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ORGANOGENESIS-STAGE CYTOCHROME P450 ISOFORMS: UTILIZATION OF PCR FOR DETECTION OF CYP1A1 mRNA IN RAT CONCEPTAL TISSUES

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SUMMARY - Utilizing the reverse transcriptase-linked polymerase chain reaction, we analyzed the capacity of three groups of rat conceptal tissues to express cytochrome P4501A1 (CYP1A1) mRNA during the dysmorphogenesis-sensitive stage of organogenesis. The visceral yolk sac, ectoplacental cone and embryo proper each were investigated on day 12 of gestation with and without prior exposure in utero to 3-methylcholanthrene as inducing agent. With two sets of discriminating oligonucleotide primers, definitive, reproducible signals were detectable only in tissues from 3-methylcholanthrene preexposed conceptuses. Signals of highest intensity were observed with visceral yolk sac tissues and signals of lowest intensity were observed with tissues of the embryo per se. Specificities of the amplified cDNAs were verified using Southern blotting with hybridization to an internal oligonucleotide probe. The results indicate that organogenesis-stage conceptal tissues of the rat will express CYP1A1 mRNA in response to environmental trans-regulating agents. © 1991 Academic Press, Inc.

Previous investigations have demonstrated that even extremely low rates of conceptal biotransformation of certain chemicals are sufficient to generate quantities of reactive metabolites that are capable of effecting grossly observable dysmorphogenesis in developing, organogenesis-stage rat embryos (1-4). The P450 cytochromes were implicated in several of the bioactivating reactions, raising questions pertaining to the identities, activities and regulation of organogenesis-stage expression of individual P450 isoforms in tissues of the conceptus per se during the earlier, sensitive stages of embryonic development in rats. Experiments with substrate probes, immunoprobes and chemical inhibitors have suggested that multiple P450 isoforms may exist in these tissues (5-7), emphasizing the need for an understanding of the biological functions of conceptal P450 hemoproteins during organogenesis and of their potential to influence the embryotoxic effects of chemicals to which conceptuses may be exposed during the most sensitive stages of development.

The purpose of the studies reported here was to investigate the possibility that cytochrome P4501A1 (CYP1A1) mRNA might be upregulated in specific rat conceptal tissues via exposure in utero to 3-methylcholanthrene, a polynuclear aromatic hydrocarbon that serves as a prototype for this class of environmental inducing agents. Other "methylcholanthrene-like" inducers include dioxins such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), planar polyhalogenated biphenyls, dibenzofurans, naphthoflavones, and methylated xanthines. These chemicals are known to be capable of acting as trans-activators for the transcription of CYP1A1 hnRNA via formation of

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complexes with various cytosolic receptors including an 8-9S protein commonly referred to as the TCDD receptor or Ah receptor (8) and a 4-4.5S protein more recently implicated as an additional receptor (9,10). "Methylcholanthrene-like" inducing agents are the only xenobiotic inducers of P450s known to act via receptor-mediated mechanisms. Tissues chosen for investigation were the visceral yolk sac, the ectoplacental cone and the embryo proper and were selected because they consititute the principal components of the currently extensively utilized whole embryo culture system. Advantage was taken of highly sensitive PCR technology for analyses of extremely small quantities of tissues.

MATERIALS AND METHODS

Tissue sources. Time-mated, pregnant rats (Sprague-Dawley, Wistar-derived) were obtained on day 4 or 5 of gestation from Tyler Laboratories, Bellevue, WA. The morning after copulation was designated as day 0 of gestation. For induction, pregnant rats received three intraperitoneal injections of 3-methylcholanthrene in corn oil (40 mg/kg) at 48, 24 and 4 hr prior to sacrifice on the morning of day 12 of gestation. Previous studies indicate that this dosing schedule should be optimal for induction of P4501A1 mRNA (11). Conceptal tissues selected for analyses were those normally explanted in whole embryo culture systems (12) and included the visceral yolk sac, the ectoplacental cone and the embryo proper. The amniotic membrane remained attached to the embryo. Decidual elements, the parietal yolk sac and Reichert's membrane were discarded.

