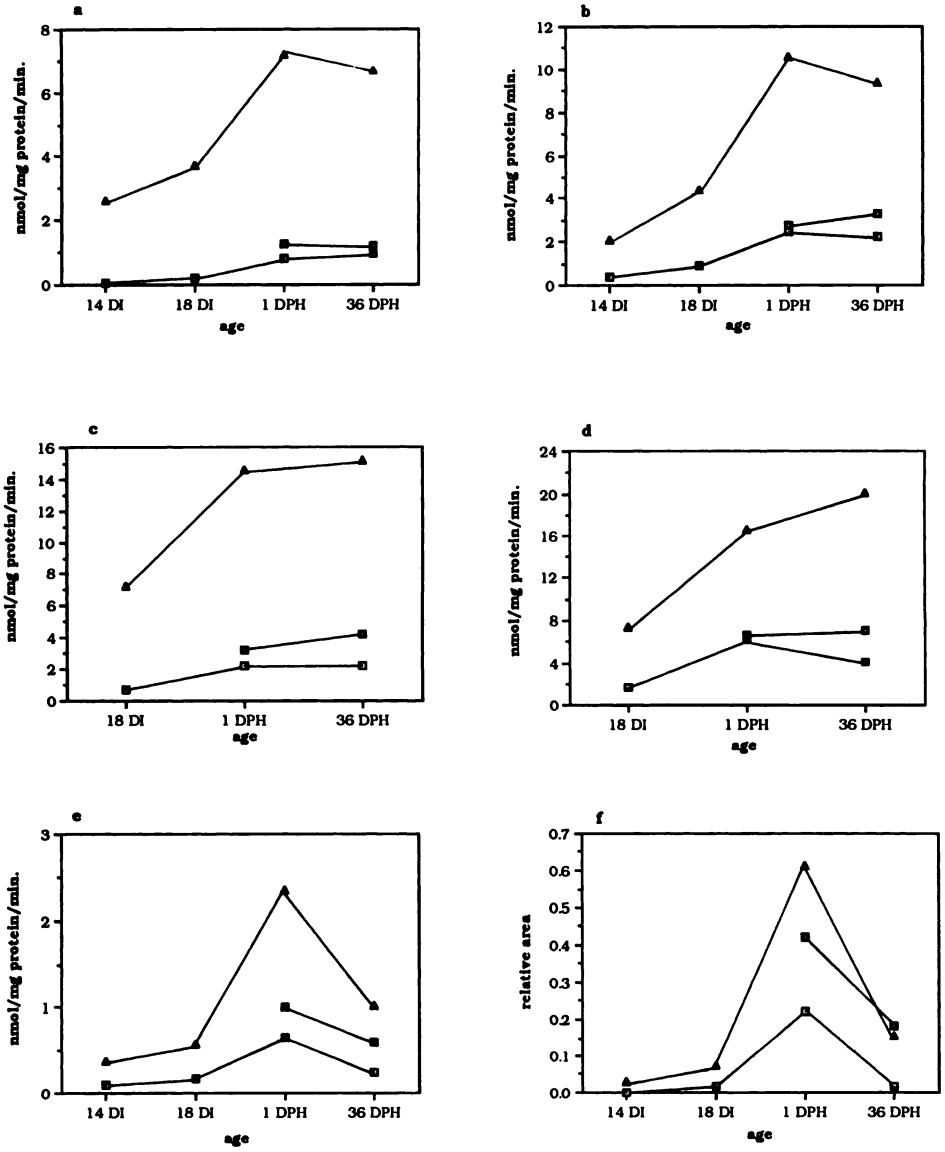

Fig. 2. Developmental profiles of aldrin epoxidase (a), benzphetamine demethylase (b), ethylmorphine demethylase (c), aminopyrine demethylase (d), erythromycin demethylase (e), and MAb 2-13-2-reactive protein (f). *DI*, days of incubation, *DPH*, days posthatch. All of these assays were performed on aliquots from a common microsomal sample for each age and treatment. □, Control; △, PB-induced; ■, dexamethasone-induced chicken liver microsomes.

one type is exemplified by erythromycin demethylase and the immunoreactive protein and the other type by aldrin epoxidase and ethylmorphine, benzphetamine, and aminopyrine demethylase. Although each of these catalytic activities probably represents the combined action of several isozymes of P450, they appear to fall into two categories. Erythromycin demethylase may be predominantly a function of a PCN-P450(s) in chicken liver whereas aldrin epoxidase, ethylmorphine demethylase, benzphetamine demethylase, and aminopyrine demethylase may be predominantly a function of P450II forms of hepatic chicken P450. Our studies suggest that it will be worthwhile to search for and then characterize the PCN-P450 gene(s) in chickens.

