Transcriptional and Post-transcriptional Regulation of the Genes Encoding Cytochromes P-450c and P-450d in Vivo and in Primary Hepatocyte Cultures*

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In both primary cell cultures of rat hepatocytes and in liver, polycyclic aromatic hydrocarbons (PAHs) were found to influence the accumulation of the cytochrome P-450c and P-450d mRNAs by both transcriptional and post-transcriptional mechanisms. Following treatment with PAHs, cytochrome P-450c mRNA levels increased approximately 100-fold in both hepatocyte cultures and in liver, while transcription rates, measured by run-on transcription of isolated nuclei, increased 3-fold in hepatocyte cultures and 10-fold in liver. The difference in the fold increases of mRNA level and transcription rate suggests that post-transcriptional, as well as transcriptional, mechanisms contributed to the regulation of cytochrome P-450c mRNA levels. Following treatment with PAHs, cytochrome P-450d mRNA levels increased 200-fold in hepatocyte cultures and 70-fold in liver, while transcription rates remained unchanged in hepatocyte cultures and increased only 1.7-fold in liver. This suggests that post-transcriptional mechanisms were of primary importance in regulating cytochrome P-450d mRNA levels. The newly developed hepatocyte primary cell culture system used in these studies differs from previously reported systems in that the cytochrome P-450d gene, as well as the cytochrome P-450c gene, were expressed in response to PAHs. In this cell culture system the regulation of these two genes was quite similar, although not identical, to that found in liver. The mechanisms controlling the tissue-specific expression of the genes encoding cytochromes P-450c and P-450d were also examined. The cytochrome P-450c mRNA was found in kidney, heart, and lung, as well as in liver, of PAH-treated rats, while the mature cytochrome P-450d mRNA was detected only in liver. The substantial increase in cytochrome P-450c mRNA in kidney in response to β-naphthoflavone was not associated with a detectable change in the transcription rate of cytochrome P-450c gene, indicating that cytochrome P-450c mRNA levels must be regulated primarily post-transcriptionally in kidney. Even though mature cytochrome P-450d mRNA could not be detected in kidney, the cytochrome P-450d gene was transcribed at a substantial rate in this tissue; therefore, the lack of accumulation of mature cytochrome P-450d mRNA in kidney must have been due to post-transcriptional control.

Cytochrome P-450c1 and P-450d from rat and the corresponding enzymes from mouse are induced by PAHs and are capable of both detoxifying these compounds and converting them to highly active carcinogens and mutagens. In recent years the transcriptional regulation of rat P-450c and its murine ortholog, P1-450, has been studied in detail (1-8). Although it has been reported (9) that post-transcriptional processes may play a role in murine P1-450 and P3-450 gene regulation, post-transcriptional regulation has not been extensively investigated in the mouse and no reports in this area have been published for rat. We have used both in vivo and cell culture approaches to investigate the contributions of transcriptional and post-transcriptional processes to the regulation of the levels of the rat P-450c and P-450d mRNAs. These studies indicate that, in liver and in cultured hepatocytes, both transcriptional and post-transcriptional mechanisms are important in controlling P-450c mRNA levels, while post-transcriptional mechanisms are of primary importance in controlling P-450d mRNA levels.

Tissue-specific expression of specific forms of P-450 may play an important role in determining tissue-specific responses of the organism to carcinogens and pharmacological agents. Evidence, primarily at the protein level, has demonstrated that many of the P-450 isozymes characterized to date are expressed in a tissue-specific and inducer-specific manner. These include the phenobarbital-induced P-450s in rabbit (10-12) and rat (13, 14) the steroid biosynthetic P-450s (15), as well as the PAH-induced P-450s in rabbit (16), mouse (9), and rat (17). We have measured the expression of the P-450c and P-450d genes in four different rat tissues. These experiments show that the mRNAs encoding P-450c and P-450d have distinct tissue-specific patterns of expression and that, at least in liver and kidney, there exist tissue-specific differences in the contributions of transcriptional and post-transcriptional mechanisms to the regulation of these genes.