RNA preparation. The tissues of 60-90 conceptuses from at least 5-8 time-mated, pregnant rats were pooled and placed in one of two categories: tissues from a control (untreated) group and tissues from a 3-methylcholanthrene-pretreated (induced) group. Tissues of the ectoplacental cone, visceral yolk sac and embryo proper were dissected and separated as rapidly as possible to minimize RNA degradation, and were immediately homogenized in 10 volumes of an ice-cold solution containing 4.0 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0) and 0.1 M 2-mercaptoethanol. The extraction of total RNA proceeded according to slight modifications of the method of Chomczynski and Sacchi (13) as follows: Tissue homogenates were passed through 20 gauge needles at least three times to shear the DNA. Sodium sarcosyl was then added to the homogenates to yield a final concentration of 0.5%. The homogenate was then subjected to RNA extraction with phenol (containing sodium acetate, pH 4.0)/chloroform as described by Chomczynski and Sacchi (13). Selection of mRNA was accomplished with a Poly(A+) Quick mRNA purification kit manufactured by Stratagene (La Jolla, CA).

First strand cDNA synthesis. Reaction mixtures for reverse transcription of conceptal mRNA contained 0.5 mM dNTP, 0.05 mM Tris buffer (pH 8.3, 42° C), 10 mM $MgCl_2$, 10 mM dithiothreitol, 50 mM KCl, 2.5 mM sodium pyrophosphate, 1 unit/μl of RNasin (Promega, Madison, WI), 0.125 μg/μl of oligo dT_{15} (Promega) and sufficient diethylpyrocarbonate-treated distilled water to yield a final volume of 40 μl. Oligo dT-selected mRNA (1-5 μg) was heated at 65° C for 5 min, quick-chilled on ice for 2 min, and then added to the reverse transcription reaction mixture described above. Finally, 5 units/μg mRNA of AMV reverse transcriptase (Life Sciences, Inc., St. Petersburg, FL) was added to the reaction mixture and the reverse transcription reaction was carried at 42° C for 1 hr. Before proceeding to the PCR reaction, the reverse transcription reaction was inactivated by heating at 95° C for 3 min.

PCR reactions. Primers used for PCR reactions were designated as primer I (5'-CCATGACCAGGAACTATGGG-3', sense), primer II (5'-TCTGGTGAGCATCCAGGACA-3', antisense) and primer III (5'-GGGGGTAGTCCTTGCAGCTT-3', sense). With primers I and II, the PCR reactions were carried out as described by Omiecinski et al. (14) to obtain a predicted target size of 341 base pairs flanking the intron/exon boundary of the 6th and 7th exons and the non-coding region immediately downstream from the coding region of the 7th exon of the P4501A1 gene. With primers II and III the PCR reaction was performed to obtain a predicted target size of 1692 base pairs flanking the 6th nucleotide of the 1st exon and the non-coding region immediately downstream from the coding region of the 7th exon. The duration of the PCR cycles for reactions with primers II and III was modified as follows: 1.5 min for denaturation, 2 min for

annealing and 4 min for extension. All PCR reactions were performed with a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT).

Agarose gel electrophoresis and Southern blotting. After amplification with PCR, 5-20 µl of the reaction mixtures were subjected to electrophoresis on 2.0% or 1.5% agarose gels which were stained with ethidium bromide for visualization of the 341 bp or 1692 bp products, respectively. Standard markers for DNA molecular weight were coelectrophoresed with each gel. The gels were then treated with 1.5 M NaCl/0.5 N NaOH to denature the cDNA. Neutralization with 1.5 M NaCl/1.0 M Tris-HCl (pH 7.4) then followed according to procedures described by Sambrook et al. (15). Southern transfer to nitrocellulose membranes was performed overnight with 20 X SCC (1 X SCC is 150 mM NaCl and 15 mM sodium citrate, pH 7.0) as transfer buffer. Prehybridization for 2 hr and overnight hybridization were according to protocols described by Ausubel et al. (16) except that both prehybridization and hybridization temperatures were 52° C. To confirm specificity for the amplified cDNA, an internal oligonucleotide probe (with a 5'-AGCCTGGAGATGCTGAGGAC-3' sequence, ref. 14) was used for hybridization. The nitrocellulose membranes were washed twice (15 min/wash) at room temperature with 1 X SCC buffer containing 0.1% SDS and then twice more (15 min/wash) at 52° C with 0.25 X SCC buffer containing 0.1% SDS. The membranes were then exposed to Kodak X-ray film XAR with intensifying screens (Fisher Scientific, Seattle, WA) for varying time periods at -70° C.