The MAb 2-13-2 was used because of its high specificity for the P450-2a/PCN-E (40) of rat liver. Radioimmunoassays showed that MAb 2-13-2 binds 60 times more strongly with the purified P450 2a/PCN-E, compared with PB-4 (P450II family) (36). MAb 2-13-2 to PCN-P450 was not found to be inhibitory to catalytic activity of reconstituted P450-2a/PCN-E or dexamethasone-induced rat liver microsomes (36). Therefore, it was not tested against chicken microsomes. Some polyclonal antibodies against rat liver PCN-P450s have been shown to inhibit rat and mouse liver microsomal activity and may be useful in demonstrating the presence and function of related forms in chicken (18, 48).

The rat liver PB- (P450II) and PCN- (P450III) inducible forms are difficult to differentiate on the basis of catalytic activity, although the P450II forms are only about 30% similar in sequence to the P450III forms (49). The related forms in chicken liver appear to have a pattern of substrate specificity and inducibility with some similarities and some differences from those in rodents. Aldrin epoxidase and ethylmorphine demethylase are induced by both PCN and PB in rat liver (26–28), but our evidence indicates that these activities are probably not efficiently catalyzed by the chicken PCN-P450(s). Erythromycin demethylase has often been used to assay for the PCN-P450 in rat liver microsomes (28, 50) and appears to also be a function of PCN-P450 in chickens. Testosterone or androstenedione-6-β-hydroxylase and (R)-warfarin-10-hydroxylase, diagnostic activities of some forms of PCN-P450 in rats and mice (18, 48), may prove to be useful in detecting PCN-P450s in chicken. Testosterone-6-β-hydroxylase is induced by PB and

2-acetylaminofluorine in chicken liver microsomes (51) but the isozymes responsible are unknown. Some PCN-P450s are induced in rat and mouse liver by PB and dexamethasone (10, 28, 48), as is the MAb 2-13-2-reactive protein in chicken. Dexamethasone may or may not induce the P450II forms in chicken; inasmuch as it is reported to induce P450II isozymes in mice but not in rats (52). These factors contribute to the difficulty in defining characteristic substrates for the PCN forms, which when purified from rat liver often lose most of their catalytic activity (10, 28).

Previous studies in this laboratory show that aminopyrine demethylase increases throughout embryonic development, peaking at 1 day posthatch (25). The specific activity of aminopyrine demethylase is 2- to 3-fold higher posthatching then embryonically. In this study, we observed a trend of increased specific activity toward all the substrates after hatching. One proposed mechanism for this increase is changes in isozymic composition posthatching. The MAb 2-13-2-reactive protein was hardly expressed during the embryonic period, although it was inducible by PB. The increase in MAb 2-13-2-reactive protein neonatally may contribute in varying degrees, to the increase in specific activities observed after hatching with all the catalytic assays. However, levels of this protein do not correlate with the sustained specific activity toward some of the substrates in control samples at 36 days posthatch (MAb 2-13-2-reactive protein falls to the level at 18 days of incubation in control samples). Other isozymes may also become elevated after hatching.

Our study lends support to the hypothesis that there are representative(s) of the PCN-P450 (P450III) family in the chicken. This contention is predicted by the early divergence of the PCN-induced form, compared with the bird-mammal divergence, based on comparisons of known PCN-P450 sequences with other P450 sequences (11). Sequencing of a chicken PCN-P450 would give further clues to the evolution of the P450 system. The chicken remains an important species for the study of P450 evolution because of its evolutionary position between mammals and other classes.

The substantial capacity of the chicken embryo for P450-associated metabolic activation of a wide spectrum of substrates recommends its continued use as a model in studies of the role of P450 in the effects of xenobiotics, including targeted tissue damage and transformation during early development.

Acknowledgments

The authors express their appreciation to Rita Misra and Ursula Nanna for their technical assistance and to Kristina Hetnarski for helpful discussion.