The research reported here makes use of a newly developed rat hepatocyte primary cell culture system in which P-450d, as well as P-450c, can be expressed in response to PAHs. This system promises to be of substantial use in the future investigation of the regulation of the P-450c and P-450d genes.

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1 The abbreviations used are: P-450, cytochrome P-450; BNF, β-naphthoflavone; 20 × SSC, 0.3 M NaCl, 0.3 M sodium citrate; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; PAH, polycyclic aromatic hydrocarbons; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
**RESULTS**

**Regulation of P-450c and P-450d mRNAs in Primary Cultures of Rat Hepatocytes and in Liver**—Previous studies have reported the inability to express rat P-450d mRNA, as well as the murine equivalent of this mRNA, in hepatocytes or hepatocyte-derived cell culture systems (28-31). We have developed a rat hepatocyte primary culture system in which the P-450d mRNA, as well as the P-450c mRNA, were expressed in response to PAHs to levels similar to those seen in vivo. We found that the PAH-induced expression of P-450d mRNA did not require the use of exotic substrate or medium components, but only required the appropriate combination of factors that have already been shown to promote differentiated hepatocyte function. These included: 1) the removal of dead and damaged cells before culture through the use of iso-denisty Percoll centrifugation (27); 2) the inclusion of insulin and dexamethasone in the culture medium. Exclusion of these two components reduced TCDD-induced P-450d mRNA accumulation by 25 and 70%, respectively; 3) the inclusion of 5% Nu-Serum in the culture medium. Replacement with 5% fetal bovine serum substantially reduced expression after the first 36 h in culture. Nu-Serum is a medium supplement containing 25% (v/v) newborn bovine calf serum, various growth factors, insulin, transferrin, and other hormones, amino acids, vitamins, and nutrients. 4) Although the studies reported herein used hepatocytes maintained on standard tissue culture plastic dishes, maintenance on a matrix of solubilized tissue basement membrane derived from Engelbreth-Holm-Swarm mouse tumors (Matrigel) further enhanced P-450d mRNA inducibility and maintained inducibility for longer periods in culture.

We (23) and others (32) have previously reported that the mRNAs for P-450c and P-450d were induced with different kinetics in the liver. As shown in Fig. 1, we have reexamined these kinetics using the hepatocyte culture system described above. In these cells, P-450c mRNA increased rapidly in response to TCDD, reaching half-maximal levels about 7 h after adding inducer and reaching maximal levels by about 11 h. In contrast, P-450d mRNA increased more slowly, reaching half-maximal levels at about 36 h. The kinetics of induction of both mRNAs were similar to those reported in vivo (23, 32).

Although it is not apparent from Fig. 1, there were small but consistent changes in the basal levels of P-450c and P-450d mRNAs 48 h after hepatocytes were plated in culture. Basal levels of P-450c mRNA were slightly similar to those found in vivo, but declined after 24 h, reaching levels that were 5% of the initial value after 3 days in culture. The reduced basal P-450d mRNA level accounts for the observation that, although the TCDD-induced levels of P-450d mRNA in this culture system were similar to those found in the livers of PAH-treated rats, the fold increase was much greater in culture, approaching 200. Whereas the basal level of P-450d mRNA decreased after placing hepatocytes in culture, that of...
were left untreated to a low but measurable level that was maintained throughout post-transcriptional as well as transcriptional mechanisms must contribute to the regulation of P-450c mRNA levels in these cultured hepatocytes. As expected, the kinetics of induction of transcription preceded those of mRNA accumulation. Run-on transcription activity of the P-450c gene reach half-maximal level within 1 h and was maximal by 3 h. Fig. 2 also shows that, despite the 200-fold increase in P-450d mRNA, shown in Fig. 1, there was no significant change in transcription rate during the first 24 h following addition of the inducer TCDD. Even at 24 h, when P-450d mRNA levels were increasing rapidly, run-on transcription activity of the P-450d gene was not significantly greater than in controls. This indicates that P-450d mRNA levels must be controlled predominantly by post-transcriptional mechanisms in primary cultures of rat hepatocytes.

