Posttranscriptional Regulation of Coumarin 7-Hydroxylase Induction by Xenobiotics in Mouse Liver: mRNA Stabilization by Pyrazole

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ABSTRACT: The induction mechanism by pyrazole or phenobarbital of coumarin 7-hydroxylase (cytochrome P450coh) was investigated in DBA/2J male mice. The P450coh mRNA in the pyrazole-induced mice was increased gradually to a 20-fold higher level within 48 h, yet transcription of the P450coh gene was not affected. The half-life of P450coh mRNA, on the other hand, was at least 4-fold longer in the pyrazole-induced DBA/2J (~6.0 h) than in control DBA/2J (~1.5 h) male mice. The stabilization of P450coh mRNA, therefore, is the primary mechanism for the induction by pyrazole of coumarin 7-hydroxylase. Phenobarbital, on the other hand, regulates the induction either translationally or posttranslationally. This drug affected neither the P450coh mRNA nor the P450coh gene's transcription levels in the DBA/2J male mice, although Western blots showed approximately a 3-fold increase of the P450coh protein in the liver microsomes of the drug-treated mice. The results indicate, therefore, that both phenobarbital and pyrazole regulate the P450coh induction posttranscriptionally; the former inducer enhances the translational efficiency of P450coh mRNA or alters the degradation rate of P450coh aproprotein, while the latter stabilizes P450coh mRNA.

Hepatic coumarin 7-hydroxylase (P450coh)[1] in mice is expressed constitutively in both genders, except that the expression is female-specific in 129/J mice (Negishi et al., 1989). Recently, Aida and Negishi (1990) have shown, using nuclear run-on assay, growth hormone dependent regulation of the sex-specific P450coh gene's transcription. It appears, therefore, that regulation by the endogenous hormone is transcriptional.

Coumarin 7-hydroxylase activity is known to be regulated also by exogenous chemicals such as phenobarbital and pyrazole (Juvonen et al., 1985; Lindberg et al., 1989; Negishi et al., 1989; Wood, 1978). Both phenobarbital and pyrazole induce the activity in mice, and moreover, increase of the activity by pyrazole is associated with that of the P450coh mRNA level (Negishi et al., 1989). There are strain differences in the induction of P450coh: DBA/2, for example, is one of the responsive strains in that coumarin 7-hydroxylase activity is increased by these xenobiotics (Wood, 1978; Lang et al., 1989). The induction mechanism, however, is not understood.

In this report, we examine the induction of the P450coh gene by pyrazole and phenobarbital in DBA/2J mice. The results indicate that P450coh mRNA is stabilized by pyrazole, while phenobarbital may enhance the translation of the mRNA or decrease the degradation rate of P450coh apoprotein. This posttranscriptional regulation of P450coh gene by these xenobiotics, therefore, is in sharp contrast with the transcriptional regulation by the hormone.

Experimental Procedures

Animals and Treatments. Two-month-old DBA/2J male mice were obtained from Jackson Laboratory (Bar Harbor, ME). Pyrazole (200 mg/kg body weight) or phenobarbital (50 mg/kg body weight) was injected intraperitoneally once every 24 h during the experiments. Livers from at least three mice were pooled for each group of the experiments, minced, and then separated into two parts from which nuclei or RNAs were obtained. When liver microsomes were prepared simultaneously in addition to nuclei and RNAs, the minced livers were divided into three parts.

Northern and Processed Northern Hybridizations. Total liver RNAs were prepared by extraction with 8 M guanidine hydrochloride (Cox, 1968). The RNAs were then enriched for poly(A^+) RNAs by an oligo(dT)–cellulose column (Aviv & Leder, 1972) prior to being used for the hybridizations. Processed Northern hybridizations were performed as described previously (Squires & Negishi, 1988): double-stranded cDNAs were synthesized, digested with the appropriate restriction enzymes, electrophoresed through a 2% agarose gel, and transferred to Nytran paper. Finally, the paper was hybridized with a ^{32}P-labeled *Cla*I–*Cla*I fragment from $P450_{15\alpha}$ cDNA as a probe, washed, and exposed to X-ray films. In addition, we used a specific oligoprobe, OP-1, (Wong et al., 1987) and a cDNA clone, pf3/46 (Noshiro et al., 1988), as probes for Northern hybridizations (Squires & Negishi, 1988) to measure C-$P450_{16\alpha}$ and P450 IIB (P450b) mRNA levels, respectively. C-$P450_{16\alpha}$ is a male-specific steroid 16α-hydroxylase in the liver of mice (Wong et al., 1987), while P450b is known to be induced by phenobarbital (Noshiro et al., 1988).