References

1. Todd, L. A., and S. E. Bloom. Differential induction of sister chromatid exchanges by indirect acting mutagen-carcinogens at early and late stages of embryonic development. *Environ. Mutagen.* **2**:435–445 (1980).
2. Juchau, M. R., C. M. Giachelli, A. G. Fantel, J. C. Greenaway, T. H. Shepard, and E. M. Faustman-Watts. Effects of 3-methylcholanthrene and phenobarbital on the capacity of embryos to bioactivate teratogens during organogenesis. *Toxicol. Appl. Pharmacol.* **80**:137–146 (1985).
3. Hamilton, J. W., and S. E. Bloom. Correlation between induction of xenobiotic metabolism and DNA damage from chemical carcinogens in the chick embryo *in vivo*. *Carcinogenesis* (Lond.) **7**:1101–1106 (1986).
4. Miller, J. A., and E. C. Miller. The metabolic activation of chemical carcinogens to reactive electrophiles, in *Biology of Radiation and Carcinogenesis* (J. M. Yuhas, R. W. Tennant, and J. D. Regan, eds). Raven Press, New York, 147–164 (1976).
5. Eskola, J., and P. Toivanen. Effect of *in ovo* treatment with cyclophosphamide on lymphoid system in chicken. *Cell. Immun.* **13**:459–471 (1974).
6. Guengerich, G. P. Enzymology of rat liver cytochrome P450s, in *Mammalian Cytochromes P450* (F. P. Guengerich, ed.), Vol. 1. CRC press, Boca Raton, FL, 1–54 (1987).
7. Nebert, D. W., M. Adesnik, M. J. Coon, R. W. Estabrook, F. J. Gonzalez, F. P. Guengerich, I. C. Gunsalus, E. F. Johnson, B. Kemper, W. Levin, I. R. Phillips, R. Sato, and M. R. Waterman. The P450 gene superfamily: recommended nomenclature. *DNA* **6**:1–11 (1987).
8. Nebert, D. W., and F. J. Gonzalez. P450 genes: structure, evolution, and regulation. *Annu. Rev. Biochem.* **56**:945–993 (1987).
9. Gonzalez, F. J., B.-J. Song, and J. P. Hardwick. Pregnenolone 16-α-carbonitrile-inducible P450 gene family: gene conversion and differential regulation. *Mol. Cell. Biol.* **6**:2969–2976 (1986).
10. Halpert, J. Multiplicity of steroid-inducible cytochromes P450 in rat liver microsomes. *Arch. Biochem. Biophys.* **263**:59–68, (1988).
11. Nelson, D. R., and H. W. Strobel. Evolution of cytochrome P-450 proteins. *Mol. Biol. Evol.* **4**:572–593 (1987).
12. Dickerson, R. E. The structure of cytochrome *c* and the rates of molecular evolution. *J. Mol. Evol.* **1**:26–45 (1971).
13. Mattschoss, L. A., A. A. Hobbs, A. W. Steggles, B. K. May, and W. Elliott. Isolation and characterization of genomic clones for two chicken phenobarbital-inducible cytochrome P-450 genes. *J. Biol. Chem.* **261**:9438–9443 (1986).
14. Hobbs, A. A., L. A. Mattschoss, B. K. May, K. E. Williams, and W. H. Elliott. The cDNA and protein sequence of a phenobarbital-induced chicken cytochrome P-450. *J. Biol. Chem.* **261**:9444–9449 (1986).
15. Ades, I. Identification and purification of a cytochrome P-450 from livers of phenobarbital-treated chick embryos. *IRCS Int. Res. Commun. Sys. Med. Sci.* **11**:302–303 (1983).
16. Oron, L., and S. Bar-Nun. Evidence that 2-allyl-2-isopropylacetamide and phenobarbital induce the same cytochrome P-450 in cultured chick embryo hepatocytes. *Biochem. Biophys. Res. Commun.* **125**:1096–1102 (1984).
17. Sinclair, P., J. Frezza, J. Sinclair, J. Bement, J. Healey, and H. Bonkovsky. Immunochemical detection of induction of different isozymes of cytochrome P450 in chick hepatocyte cultures. *Fed. Proc.* **45**:1663 (1986).