**Tissue-specific Regulation of the P-450c and P-450d mRNAs**—Previous studies (23, 32) have shown that the P-450c and P-450d mRNAs were noncoordinately regulated in three other tissues. The Northern blot in Fig. 3, upper panel, shows that the 2.9-kilobase P-450c mRNA was present in measurable amounts in kidney, lung, and cardiac muscle, as well as in liver, from PAH-treated rats, but was not detected in tissues of untreated rats. The P-450c mRNA was induced to 5-fold higher levels in liver than in other tissues, with the exception of kidney from TCDD-treated rats in which this mRNA reached levels equal to those in liver. Fig. 3, lower panel shows that, in contrast to P-450c mRNA, the 2.1-kilobase P-450d mRNA was detectable only in liver, not in kidney, lung, or cardiac muscle, even in animals treated with high levels of TCDD. Similar results were obtained using MC as inducer (data not shown). Long exposures
Differential Regulation of P-450c and P-450d mRNA Levels

Li Ki Lu

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Table I

<table>
<thead>
<tr>
<th>Tissue</th>
<th>mRNA level</th>
<th>Relative transcription rate</th>
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<tbody>
<tr>
<td></td>
<td>O.D. units</td>
<td>Control</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
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<tr>
<td>β-Fibrinogen</td>
<td>9.6 ± 2.1</td>
<td>59.8 ± 10.4</td>
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<tr>
<td>P-450d</td>
<td>1.0 ± 0.04</td>
<td>71.3 ± 21</td>
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<tr>
<td>P-450c</td>
<td>ND b</td>
<td>39.1 ± 5.7</td>
</tr>
<tr>
<td>Albumin</td>
<td>126 ± 34</td>
<td>54 ± 14</td>
</tr>
<tr>
<td>Kidney</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Fibrinogen</td>
<td>ND</td>
<td>0.2 ± 0.01</td>
</tr>
<tr>
<td>P-450d</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>P-450c</td>
<td>ND</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>Albumin</td>
<td>ND</td>
<td>ND</td>
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a X, P-450d, P-450c, β-fibrinogen, or albumin.
bND, not detectable. The lower limit of detection on Northern blots was <0.02 O.D. units.

Table I shows, first, that there were differences between liver and kidney in the levels of P-450c and P-450d mRNAs, reflecting the noncoordinate, tissue-specific expression of the P-450c and P-450d mRNAs and, second, that there were distinct, tissue-specific differences in the contributions of transcriptional and post-transcriptional processes to the regulation of the levels of these two mRNAs. Fig. 4 presents an autoradiogram from an experiment representative of those included in Table I. In these experiments nuclei and total RNA were isolated in parallel from liver and kidney of untreated and BNF-treated rats. The total RNA preparations were used to quantitate mRNA levels by Northern hybridization and the nuclei were used to measure run-on transcription rates. Messenger RNA levels were expressed as average arbitrary densitometric units ± S.D. (n = 3). The abundance of a specific mRNA can be compared between liver and kidney, but comparisons between different mRNAs are not valid because of differences in probe specific activities. Run-on transcription data was normalized to actin and is expressed as average arbitrary densitometric units ± S.D. (n = 3). The transcription rates of different genes cannot be directly compared, nor can these rates be directly compared between tissues.
important in liver. Table 1 shows that the P-450d gene was actively transcribed in both kidney and liver, although mature P-450d mRNA was present only in liver and not in kidney, where the abundance of this mRNA increased 70-fold following BNF treatment. In both tissues run-on transcription rates changed minimally following BNF treatment, 1.5-fold increase in liver and no significant change in kidney. The observation of P-450d transcription in kidney was supported by the finding that although mature P-450d mRNA was not detectable in kidney, lung, or cardiac muscle, long exposures of Northern blots showed the presence of large mRNA species, presumably precursors to this mRNA, in kidney and lung from untreated and PAH-treated rats. These findings suggest that post-transcriptional regulation was primarily responsible both for the lack of expression of the P-450d mRNA in kidney and for the PAH-induced increase in this mRNA in liver.