Nuclear Run-On Assays. Liver nuclei were prepared from each group of mice according to the method of Blobel and Potter (1966). We performed the nuclear run-on assay as described by McKnight and Palmiter (1979) with some modifications. Liver nuclei were prepared from at least three male DBA/2J mice and washed first with 10 mM Tris-HCl, pH 8.0, containing 2.2 M sucrose, 5 mM $MgCl_2$, and 0.2%

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[1] P450coh and $P450_{15\alpha}$ belong to the P450 IIA subfamily, P450b the IIB subfamily, and C-$P450_{16\alpha}$ the IID subfamily.

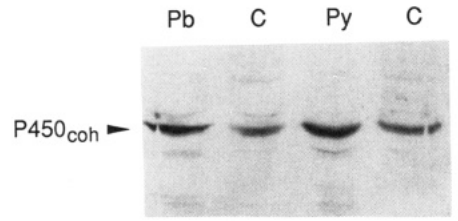

FIGURE 1: Western blot analysis of liver microsomes from the pyrazole- or phenobarbital-treated DBA/2J male mice. The liver microsomes were prepared from the control DBA/2J mice (C) or the mice injected once every day with pyrazole (Py) or phenobarbital (Pb) for 2 days. Four micrograms of the microsomes was electrophoresed through an SDS–polyacrylamide gel (8%), transferred to nitrocellulose paper, and then immunostained with anti-$P450_{15\alpha}$. Because P450coh and $P450_{15\alpha}$ differ by only 11 amino acids within their 494 residues (Lindberg et al., 1989), polyclonal antibody made against a purified $P450_{15\alpha}$ indistinguishably cross-reacts to P450coh (Negishi et al., 1989).

Triton X-100 and then the storage buffer (40% glycerol, 5 mM $MgCl_2$, 2 mM DTT, and 50 mM Hepes, pH 8.0) and resuspended in the storage buffer. The in vitro transcription mixture consisted of isolated liver nuclei (400 μg of DNA) in 100 μL of 50 mM Hepes, pH 8.0, containing 16% glycerol, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 150 mM KCl, 0.5 mM each of ATP, CTP, and GTP, 250 μCi of [^{32}P]UTP (800 Ci/mol, Amersham Corp, IL), and 40 units of RNasin, and was incubated at 26 °C for 60 min. After the treatment with RNase-free DNase I (5 μg/100 μL) at 30 °C for 5 min, the pelleted nuclei were lysed in 10 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA, 0.3% SDS, and 50 μg/mL yeast tRNA, digested with proteinase K (200 μg/mL) at 30 °C for 15 min, and finally extracted with phenol/chloroform. Extracted RNA transcripts were precipitated with 2 volumes of ethanol and dried. Dried [^{32}P]RNAs were dissolved in 10 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA, treated again with the DNase I (50 μg/100 μL), extracted with phenol/chloroform, precipitated with ethanol, dried, and finally redissolved in the same buffer. For filter hybridization, cDNAs were alkaline-denatured and blotted on Nytran paper and hybridized with 2×10^7 cpm of ^{32}P-labeled RNA. The filters were washed with 0.1 × SSPE containing 0.1% SDS twice at 52 °C for 20 min, and then with 10 mM Tris-HCl, pH 7.5, which contains 0.3 M NaCl and 2 mM EDTA, following digestion with RNase A (10 μg/mL) and RNase T1 (1 μg/mL) in the same buffer at 37 °C for 30 min. They were finally washed with 10 mM Tris-HCl, pH 7.5, containing 0.3 M NaCl, 2 mM EDTA, and 0.1% SDS at 42 °C for 30 min and dried prior to exposure to X-ray film. The cDNAs used as the probes were p15α-2 (Lindberg et al., 1989), p16α-14 (Ichikawa et al., 1989), and pf3/46 (Noshiro et al., 1988) for transcriptions of P450coh, C-$P450_{16\alpha}$, and P450IIB (P450b) genes, respectively. Additionally, the mouse major urinary protein (MUP) (a gift from Dr. W. A. Held, Roswell Park Cancer Institute) and γ-actin cDNAs were used as control probes for transcription in mouse livers. The intensity of each band was measured by densitometry and normalized to γ-actin in order to determine the transcription level of the corresponding gene.

