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Structural Gene Products of the *Ah* Locus

Transcriptional Regulation of Cytochrome P_1-450 and P_3-450 mRNA Levels by 3-Methylcholanthrene

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SUMMARY

Mouse liver cytochromes P_1-450 and P_3-450 represent those forms of polycylic hydrocarbon-induced P-450 most closely associated with induced aryl hydrocarbon (benzo[*a*] pyrene) hydroxylase and acetanilide 4-hydroxylase activity, respectively. These two proteins are controlled by the *Ah* receptor: C57BL/6N mice possess the high-affinity receptor; DBA/2N mice, the poor-affinity receptor. 3-Methylcholanthrene at the highest dose technically possible induces both proteins in C57BL/6N but not DBA/2N mice, whereas sufficiently high doses of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) induce both proteins in both inbred mouse strains. Plasmids containing DNA complementary to P_1-450 and P_3-450 mRNA, respectively, were used in an *in vitro* nuclear transcription assay to determine the mechanism of the induction response. In C57BL/6N mice, transcriptional rates of the P_1-450 and P_3-450 genes increase dramatically as early as 3 hr after 3-methylcholanthrene treatment and at 12 hr reach maximal levels of 20- and 15-fold, respectively, above control values. In contrast, no increase in either gene is found in 3-methylcholanthrene-treated DBA/2N mice. Following TCDD administration, both P_1-450 and P_3-450 gene transcription rates are elevated in DBA/2N mice. There is a 3- to 6-hr lag period between the early onset of enhanced transcription rates and the later rise in P_1-450 and P_3-450 mRNA. Basal and induced levels of P_3-450 mRNA are about 5-fold greater than those of P_1-450 mRNA. These data confirm that the 3-methylcholanthrene and TCDD induction responses, governed by the *Ah* receptor, are mediated principally through an increase in specific gene transcription.

INTRODUCTION

The induction of drug-metabolizing enzymes by foreign chemicals such as 3-methylcholanthrene, TCDD,[1] and benzo[*a*]pyrene is controlled by the *Ah* locus (reviewed in refs. 1 and 2). The induction response is the result of an increase in several gene products associated with the metabolism of drugs and carcinogens chemically related in structure to the inducers. These gene products include cytochrome P_1-450 (3), P_2-450 (4), P_3-450 (2, 3), and UDP-glucuronosyltransferase (6).

With the use of mouse genetics and recombinant DNA technology, a wealth of information is accumulating with regard to understanding the mechanism of the *Ah* locus induction response (1, 2). The B6 inbred mouse strain has a cytosolic high-affinity *Ah* receptor that specifically binds inducing compounds (7–9), whereas the D2 inbred mouse strain has an *Ah* receptor with at least 10-fold poorer affinity. Inheritance of this receptor defect follows

[1] The abbreviations used are: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; B6, the C57BL/6N inbred strain; D2, the DBA/2N inbred strain; pre-mRNA, large molecular weight precursor mRNA in nucleus; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. The term "P-450" is used to designate any or all forms of microsomal cytochrome P-450. Mouse "P_1-450" and "P_3-450" are defined as those forms of 3-methylcholanthrene-induced P-450 in B6 liver having the highest turnover number for induced aryl hydrocarbon hydroxylase and acetanilide 4-hydroxylase activity, respectively (1–3). Mouse "P_2-450" is defined as that form of isosafrole-induced P-450 in D2 liver having the highest turnover number for isosafrole metabolism (4). The size of all three proteins is 55,000 M_r, as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Soret peaks of the reduced hemoprotein CO complex for P_1-450, P_2-450, and P_3-450 are 449, 448, and 448 nm, respectively (3, 4). Because we have now characterized two proteins with a spectral maximum of 448 nm, we have changed the name of "P-448" studied earlier (3) to "P_3-450." The concentration of P_3-450 protein in control mouse microsomes is at least 5 times greater than that of P_1-450; after 3-methylcholanthrene induction of B6 liver, P_3-450 is the major induced form, being several times greater in concentration than P_1-450 (3, 5). We believe that mouse P_1-450 and P_3-450 correspond to rat P-450c and P-450d, respectively, and rabbit form 6 and form 4, respectively (1).

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Mendelian genetics (1). Following formation of the inducer-receptor complex in the cytosol, the complex is translocated to the nucleus, where it is believed to interact with chromatin components to stimulate gene activity (10, 11). With use of a cloned probe for P_1-450, it was recently demonstrated that translocation of the inducer-receptor complex to the nucleus is highly correlated with the accumulation of P_1-450 mRNA (11). The increase in a high-molecular weight mRNA precursor (pre-mRNA) during the P_1-450 induction process (12, 13) suggests that this induction process is associated with an increase in transcriptional activity, stabilization of existing mRNA, or some combination of the two.