**Assessment of Run-on Transcription Assay**—Several controls and comparisons were carried out to evaluate the accuracy and reliability of the run-on transcription data. First, the addition of α-amanitin (1 μg/ml) to run-on transcription reactions decreased all gene-specific sequences by approximately 80% in both liver and kidney (data not shown). Second, based on the run-on transcription of actin sequences, the transcriptional activity of kidney nuclei and cultured hepatocyte nuclei was no less than half that of liver nuclei. This indicates that the nuclei isolated from all three sources were consistently active in run-on transcription. Third, the P-450c, P-450d, and β-fibrinogen transcription signals observed in kidney did not seem to be due to background, since run-on transcription reactions using kidney nuclei did not yield sequences that could hybridize either to pUC18 sequences or to albumin-specific sequences. We had expected that the expression of the β-fibrinogen gene would, like that of the albumin gene, be strictly liver-specific, however, β-fibrinogen mRNA levels and run-on transcription activities were consistently detected in kidney as well as in liver from BNF-treated rats. The level of β-fibrinogen mRNA in kidney was 0.5% of that in liver. The liver mRNA levels and run-on transcription activities for both albumin and β-fibrinogen changed in parallel indicating that PAHs influence the expression of these two mRNAs primarily at the transcriptional level. The PAH-induced decrease in liver albumin mRNA levels has been previously reported (33). The PAH-induced increase in β-fibrinogen mRNA levels, shown in Table 1, will be described in detail elsewhere.

**DISCUSSION**

It has been reported that, although P-450c and P-450d in rat (23, 32) and P-450 and P3-450 in mouse (9) are induced by the same classes of compounds, they are not induced in a coordinate manner. Our observations in hepatocyte primary cell cultures and in liver show that the noncoordinate regulation of the levels of these two mRNAs results from the fact that both transcriptional and post-transcriptional mechanisms are important in controlling P-450c mRNA levels, while post-transcriptional mechanisms are of primary importance in regulating P-450d mRNA levels. Our findings for P-450c gene regulation correspond reasonably well to those reported for P-450 in mouse (9); however, our findings for P-450d gene regulation differ significantly from those for mouse, where it was reported that P-450d mRNA levels in liver were controlled entirely by transcriptional regulation (9). Although we suspect there exists a real difference in the regulation of the P-450d and P-450c genes, our experiments are not directly comparable to those carried out in mouse because different inducers were used.

It is generally understood that the PAH induction of P-450s is mediated by a receptor, which participates in nuclear events that activate the transcription of the P-450 genes (reviewed in Ref. 34). The observations reported here indicate that PAHs must also influence post-transcriptional events. Our present results do not allow us to identify this post-transcriptional process more precisely; possibilities include regulation of mRNA processing, mRNA transport from the nucleus, or regulation of mRNA stability. It is currently not clear whether PAHs influence this post-transcriptional process directly or whether another gene product (or products), whose expression is, itself, controlled by the PAH receptor, might mediate this post-transcriptional regulation of the P-450c and P-450d genes.

The findings presented here indicate that the previously reported, tissue-specific expression of the cytochrome P-450c and P-450d proteins (17) was probably a consequence of tissue-specific differences in the regulation of P-450c and P-450d mRNA levels. In agreement with our observations regarding the levels of the P-450c and P-450d mRNAs in the tissues of PAH-treated rats, it has been reported (17) that P-450c can be detected in several tissues, including kidney, lung, and liver, while P-450d can be detected only in the liver. These observations in the rat are quite different from mouse, where it was reported that the murine equivalents of the P-450c and P-450d mRNAs (P1-450c and P1-450d, respectively) were both expressed in kidney and lung, as well as in liver (9). The P1-450c mRNA was reported not only to be expressed in these tissues, but to be induced by lower concentrations of TCDD than were required to induce the P1-450m RNA. Moreover, P1-450m mRNA levels in kidney were reported to be 25% of those found in livers of TCDD-treated mice (9). Our data show that we could have easily detected P-450d mRNA if it were present in non-hepatic tissues of the rat at the levels reported in the mouse.