18. Waxman, D. J., G. A. Dannan, and F. P. Guengerich. Regulation of rat hepatic cytochrome P-450; age-dependent expression, hormonal imprinting and xenobiotic inducibility of sex-specific isoenzymes. *Biochemistry* **24**:4409–4417 (1985).
19. Giachelli, C. M., and C. J. Omiecinski. Developmental regulation of cytochrome P-450 genes in the rat. *Mol. Pharmacol.* **31**:477–484 (1987).
20. Cresteil, T., P. Beaune, P. Kremers, C. Celier, F. P. Guengerich, and J.-P. Leroux. Immunoquantification of epoxide hydrolase and cytochrome P-450 isozymes in fetal and adult human liver microsomes. *Eur. J. Biochem.* **151**:345–350 (1985).
21. Wrighton, S. A., D. T. Molowa, and P. S. Guzelian. Identification of a cytochrome P-450 in human fetal liver related to glucocorticoid-inducible cytochrome P-450 HLp in the adult. *Biochem. Pharmacol.* **37**:3053–3055 (1988).
22. Bloom, S. E. Detection of sister chromatid exchanges *in vivo* using avian embryos, in *Cytogenetic Assays of Environmental Mutagens* (T. C. Hsu, ed.). Allanheld Osmun, Totowa, NJ, 137–159 (1983).
23. Brunstrom, B. Activities in chick embryos of 7-ethoxycoumarin *O*-deethylase and aryl hydrocarbon (benzo[*a*]pyrene) hydroxylase and their induction by 3,3′,4,4′-tetrachlorobiphenyl in early embryos. *Xenobiotica* **16**:865–872 (1986).
24. Hamilton, J. W., M. S. Denison, and S. E. Bloom. Development of basal and induced aryl hydrocarbon (benzo[*a*]pyrene) hydroxylase activity in the chicken embryo *in ovo*. *Proc. Natl. Acad. Sci. USA* **80**:3372–3376 (1983).
25. Lorr, N. A., and S. E. Bloom. Ontogeny of the chicken cytochrome P-450 enzyme system: expression and development of responsiveness to phenobarbital induction. *Biochem. Pharmacol.* **36**:3059–3067 (1987).
26. Newman, S. L., and P. S. Guzelian. Identification of the cyanopregnenolone-inducible form of hepatic cytochrome P-450 as a catalyst of aldrin epoxidation. *Biochem. Pharmacol.* **32**:1529–1531 (1983).
27. Elshourbagy, N. A., and P. S. Guzelian. Separation, purification and characterization of a novel form of hepatic cytochrome P-450 from rats treated wtih pregnenolone-16-α-carbonitrile. *J. Biol. Chem.* **255**:1279–1285 (1980).
28. Wrighton, S. A., P. Maurel, E. G. Schuetz, P. B. Watkins, B. Young, and P. S. Guzelian. Identification of the cytochrome P-450 induced by macrolide antiboitics in rat liver as the glucocorticoid responsive cytochrome P-450p. *Biochemistry* **24**:2171–2178 (1985).
29. Gelboin, H. V., and F. K. Friedman. Monoclonal antibodies for studies on xenobiotic and endobiotic metabolism. *Biochem. Pharmacol.* **34**:2225–2234 (1985).
30. Thomas, P. E., S. Bandiera, L. M. Reik, S. L. Maines, D. E. Ryan, and W. Levin. Polyclonal and monoclonal antibodies as probes of rat hepatic cytochrome P-450 isozymes. *Fed. Proc.* **46**:2563–2566 (1987).
31. Cheng, K.-C., H. V. Gelboin, B.-J. Song, S. S. Park, and F. K. Friedman. Detection and purification of cytochromes P-450 in animal tissues with monoclonal antibodies. *J. Biol. Chem.* **259**:12279–12284 (1984).
32. Song, B.-J., T. Fujino, S. S. Park, F. K. Friedman, and H. V. Gelboin. Monoclonal antibody-directed radioimmunoassay of specific cytochromes P-450. *J. Biol. Chem.* **259**:1394–1397 (1984).
33. Thomas, P. E., J. Reidy, L. M. Reik, D. E. Ryan, D. R. Koop, and W. Levin. Use of monoclonal antibody probes against rat hepatic cytochromes P-450c and P-450d to detect immunochemically related isozymes in liver microsomes from different species. *Arch. Biochem. Biophys.* **235**:239–253 (1984).