In the present report an *in vitro* nuclear transcription system was utilized to examine the rates of transcription of the P_1-450 and P_3-450 genes in 3-methylcholanthrene- or TCDD-treated B6 and D2 mice. These results were extended to include measurements of P_1-450 and P_3-450 mRNA levels following a single dose of 3-methylcholanthrene or TCDD.

EXPERIMENTAL PROCEDURES

Animals. B6 and D2 (weanling, either sex) mice were given a single i.p. injection of 3-methylcholanthrene (250 mg/kg) dissolved in corn oil (25 ml/kg). Controls were treated with the vehicle alone. TCDD (25 μg/kg) was injected with dimethyl sulfoxide (50 μl/kg) as the vehicle. Groups of six animals for each time point were killed, and their livers were homogenized in 0.25 M sucrose, 50 mM Tris/HCl (pH 7.5), 25 mM KCl, and 5 mM $MgCl_2$. Half of the homogenate was used to isolate nuclei, as described previously (14). The remaining homogenate was used to extract RNA by the guanidine HCl procedure (15).

Transcription with nuclei in vitro. Nuclear transcription assays were performed essentially as described by McKnight and Palmiter (16). Purified nuclei (14) were washed with 0.2% Triton X-100 in the liver homogenization buffer, suspended in 40% glycerol, 50 mM Hepes (pH 8.3), 5 mM $MgCl_2$, and 2 mM dithiothreitol and stored at −70°. Transcription was carried out in a final volume of 0.5 ml containing 16% glycerol, 50 mM Hepes (pH 8.0), 5 mM $MgCl_2$, 1.5 mM $MnCl_2$, 2 mM dithiothreitol, 3 mM spermidine, 150 mM KCl, 0.5 mM ATP, 0.5 mM GTP, 0.5 mM CTP, 250 μCi of [^{32}P]UTP (specific activity 2000 Ci/mmole; ICN Pharmaceuticals Inc., Irvine, Calif.), and 2.5×10^7 nuclei. After incubation at 26° for 60 min, DNase was added to a final concentration of 100 μg/ml (17). Incubation was continued for 30 min at 26°, and total nucleic acid was extracted with phenol/chloroform (1:1, v/v). RNA was recovered by trichloroacetic acid precipitation on nitrocellulose filters as described previously (17). Hybridizations were performed as detailed for poly(A^+)-enriched RNA (18). The efficiency of hybridization was determined to be approximately 85% (18). This was accomplished by including trace amounts of [^{3}H]RNA, transcribed from each plasmid using *Escherichia coli* RNA polymerase in ^{32}P-containing hybridization reactions, and by denaturing and recovering hybridized transcripts and then rehybridizing these with fresh cDNA-containing filters (19). The hybridization was linear with increasing RNA input, and the sensitivity of mRNA transcripts to α-amanitin was 85%–100% (19). Transcriptional activity was expressed as parts per million of the total RNA (disintegrations per minute) hybridized, minus parts per million adsorbed to pBR322.

Quantitation of mRNA levels. Poly(A^+)-enriched RNA was purified by oligo(dT)-cellulose (20) (Type 7; Collaborative Research Inc., Lexington, Mass.) via two passes with an intermediate heat step (14). Specific mRNA sequences were quantitated by filter hybridization as previously described (18). For a brief description, poly(A^+)-containing RNA was partially base-hydrolyzed to 100–150 nucleotides and labeled with ^{32}P by use of polynucleotide kinase. After separating the RNA from [^{32}P]ATP, we hybridized the RNA to separate nitrocellulose filters (18) containing $pP_1$450-57, $pP_3$450-21, and pBR322 DNA. Clone $pP_1$450-57 contains 1750 nucleotides complementary to the 3′ portion of P_1-450 mRNA (21) and overlaps the previously characterized clone 46 that is 1100 nucleotides in length (22). $pP_3$450-21 contains a DNA insert[2] of 1710 base pairs complementary to P_3-450 mRNA (21). The percentage of total RNA hybridizing to each filter was then determined after the radioactivity adsorbing to pBR322 controls had been subtracted (18).