RESULTS

Results of experiments in which aliquots of the PCR reaction mixtures were subjected to agarose gel electrophoresis and ethidium bromide staining are presented in Figs. 1A and 2A. Positive signals were obtained for each of the three conceptal tissues analyzed and with each of the two sets of primers employed, but only if the conceptuses had been pretreated in utero with 3-methylcholanthrene as inducer (see Materials and Methods). No signals were detected in any of the tissues of uninduced conceptuses nor in tissues of induced conceptuses if reverse transcriptase was omitted from reverse transcription reaction mixtures. For induced conceptuses, visceral yolk sac tissues produced signals of greatest intensity in all experiments performed and tissues of the

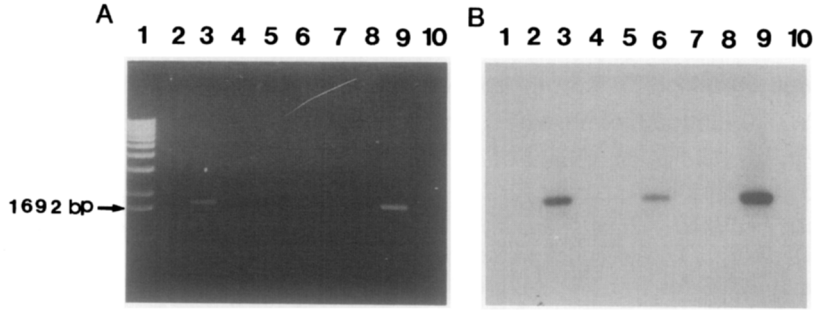

Figure 1. Rat conceptal P4501A1 PCR products generated with primers II and III (See Materials and Methods). **A.** Ethidium bromide stained agarose gel shows PCR products from the amplification of cDNA synthesized from oligo dT-selected mRNA of conceptal tissues. 5 µl of PCR reaction mixture were loaded onto the gel. Lane 1, DNA size markers; Lane 2, ectoplacental cone of control rats; Lane 3, ectoplacental cone of 3-methylcholanthrene-treated rats; Lane 4, same as Lane 3 except without reverse transcriptase in the reverse transcription reaction mixture; Lane 5, embryo proper of control rats; Lane 6, embryo proper of 3-methylcholanthrene-treated rats; Lane 7, same as Lane 6 but without reverse transcriptase; Lane 8, visceral yolk sac of control rats; Lane 9, visceral yolk sac of 3-methylcholanthrene-treated rats; Lane 10, same as Lane 9 but without reverse transcriptase. **B.** Southern blotting of the same gel. Hybridization was performed with a ^{32}P-labeled internal probe (See Materials and Methods). The arrow indicates the migration position of the PCR product at a 1692-bp size as predicted from usage of the described primer pair.