34. Park, S. S., T. Fujino, D. West, F. P. Guengerich, and H. V. Gelboin. Monoclonal antibodies that inhibit enzyme activity of 3-methylcholanthrene-induced cytochrome P-450. *Cancer Res.* **42**:1798–1808 (1982).
35. Park, S. S. I. Y. Ko, C. Patten, C. S. Yang, and H. V. Gelboin. Monoclonal antibodies to ethanol-induced cytochrome P-450 that inhibit aniline and nitrosamine metabolism. *Biochem. Pharmacol.* **35**:2855–2858 (1986).
36. Park, S. S., D. J. Waxman, H. Miller, R. Robinson, C. Attisano, F. P. Guengerich, and H. V. Gelboin. Preparation and characterization of monoclonal antibodies to pregnenolone 16-α-carbonitrile-inducible rat liver cytochrome P-450. *Biochem. Pharmacol.* **35**:2859–2867 (1986).
37. Park, S. S., T. Fujino, H. Miller, F. P. Guengerich, and H. V. Gelboin. Monoclonal antibodies to phenobarbital-induced rat liver cytochrome P-450. *Biochem. Pharmaco.* **33**:2071–2081 (1984).
38. Fujino, T., D. West, S. S. Park, and H. V. Gelboin. Monoclonal antibody-directed phenotyping of cytochrome P-450-dependent arylhydrocarbon hydroxylase and 7-ethoxycoumarin deethylase in mammalian tissues. *J. Biol. Chem.* **259**:9044–9050 (1984).
39. Friedman, F. K., R. C. Robinson, B.-J. Song, S. S. Park, and H. V. Gelboin. Preparation of catalytically active cytochromes P-450 by antigen exchange on monoclonal antibody based immunoadsorbents. *Biochemistry* **24**:7044–7048 (1985).
40. Guengerich, F. P., G. A. Dannan, S. T. Wright, M. V. Martin, and L. S. Kaminsky. Purification and characterization of liver microsomal cytochromes P-450: electrophoretic, spectral, catalytic, and immunochemical properties and inducibility of eight isozymes isolated from rats treawted with phenobarbital or β-naphthoflavone. *Biochemistry* **21**:6019–6030 (1982).
41. Cole, R. F., and F. B. Hutt. Selection and heterosis in Cornell white leghorns: a review, with special consideration of interstrain hybrids. *Anim. Breed. Abstr.* **41**:103–118 (1973).
42. Bloom, S. E., and T. C. Hsu. Differential fluorescence of sister chromatids in chick embryos exposed to 5-bromodeoxyuridine. *Chromosoma* (Berl.) **51**:261–267 (1975).
43. Nash, T. The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. *Biochem. J.* **55**:416–421 (1953).
44. Matsubara, T., M. Koike, A. Touchi, Y. Tochino, and K. Sugeno. Quantitative determination of cytochrome P-450 in rat liver homogenate. *Anal. Biochem.* **75**:596–603 (1976).
45. Krieger, R. I., and C. F. Wilkinson. Microsomal mixed-function oxidases in insects. I. *Biochem. Pharmacol.* **18**:1403–1415 (1969).
46. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of the bacteriophage T_4. *Nature* (Lond.) **277**:680–685 (1970).
47. Towbin, H., T. Staehelin, and J. Gordon. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. *Proc. Natl. Acad. Sci. USA* **76**:4350–4354 (1979).
48. Kaminsky, L. S., G. A. Kannan, and F. P. Guengerich. Composition of cytochrome P-450 isozymes from hepatic microsomes of C57BL/6 and DBA/2 mice assessed by warfarin metabolism, immunoinhibition and immunoelectrophoresis with anti-(rat cytochrome P-450). *Eur. J. Biochem.* **141**:141–148 (1984).
49. Gonzalez, F. J., D. W. Nebert, J. P. Hardwick, and C. B. Kasper. Complete cDNA and protein sequence of a pregnenolone-16-α-carbonitrile induced cytochrome P-450. *J. Biol. Chem.* **260**:7435–7441 (1985).
50. Decker, C., K. Sugiyama, M. Underwood, and M. A. Correia. Inactivation of rat hepatic cytochrome P-450 by spironolactone. *Biochem. Biophys. Res. Commun.* **136**:1162–1169 (1986).
51. Darby, N. J., A. Lodola, and F. Burnet. Testosterone metabolite profiles reveal differences in the spectrum of cytochrome P-450 isozymes induced by phenobarbitone, 2-acetylaminofluorene and 3-methylcholanthrene in the chick embryo liver. *Biochem. Pharmacol.* **35**:4073–4076 (1986).
52. Meehan, R. R., L. M. Forrester, K. Stevenson, N. D. Hastie, A. Buchmann, H. W. Kunz, and C. R. Wolf. Regulation of phenobarbital-inducible cytochrome P-450s in rat and mouse liver following dexamethasone administration and hypophysectomy. *Biochem. J.* **254**:789–797 (1988).

Send reprint requests to: Dr. N. A. Lorr, Department of Poultry and Avian Sciences, Cornell University, 213 Rice Hall, Ithaca, NY 14853.