Determination of mRNA Half-Life. Mice were induced by intraperitoneal injection of pyrazole (200 mg/kg body weight) for 24 h prior to intraperitoneal injection of actinomycin D (7.5 mg/kg body weight). At least three mouse livers were pooled for each time point after actinomycin D treatment and dissolved in 8 M guanidine hydrochloride to extract total RNAs. The total RNAs were subjected to Northern and processed Northern hybridizations, and the radioactivity of hybridized bands was counted to measure the relative P450 mRNA contents. In order to determine normal half-lives, mice were injected with saline (vehicle for pyrazole) prior to the actinomycin D. Although actinomycin D was dissolved in 0.9% NaCl containing 10% ethanol, a preliminary study showed that the amount of ethanol used had no detectable effect on P450coh mRNA half-lives (not shown).

Other Analytical Methods. We prepared liver microsomes using the previous methods (Harada & Negishi, 1984) and measured coumarin 7-hydroxylase activity by the method of Kaipainen et al. (1985). Western blots were performed as described (Lindberg et al., 1989).

RESULTS

Induction by Phenobarbital of P450coh. Coumarin 7-hydroxylase activity was increased approximately 3-fold from 0.35 nmol min^{-1} (mg of protein)$^{-1}$ in control microsomes to 0.95 nmol min^{-1} (mg of protein)$^{-1}$ in microsomes from the phenobarbital-treated mice at 48-h posttreatment. Concurrently, the P450coh protein was elevated approximately 3-fold (Figure 1). Also, pyrazole increased the P450coh similarly (Figure 1). Neither the P450coh mRNA level nor the P450coh gene transcription, on the other hand, was increased by phenobarbital (Figure 2A and 3). We used mouse P450b to verify that this phenobarbital treatment could induce a P450 mRNA and its gene transcription. Indeed, P450b mRNA and its gene transcription quickly increased to maximum levels within 8 h of the phenobarbital injection (Figures 2B and 3). Phenobarbital, therefore, activated transcription of the P450b gene, while induction by phenobarbital of the P450coh is regulated either translationally or posttranslationally. Neither transcription of the C-$P450_{16\alpha}$ gene nor the P450 mRNA level was affected by phenobarbital (Figure 2B and 3).