$pP_1$450-57 and $pP_3$450-21 mRNA share sequence homology over 100–200 nucleotides (21). The region of $pP_3$450-21 that is homologous with $pP_1$450-57 is not present in other P-450 mRNA forms. Because of the nature of the hybridization technique, which includes an RNase treatment (18), this small amount of homology does not significantly affect quantitation of P_1-450 or P_3-450 mRNA or rate of gene transcription.

RESULTS

Transcription rate. B6 and D2 hepatic nuclei were purified at various times after a single dose of 3-methylcholanthrene. Transcription was carried out in the presence of [^{32}P]UTP, as detailed under Experimental Procedures. Newly synthesized P_1-450 and P_3-450 pre-mRNA transcripts were then quantitated by filter hybridization to $pP_1$450-57 and $pP_3$450-21 probes, respectively. This transcription assay measures elongation of the RNA chains initiated *in vivo* (24). The levels of specific transcripts detected therefore reflect the number of RNA polymerase molecules in the process of transcribing the P_1-450 and P_3-450 genes.

Rapidly elevated transcription rates for both the P_1-450 and P_3-450 genes were noted 3 hr after 3-methylcholanthrene treatment (Fig. 1). In B6 mice, maximal rates of 20- and 15-fold for the P_1-450 and P_3-450 genes, respectively, were attained 12 hr after the single 3-methylcholanthrene dose. Transcription rates remained elevated at 24 hr. In contrast, D2 mice showed no increase in P_1-450 transcription rate (Fig. 1). These data demonstrate that the lack of P_1-450 induction (induced aryl hydrocarbon hydroxylase activity) is D2 mice is due to the absence of transcriptional activation after 3-methylcholanthrene administration. Furthermore, this is substantiated by the findings that the D2 P_3-450 gene exhibited 23 and 18 ppm transcription rates (average of duplicate determinations) after 0 and 16 hr of 3-methylcholanthrene treatment, respectively.

The potent inducer TCDD, at sufficiently high doses, is able to overcome the receptor defect of D2 mice (11, 25), and appearance of the TCDD-receptor complex in D2 liver nuclei has been closely correlated with P_1-450 mRNA induction (11). Treatment of D2 mice with a sufficiently high dose of TCDD (Table 1) readily increased the P_1-450 gene transcription rate.

Messenger RNA levels. Levels of P_1-450 and P_3-450 mRNA (Fig. 2) increased after 3-methylcholanthrene administration to B6 mice; a lag period of 3–6 hr was noted between the increase in transcription rate (Fig. 1) and the later rise in mRNA levels (Fig. 2). This finding indicates that, during the early phase of induction following 3-methylcholanthrene treatment of B6 mice, a burst in the rate of transcription of both genes occurs, followed

[2] The complete nucleotide sequence of mouse P_3-450 cDNA and its corresponding protein has been reported (23).

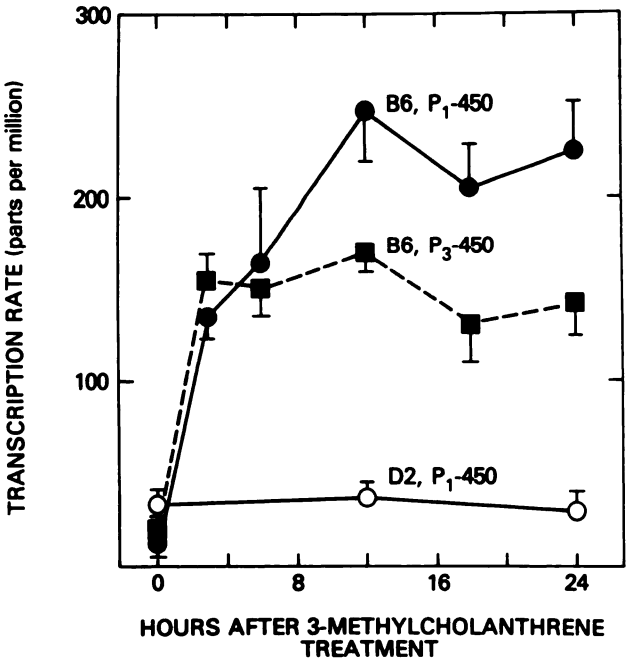

FIG. 1. *Transcription rates of the mouse P_1-450 and P_3-450 genes following 3-methylcholanthrene treatment of B6 and D2 mice*