The data regarding mRNA levels and transcription rates in kidneys provide new insights regarding the contributions of transcriptional and post-transcriptional regulation to the tissue-specific expression of the P-450c and P-450d genes. It can be concluded from the similarities in the run-on transcription activities of nuclei isolated from both control and BNF-induced kidney and liver that the P-450d gene is being transcribed in both tissues, whether treated or not treated with BNF; therefore, the absence of mature P-450d mRNA in kidney is not due to lack of transcription of this gene but must be due to some, as yet unidentified, post-transcriptional mechanism. A second line of evidence that the P-450d gene is transcribed in non-hepatic tissue is that large RNA species, which are homologous to the P-450d mRNA and which are presumably P-450d mRNA precursors, are detected on long exposures of Northern blots of RNA from kidney and lung of untreated and BNF-treated rats at levels similar to those seen in liver from untreated rats. It is interesting to note that in the kidney from TCDD-treated mice, P-450m mRNA appears to accumulate as a result of predominantly post-transcriptional mechanisms (9). Run-on transcription of the P-450c gene did not respond to BNF in kidney, while it increased 10-fold in liver. Thus, transcriptional regulation contributes substantially to the large, greater than 100-fold increase in P-450c mRNA observed in liver, while the modest 6-fold increase in P-450c mRNA observed in kidney must result primarily from post-transcriptional regulation.

The use of cell culture systems has led to rapid advances in understanding the regulation of the rat P-450c and mouse P-450d.
450 genes. The study of P-450d and P-450 gene regulation has, however, been limited by the lack of cell culture systems in which these genes could be expressed (29–31). We have described in this paper a primary cell culture system for rat hepatocytes in which the P-450d gene is expressed and which can now be used to study P-450d gene regulation in more depth. Based upon our findings, four points can be made that are pertinent to considering the usefulness of this cell culture system as a model for studying liver P-450c and P-450d gene regulation. First, PAHs induce the P-450c and P-450d mRNAs to approximately the same levels both in the liver and in cultured hepatocytes. Second, there were small but consistent changes in the basal levels of the P-450c and the P-450d mRNAs after hepatocytes were placed in culture. Third, the regulation of both the P-450c and P-450d genes was found to be similar to the hepatocyte culture system to that observed in vivo. Both in vivo and in the hepatocyte culture system, PAH-induced accumulation of P-450c mRNA was found to result from both transcriptional and post-transcriptional processes, while that of P-450d mRNA was found to be the result of predominantly post-transcriptional processes. Fourth, the extent of transcriptional activation of the P-450c and P-450d genes in culture is somewhat less than that observed in vivo (3-versus 10-fold for P-450c and no change versus 1.7-fold for P-450d). This could be due either to a higher basal level of transcription in culture, possibly resulting from the presence of an inducer-like factor or culture condition, or to attenuation of transcriptional responsiveness to PAHs. It has been shown previously that the regulation of some liver-specific genes can be significantly altered when hepatocytes are placed in culture; transcriptional regulation is lost or greatly decreased, resulting in the predominance of post-transcriptional regulation (35). It has also been shown that the addition of proteoglycans, glycosaminoglycans, or other polyanions can restore the transcriptional regulation of these genes (36). In preliminary experiments we have observed that when hepatocytes are maintained on a matrix of solubilized tissue basement membrane, derived from Engelbreth-Holm-Swarm mouse tumors (Matrigel), transcriptional responsiveness approaches that of the liver, increasing 8-fold in the presence of TCDD. The first and third points indicate that the hepatocyte primary cell culture system that we have developed reflects, relatively accurately, the most important features of P-450c and P-450d gene regulation in cell culture and should, therefore, be of immediate use in the study of the regulation of these genes. The second and fourth points indicate that minor differences do exist between this cell culture system and liver in the regulation of the P-450c and P-450d genes, but that further development should improve the usefulness of this cell culture system for the study of P-450c and P-450d gene regulation as well as for the study of other liver-specific genes.

REFERENCES