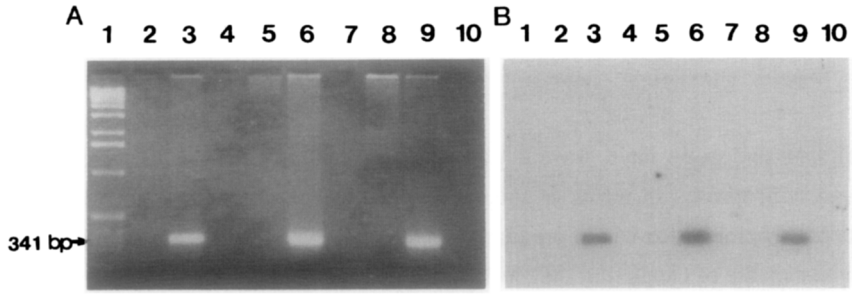

Figure 2. Rat conceptal P4501A1 PCR products generated with primers I and II (See Materials and Methods). **A.** Ethidium bromide stained agarose gel shows PCR products from the amplification of cDNA synthesized from oligo dT-selected mRNA of conceptal tissues. 5 µl of PCR reaction mixture were loaded onto the gel except for the embryo proper (20 µl). Lane 1, DNA size markers; Lane 2, ectoplacental cone - controls; Lane 3, ectoplacental cone - 3-methylcholanthrene-treated; Lane 4, same as Lane 3 except without reverse transcriptase; Lane 5, embryo proper - controls; Lane 6, embryo proper - 3-methylcholanthrene-treated; Lane 7, same as Lane 6 except without reverse transcriptase; Lane 8, visceral yolk sac - controls; Lane 9, visceral yolk sac - 3-methylcholanthrene-treated; Lane 10, same as Lane 9 except without reverse transcriptase. **B.** Southern blotting of the same gel. Hybridization was performed with a ^{32}P-labeled internal probe (see Materials and Methods). The arrow indicates the migration position of the PCR product at a 341-bp size as predicted from usage of the described primer pair.

embryo proper consistently elicited the weakest signals. For the embryo proper, increased quantities of material were applied to the agarose gel (Fig 2A) in order to adequately visualize the signal. Signals produced by tissues of the ectoplacental cone were intermediate in intensity. No attempt was made to further quantify the intensity of the signals.

Results of experiments with Southern blotting are presented in Figs. 1B and 2B and verified that the amplified cDNA generated in the experiments with PCR (described above) was in fact complementary to P4501A1 mRNA. These figures are shown in alignment with Figs. 1A and 2A respectively, for visualization purposes. Again, signals of greatest intensity were observed with the visceral yolk sac followed by the ectoplacental cone and the embryo proper. As indicated above, increased quantities of material from the embryo proper were applied to the agarose gel for adequate visualization of the signal on the Southern blot (Fig 2B).

DISCUSSION

These investigations have provided strong evidence that P4501A1 mRNA can be upregulated in several tissues of organogenesis-stage rat conceptuses via preexposure to 3-methylcholanthrene, a polynuclear aromatic hydrocarbon and prototypic inducing agent. Organogenesis is commonly regarded as the stage of development most sensitive to chemically-elicited dysmorphogenesis (17). Based on past studies of mechanisms whereby "methylcholanthrene-like" compounds elicit P4501A1 induction (18-20), the results also suggest that the gene coding for P4501A1 hnRNA can be transcriptionally activated via exposure to 3-methylcholanthrene and similar inducing agents during this early period of development, although further work will be needed to confirm this tentative conclusion. The visceral yolk sac appeared to be the most responsive to induction and the embryo proper the least responsive. These results are

consistent with previously published immunoquantitative and enzymic analyses of the same three tissues at the same stage of gestation in rats (5, 6) and with *in situ* hybridization studies in mice (21).

The results of the study have several potential implications for the welfare of developing mammalian conceptuses in terms of their responses to the potentially deleterious effects of environmental chemicals to which pregnant animals may be exposed. Cytochrome P4501A1 is known to be capable of catalyzing the bioactivation of numerous procarcinogenic, promutagenic, procytotoxic and prodysmorphogenic P4501A1 substrates to their proximate/ultimate forms (1, 22). Therefore, unless conceptal inactivating sytems and/or other defense mechanisms are concomitantly increased, such upregulation of conceptal P4501A1 would be expected to increase the likelihood of carcinogenic, mutagenic, cytotoxic and dysmorphogenic effects in utero. The actual testing of these ideas would appear to merit a high priority in view of the ubiquitous distribution of and increasing exposure to toxic and potentially toxic P4501A1 substrates in our current environment.