Nuclei were isolated from B6 and D2 liver at various times following a single dose of 3-methylcholanthrene. *In vitro* nuclear transcription was carried out and the newly labeled RNA was quantitated as described under Experimental Procedures. Approximately 4×10^6 to 6×10^6 dpm from each transcription reaction were hybridized in duplicate with each cDNA-containing filter and a pBR322 filter. Transcription rates are expressed as parts per million of the total disintegrations per minute hybridized, minus the parts per million adsorbed to filters containing pBR322 (16, 19). The radioactivity adsorbing to pBR322-bound filters was between 7 and 14 ppm of the total disintegrations per minute hybridized. *Symbols* and *brackets* denote means ± standard deviations for three duplicate determinations. pBR322-corrected control values used were 8 ± 4 and 16 ± 7 ppm for B6 and D2 P_1-450 gene transcription, respectively, and 10 ± 4 ppm for B6 P_3-450 gene transcription. The control values, expressed in parts per million per kilobase pair of cDNA insert, were 4.7 and 9.4 for B6 and D2 P_1-450, respectively, and 5.9 for B6 P_3-450 gene transcription.

by an accumulation of P_1-450 and P_3-450 messengers. The lag period between the increase in transcription rate and increase in mRNA levels is similar to that observed for rat epoxide hydrolase, NADPH P-450 oxidoreductase, and cytochrome P-450*b* mRNAs after phenobarbital treatment (19). This phenomenon is probably due to the fact that transcriptional activity must be elevated for a certain period of time before an accumulation of mRNA content can be detected.

The lag in P_3-450 mRNA induction is larger than the lag (if any) in P_1-450 induction (Fig. 2). Fouts and coworkers (26) noted in 3-methylcholanthrene-treated B6 mice that induced aryl hydrocarbon hydroxylase activity (P_1-450) is detectable at least 6 hr before the blue shift in the Soret peak of the reduced cytochrome CO complex from 450 to 448 nm (P_3-450) is detectable. These induction kinetics are confirmed at the mRNA level in Fig. 2. In view of a very similar burst in transcription rates for P_1-450 and P_3-450 (Fig. 1), the difference in the kinetics of P_1-450 and P_3-450 mRNA induction (Fig. 2) might

TABLE 1

P_1-450 gene transcription rate and mRNA levels in D2 liver following TCDD treatment

Treatment of D2 mice with TCDD and determination of the transcription rate and mRNA levels are detailed under Experimental Procedures. For each transcription reaction, duplicate aliquots of approximately 4×10^6 to 6×10^6 dpm of *in vitro* ^{32}P-labeled RNA were subjected to hybridization with each cDNA-containing filter and a pBR322-containing filter. Levels of nuclear transcripts adsorbing to pBR322-containing filters were consistently 7–14 ppm. This was subtracted from the parts per million hybridized to each cDNA-containing filter to give transcription rates. Background values (RNA adsorbing to pBR322 filters) for mRNA quantitations were approximately 0.002–0.003%. Values are expressed as the means ± standard deviations for three duplicate determinations.

Time after TCDD administration	Transcription rate	mRNA levels
hr	*ppm*	*% hybridized*
0	20 ± 5.0	0.020 ± 0.005
2	49 ± 12	0.025 ± 0.007
12	90 ± 20	0.086 ± 0.010

reflect some component of posttranscriptional regulation such as differences in pre-mRNA processing or mRNA stabilization. Further studies are needed to test this hypothesis.

The basal level of P_3-450 mRNA was more than 5 times greater than that of P_1-450 mRNA (Fig. 2). The maximal level of induced P_3-450 mRNA was about 5 times greater than that of induced P_1-450 mRNA in B6 mice (Fig. 2). This same order of magnitude difference between both the basal and the induced P_3-450 and P_1-450 membrane-bound proteins has been observed by immunoprecipitable radioactivity experiments (5) and catalytic activity studies during acetanilide 4-hydroxylase and aryl hydrocarbon hydroxylase induction (3).

As expected from Fig. 1, no increase in either mRNA was detected in 3-methylcholanthrene-treated D2 mice (Fig. 2). P_1-450 mRNA levels were elevated, however, in TCDD-treated D2 mice (Table 1). The transcription rate of the P_3-450 gene in TCDD-treated D2 mice was not quantitated, although P_3-450 mRNA levels were clearly found to be elevated from 0.18% to 0.62% hybridized (average of duplicate determinations) after 0 and 12 hr of TCDD treatment, respectively. In D2 mice treated with this dose of TCDD, sufficient amounts of the inducer-receptor complex are known (11) to enter the nucleus and trigger the P_1-450 induction response. In other words, TCDD can overcome the *Ah* receptor defect in D2 mice (1).