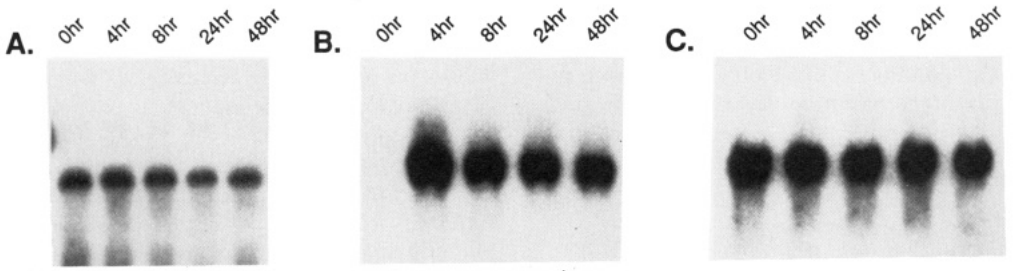

FIGURE 2: Effects of phenobarbital on P450coh mRNA levels. Liver poly(A^+) RNAs were prepared from the phenobarbital-treated DBA/2J male mice. (A) From 5 μg of poly(A^+) RNAs, double-stranded cDNAs were synthesized, digested with *Cla*I and *Pst*I, electrophoresed through a 2% agarose gel, and then transferred to Nytran paper. The Nytran was hybridized with the ^{32}P-labeled *Cla*I–*Cla*I fragment from $P450_{15\alpha}$ cDNA. Since the two *Cla*I sites are common in P450coh and $P450_{15\alpha}$, the same 950 bp fragment is generated from both types of cDNA. However, a unique *Pst*I site cut the *Cla*I–*Cla*I fragment of P450coh into two fragments of 700 and 250 bp. The intensities of bands at 950 and 700 bp, therefore, represent the relative amounts of $P450_{15\alpha}$ and P450coh mRNAs in poly(A^+) RNA samples, respectively. (B, C), Five micrograms of poly(A^+) RNAs was electrophoresed through the presence of 2.2 M formamide, transferred to Nytran paper, and hybridized with cDNA clone pf3/46 for the detection of P450IIB (B) and oligoprobe OP-1 for the C-$P450_{16\alpha}$ (C).

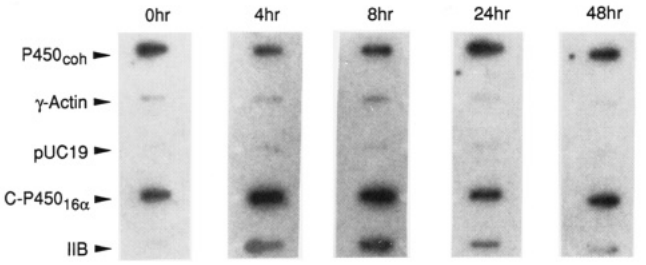

FIGURE 3: Effects of phenobarbital on P450coh, C-$P450_{16\alpha}$, and mouse P450b transcription rates. Liver nuclei were isolated from the phenobarbital-treated DBA/2J male mice and subjected to the run-on assay. The newly synthesized transcripts were then hybridized with P450coh cDNA, C-$450_{16\alpha}$ cDNA, γ-actin cDNA, and plasmid pUC19 previously bound to a Nytran filter according to the methods described under Experimental Procedures. The hybridizations were visualized by autoradiography.

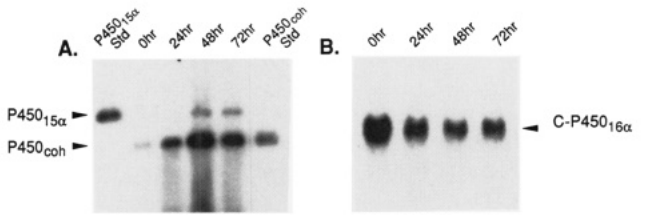

FIGURE 4: Effects of pyrazole on P450coh, $P450_{15\alpha}$, and C-$P450_{16\alpha}$ mRNA levels in DBA/2J male mice. Liver poly(A^+) RNAs were prepared at each time point from pyrazole-treated DBA/2J male mice which received the inducer once every 24 h. (A) Five micrograms of poly(A^+) RNAs were subjected to processed Northern hybridization as described under Experimental Procedures and in the legend for Figure 2. (B) Five micrograms of poly(A^+) RNAs were subjected to Northern blotting in the presence of 2.2 M formamide and hybridized with oligoprobe OP-1 which was specific for C-$P450_{16\alpha}$.

Slow Increase by Pyrazole of P450coh mRNA. Figure 4A shows a time-dependent increase of P450coh and $P450_{15\alpha}$ mRNAs in the pyrazole-treated DBA/2J male mice. The slow induction of the P450coh mRNA took 48 h to reach maximum levels. As a result, the mRNA level was elevated more than 20-fold compared to that seen in control male mice. This slow mRNA increase by pyrazole was in sharp contrast with the rapid induction by phenobarbital of P450b mRNA. In a earlier section of this paper, it was shown that P450b mRNA in DBA/2J male mice was increased to its maximum level within 8 h after the phenobarbital injection.