The data presented here also evoke several important questions as follows: 1. Is the conceptal P4501A1 identical in structure and function to the isoform present in hepatic tissues of induced adult rats or do posttranscriptional and/or posttranslational processing perhaps alter these parameters? 2. Will conceptuses of all mammalian species exhibit similar responses to "methylcholanthrene-type" inducers? 3. Will conceptuses respond to other members of this class of inducers (e.g., dioxins, flavones, methylated xanthines, etc.)? 4. What other factors/agents will contribute the regulation of conceptal P4501A1 (e.g., glucocorticoids, thyroid hormone, etc.)? 5. Is the upregulation observed primarily the result of receptor-mediated increases in rates of P4501A1 hnRNA transcription? This seems likely but is by no means certain at this point. 6. What are the identities of other P450 isoforms expressed in organogenesis-stage conceptal tissues and what are their functions? These and other pertinent questions must await further studies.

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REFERENCES

1. Juchau, M.R. (1989) Ann. Rev. Pharmacol. Toxicol. 29, 165-187.
2. Harris, C., Stark, K.L., Luchtel, D.L., and Juchau, M.R. (1989) Toxicol. Appl. Pharmacol. 101, 432-447.
3. Stark, K.L., Lee, Q.P., Namkung, M.J., Harris, C., and Juchau, M.R. (1990) J. Pharmacol. Exp. Ther. 255, 74-83.
4. Juchau, M.R., Harris, C., Stark, K.L., Lee, Q.P., Yang, H.L., Namkung, M.J., and Fantel, A.G. (1991) Reprod. Toxicol., in press.
5. Yang, H.Y.L., Namkung, M.J., and Juchau, M.R. (1988) Mol. Pharmacol. 34, 67-74.

6. Yang, H.L., Namkung, M.J., and Juchau, M.R. (1989) Biochem. Pharmacol. 38, 4027-4037.
7. Lee, Q.P., Yang, H.L., Namkung, M.J., and Juchau, M.R. (1991) Reprod. Toxicol., in press.
8. Fisher, J.M., Wu, L., Denison, M.S., and Whitlock, J.P., Jr. (1990) J. Biol. Chem. 265, 9676-9681.
9. Raha, A., Reddy, V., Houser, W., and Bresnick, E. (1990) J. Toxicol. Env. Health 29, 339-355.
10. Forkert, P.G., Barton, H.A., Costantini, M.G., and Marletta, M.A. (1990) Carcinogen. 11, 1831-1835.
11. Miller, M.S., Jones, A.B., Chauhan, D.P., Park, S.S., and Anderson, L.M. (1989) Carcinogenesis 10, 875-883.
12. New, D.A.T. (1973) In The Mammalian Fetus In Vitro (C.R. Austin, Ed.), pp. 15-54, Chapman and Hall, London.
13. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156-159.
14. Omiecinski, C.J., Redlich, C.A., and Costa, P. (1990) Cancer Res. 50,4315-4321.
15. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) Cloning, A Laboratory Manual, 2nd Ed., pp. 9.38-9.40, Cold Spring Harbor Laboratory Press, New York.
16. Schatz, D.G. (1989, Suppl. 1990) In Current Protocols in Molecular Biology (F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl, Eds.), Vol. 1, pp. 2.9.1-2.9.13, Greene Publishing Assoc. and Wiley-Interscience, New York.
17. Fritz, H., and Giese, K. (1990) Pharmacology 40, 1-28.
18. Okey, A.B. (1990) Pharmacol. Therap. 45, 241-298.
19. Nebert, D.W., and Jones, J.E. (1989) Int. J. Biochem. 21, 243-252.
20. Fisher, J.M., Jones, K.W., and Whitlock, J.P., Jr. (1989) Mol. Carcinogen. 1, 216-221.
21. Dey, A., Westphal, H., and Nebert D.W. (1989) Proc. Natl. Acad. Sci. USA 86, 7446-7450.
22. Lewis, D.F.V., Ioannides, C., and Parke, D.V. (1987) Chem. Biol. Interact. 64, 39-60.