DISCUSSION

Our understanding of the mechanism by which polycyclic aromatic compounds induce drug-metabolizing enzyme activities has become more complete than it was several years ago. The cytosolic *Ah* receptor binds avidly to ligands capable of delivering an induction response. These ligands are usually large, relatively planar, foreign chemicals. the possibility of *Ah* receptor heterogeneity (i.e., two or more proteins encoded by two or more genes) has not been rigorously ruled out (27). In B6 mice, the *Ah* receptor has a high affinity for 3-methylcholanthrene,

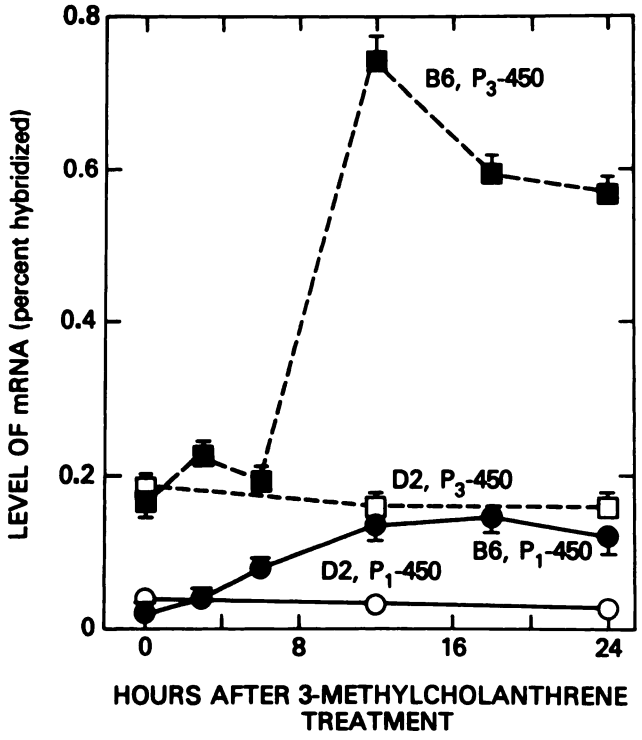

FIG. 2. *Hepatic P_1-450 and P_3-450 mRNA levels following 3-methylcholanthrene treatment of B6 and D2 mice*

Total poly(A$^+$)-enriched RNA was isolated from B6 and D2 liver at various times following 3-methylcholanthrene treatment. RNA was quantitated as described under Experimental Procedures, and the results are expressed as the percentage of total disintegrations per minute hybridized. *Symbols* and *brackets* denote means ± standard deviations for three duplicate determinations.

whereas a poor-affinity receptor exists in D2 mice (1). This receptor defect accounts for the absence of an appreciable P_1-450 induction response following 3-methylcholanthrene treatment of D2 mice. TCDD, on the other hand, is a sufficiently potent inducer to overcome the receptor defect in D2 mice. Following complexation in the cytoplasm, the ligand-receptor complex enters the nucleus to interact with chromatin components; the net result is the activation of several P-450 genes, the induction of several forms of P-450 mRNA, and rises in their associated membrane-bound proteins and catalytic activities (3–5).

The studies reported herein demonstrate conclusively that 3-methylcholanthrene activates transcription of the P_1-450 and P_3-450 genes. This stimulation was further shown to be absent in 3-methylcholanthrene-treated D2 mice, yet overcome by TCDD in D2 mice; these data suggest strongly that the induction process is mediated by the *Ah* receptor. Transcriptional regulation is the primary mode of control in other hormonal and drug induction systems. Examples of elevated transcription rates include (a) the conalbumin and ovalbumin genes in hen oviduct stimulated by estrogen and progesterone (16, 17, 28); (b) the rat liver α_{2U}-globulin (29) and mouse mammary tumor virus genomes (30–33) by glucocorticoids; (c) the prolactin gene in pituitary cell nuclei (34); (d) the mouse metallothionein gene by cadmium and glucocorticoids (35); and (e) the rat epoxide hydrolase, NADPH P-450 oxidoreductase, and cytochrome P-450*b* genes by phenobarbital (19).

Although involvement of an inducer-receptor complex in the activation of gene transcription is now well established, much remains to be learned about the inducer-receptor-chromatin interaction and how this reaction triggers the enhancement of RNA polymerase II transcription rates. This report demonstrates the value of the *Ah* locus as another interesting model system for studying receptor-chromatin interaction and gene expression.

Note added in proof. A similar finding of transcriptional activation of the mouse P_1-450 gene alone has just appeared (36). These data, with TCDD-treated mouse hepatoma cell cultures, confirm our results in the intact mouse.

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