A small increase of $P450_{15\alpha}$ mRNA also was observed in the pyrazole-treated DBA/2J male mice. A maximum level of $P450_{15\alpha}$ mRNA was at least 20-fold lower than that of P450coh mRNA in the pyrazole-treated mice. In other experiments, however, the pyrazole treatment resulted in no increase of $P450_{15\alpha}$ mRNA. This suggested that pyrazole is a weak inducer for $P450_{15\alpha}$ mRNA in DBA/2J male mice. Conversely, the male-specific C-$P450_{16\alpha}$ mRNA was decreased by pyrazole in DBA/2J male mice (Figure 4B). The mRNA level decreased slowly in the pyrazole-treated mice: a small change was detected within the first 12 h (data not shown), and an approximate 10-fold decrease occurred within the next 48 h.

No Effect by Pyrazole on P450coh Gene Transcription. In order to investigate whether the P450coh induction by pyrazole resulted from the transcriptional activation of the gene, we performed a run-on assay with liver nuclei from both the pyrazole-treated and control DBA/2J male mice (Figure 5). Because of their nucleotide sequence homology, the run-on assay could have included both $P450_{15\alpha}$ and P450coh genes' transcription. The transcription of the $P450_{15\alpha}$ gene, however, appeared to be below a detectable level in the normal DBA/2J

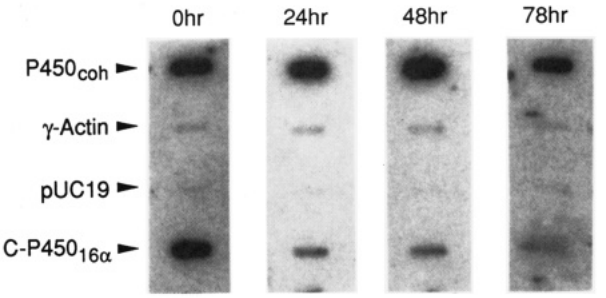

FIGURE 5: Effects of pyrazole on P450coh and C-$P450_{16\alpha}$ transcription rates. Liver nuclei were isolated at each time point from the pyrazole-treated DBA/2J male mice which received the inducer once every 24 h. The nuclei samples were then used to perform the run-on assay as described under Experimental Procedures.

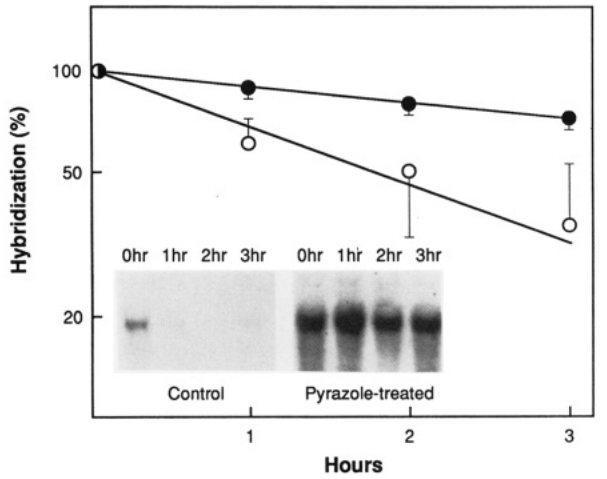

FIGURE 6: Decay of P450coh mRNA. DBA/2J male mice were treated only once with pyrazole or vehicle alone as described under Experimental Procedures. Twenty-four hours after the treatment, the mice were injected intraperitoneally with actinomycin D (7.5 mg/kg body weight) and sacrificed at various intervals. Total RNAs were extracted and analyzed by processed Northern hybridization. The radioactivity of hybridized bands was counted to measure the relative mRNA contents in the pyrazole-treated (●) and in normal mice (○). The results are the means from three independent experiments. Each value of control and pyrazole-treated mice at time zero was taken as 100%. (Inset) Autoradiogram of a processed Northern hybridization.

mice and negligible in the pyrazole-treated DBA/2J mice as judged from the $P450_{15\alpha}$ mRNA levels. The present run-on assay, therefore, accounted for the transcription of the P450coh gene. The gene transcription was neither increased nor decreased at any time points when the increase of its mRNA was observed. Furthermore, the transcription was not affected at earlier time points (4, 8, and 12 h) following pyrzole treatment (data not shown). The results indicate, therefore, that the induction by pyrazole of P450coh is regulated post-transcriptionally.

Transcription of the C-$P450_{16\alpha}$ gene, on the other hand, was decreased to approximately a 10-fold lower level within 4 h after the injection of pyrazole in DBA/2J male mice (Figure 5). The decrease of C-$P450_{16\alpha}$ mRNA in the pyrazole-treated mice, therefore, resulted from transcriptional repression of its gene. In addition, pyrazole repressed transcription of the mouse MUP gene (data not shown) but not the γ-actin gene (Figure 5).

Stabilization by Pyrazole of P450coh mRNA. Taking into consideration that induction of P450coh mRNA was slow and the P450coh gene transcription was not increased, P450coh mRNA appeared to be stabilized by pyrazole. We determined, therefore, the half-life of the mRNA in both the pyrazole-

treated and control DBA/2J male mice (Figure 6). Pyrazole prolonged the half-life of mRNA 4-fold, with times of approximately 1.5 h in control mice and 6 h in the pyrazole-treated mice. Further, we measured the half-life of C-P450$_{16\alpha}$ mRNA in both the pyrazole-treated and control mice (data not shown). The half-life of C-P450$_{16\alpha}$ mRNA was approximately 4 h and, moreover, was not changed in the pyrazole-treated mice. The results indicate, therefore, that stabilization by pyrazole is specific to the P450coh mRNA.

Discussion

This paper has demonstrated that the stabilization of P450 mRNA is a major mechanism by which P450coh is induced by pyrazole. Transcription of the P450coh gene in the pyrazole-treated DBA/2J mice remains at the same level seen in control mice, while the P450coh mRNA was increased more than 20-fold in the pyrazole-treated mice. This increase results from the stabilization of the mRNA: the half-life of the P450coh mRNA is approximately 4-fold longer in the pyrazole-treated versus control DBA/2J mice. Stabilization of mRNA has been proposed as an induction mechanism of certain P450s, for instance, the induction of rat P450b+e by dexamethasone (Simmons et al., 1987); rabbit P4503c by triacetyloleandomycin, erythromycin, and rifampine (Dalet et al., 1986); and P450j in diabetic rats (Hong et al., 1987; Song et al, 1987) and P450c or P450d (IA P450s) by TCDD or 3MC (Gonzalez et al., 1984; Kimura et al., 1986; Soderkvist et al., 1988; Pasco et al., 1988; Silver & Krauter, 1988; Silver et al., 1990). These previous reports, however, did not provide direct evidence which indicates that the corresponding mRNAs are, indeed, stabilized by the inducers. Recent studies by Silver and Krauter (1990), on the contrary, showed that rat P450d mRNA is not stabilized by an inducer of aryl hydrocarbon. Their results indicate that induction of rat P450d is due to an increased processing of the RNA precursor. The longer half-life of P450coh mRNA in the pyrazole-treated mice, therefore, has proved for the first time that mRNA stabilization is an induction mechanism of the P450.

Recently, increasing attention has been given to the stabilization of mRNA as a mechanism of gene regulation (Brawerman, 1989; Cleveland & Yen, 1989; Nielsen & Shapiro, 1990). Although the 5′-noncoding regions or exon sequences have been reported to contain necessary information for mRNA stabilization, most studies have been focused on information within the 3′-untranslated regions. Stem and loop structure, AU-rich sequences, and a poly(A^+) tail, independently or cooperatively, play a role in determining the stability of mRNAs. The best-known example is the iron-responsive elements (IREs) in the 3′-untranslated region of the transferrin receptor mRNA (Müllner & Kühn, 1988; Casey et al., 1989). The IREs form stem and loop structures and serve as a recognition signal for a cytoplasmic stabilization factor which recently has been characterized (Neupert et al., 1990; Hentze et al., 1989; Rouault et al., 1989, 1990). The nucleotide sequence of P450coh cDNA (Squires & Negishi, 1988) indicates that its mRNA possesses in its 3′-untranslated region a potential stem and loop structure that overlaps with the poly(A^+) signals. This region of the P450coh mRNA also contains one AUUUA or UUAUUUA motif in the potential stem and loop structure, which is known to regulate mRNA stability (Shaw & Kamen, 1986). Further studies, therefore, are required to elucidate whether this 3′-untranslated region is responsible for the stabilization of P450coh mRNA by pyrazole.

Phenobarbital is known to activate transcription of a P450IIB gene in rats (Hardwick et al., 1983). Our finding indicates that a mouse IIB gene is also activated transcriptionally by this drug. Surprisingly, we found that phenobarbital affects neither transcription of the P450coh gene nor the level of the P450coh mRNA within 48 h after the drug induction. Both coumarin 7-hydroxylase activity and P450coh apoprotein, however, are increased at least 3-fold during the induction. It appears, therefore, that phenobarbital regulates induction either by increasing translational efficiency of P450coh mRNA or by decreasing degradation of P450coh apoprotein. It has been suggested that the induction of several P450s is the result of posttranslational regulations; examples include the induction of rat P450j by pyrazole, ethanol, and acetone (Song et al., 1986), P450p by triacetyloleandomycin in rats and in rat hepatocytes (Watkins, 1986), and P450d by isosafrole in rate hepatocytes (Steward et al., 1985; Voorman & Aust, 1988). In fact, Song et al. (1989) measured the half-life of P450j apoprotein and found that it was degraded biphasically with half-lives of 7 and 37 h in control rats and monophasically degraded with a half-life of 37 h in the acetone-treated rats.

In conclusion, the induction of P450coh by pyrazole or phenobarbital is regulated posttranscriptionally. The former inducer stabilizes P450coh mRNA, while the latter either stabilizes P450coh apoprotein or increases the translational efficiency of P450coh mRNA. The regulation mechanisms by xenobiotics are in sharp contrast to the transcriptional regulation of P450coh by growth hormone in control male mice. Further studies are required to elucidate the stabilization mechanism of P450coh mRNA by pyrazole.

Acknowledgments

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Effect of Cobalamin Derivatives on in Vitro Enzymatic DNA Methylation: Methylcobalamin Can Act as a Methyl Donor†

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ABSTRACT: 5-Methylcytosine synthesis in DNA involves the transfer of methyl groups from *S*-adenosylmethionine to the 5'-position of cytosine through the action of DNA (cytosine-5)-methyltransferase. The rate of this reaction has been found to be enhanced by cobalt ions. We therefore analyzed the influence of vitamin B_{12} and related compounds containing cobalt on DNA methylation. Vitamin B_{12}, methylcobalamin, and coenzyme B_{12} were found to enhance significantly the de novo DNA methylation in the presence of *S*-adenosylmethionine for concentrations up to 1 μM, but at higher concentrations these compounds were found to inhibit DNA methylation. Methylcobalamin behaves as a competitive inhibitor of the enzymatic methylation reaction (K_i = 15 μM), the K_m for *S*-adenosylmethionine being 8 μM. In addition, the use of radioactive methylcobalamin shows that it can be used as a methyl donor in the de novo and maintenance DNA methylation reactions. Thus, two DNA methylation pathways could exist: one involving methylation from *S*-adenosylmethionine and a second one involving methylation from methylcobalamin.

The base 5-methylcytosine (m^5C) appears to be so far the only modified base that has been found in vertebrate DNAs. The extent and pattern of the genomic DNA methylation is not random, but is species- and tissue-specific (Gama-Sosa & Ehrlich, 1983). It occurs mostly in 5'-CpG-3' dinucleotides that are often clustered in the 5'-region of eukaryotic genes (Gardiner-Garden & Frommer, 1987). Many studies have shown that undermethylation of the transcriptional control regions of genes is correlated with the expression of these genes (Holliday, 1987; Cedar, 1988; Dynan, 1989; Michalowsky & Jones, 1989; Doerfler et al., 1990). In addition, de novo methylation is correlated with transcriptional inactivity (Gasson et al., 1983; Jahner & Jaenisch, 1984; Tasseron et al., 1989; Doerfler, 1990). Some authors have suggested that the inactivation of gene expression by CpG methylation could

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