DETECTION OF CHEMICAL-INDUCED DIFFERENTIAL expression of RAT HEPATIC CYTOCHROME P450 mRNA TRANSCRIPTS USING BRANCHED DNA SIGNAL AMPLIFICATION TECHNOLOGY

DYLAN P. HARTLEY AND CURTIS D. KLAASSEN

Environmental and Occupational Medicine Center, Department of Pharmacology, Toxicology, and Therapeutics, University of Kansas Medical Center, Kansas City, Kansas

(Received September 3, 1999; accepted January 21, 2000)

This paper is available online at http://www.dmd.org

ABSTRACT:

The importance of the cytochrome P450 (CYP) enzyme family in xenobiotic metabolism, as well as their differential expression and activity in response to a wide range of environmental chemicals and pharmaceuticals, is well documented. The objective of this study was to evaluate the specificity of the branched DNA (bDNA) signal amplification technique for the detection of multiple rat CYPs from hepatocellular RNA. Oligonucleotide probe sets were designed to various chemically inducible rat CYP mRNA transcripts, including CYP1A1, CYP1A2, CYP2B1/2, CYP2E1, CYP3A1/23, and CYP4A2/3. The robustness of the bDNA assay was assessed with the CYP2B1/2-specific probe set, and total RNA was isolated from control and phenobarbital (PB)-treated rats. Analysis of these RNA samples by bDNA signal amplification resulted in a linear quantifiable range of RNA detection that spanned three orders of magnitude (0.1–100 μg of total RNA). The fidelity of the bDNA assay was evaluated within a single assay and between assays where repeated measurements of a single sample were reproduced reliably. The specificity of individual CYP probe sets was evaluated with five typical CYP-inducing chemicals on the expression of specific hepatic CYP mRNA transcripts. Male Sprague-Dawley rats were administered 3-methylcholanthrene, PB, isoniazid, pregnenolone-16α-carbonitile, or clofibric acid to induce transcription of CYP1A1, CYP1A2, CYP2B1/2, CYP2E1, CYP3A1/23, and CYP4A2/3 mRNA, respectively. Analysis of chemical-induced differences in gene expression by bDNA signal amplification indicated that 3-methylcholanthrene induced CYP1A1 and CYP1A2 mRNA levels 670- and 11-fold, respectively; PB induced CYP2B1/2 expression 71-fold; pregnenolone-16α-carbonitile induced CYP3A1/23 expression 34-fold; and clofibric acid induced CYP4A2/3 expression 4.7-fold. Overall, these data support the use of bDNA signal amplification technology as a robust, reproducible, and efficient means of monitoring the differential expression of multiple isoforms of the CYP enzyme family.

In two decades, hundreds of cytochrome P450 (CYP)1 enzymes have been cloned, isolated, and functionally characterized from numerous species, including humans. The efforts of scientists in the CYP field have given rise to advances by other drug metabolism researchers in the identification of novel proteins that play an essential role in the absorption, activation, deactivation, excretion, and toxicity of various chemicals. This wealth of knowledge has led to the realization that many enzymes cannot be categorized according to substrate specificity, due to the multiplicity of enzymes/proteins with apparent functional redundancy. However, when compared at the level of amino acid sequence, proteins can be categorized into large families of related molecules, such as the CYPs, UDP-glucuronosyltransferases, sulfotransferases, organic-anion transporters, multidrug resistance proteins, etc. Each of these encompassing enzyme families contains multiple enzyme subfamilies; each of these may contain numerous and highly similar homologous genes. As a result, it becomes increasingly clear that new and novel methods that allow us to glean new information on the differential regulation of homologous genes and their respective protein products.

One technology that will assist in the concerted effort of scientists to evaluate the CYPs at the mRNA level, as well as many other gene products, is the branched DNA (bDNA) signal amplification assay. The bDNA signal amplification assay is a nonpolymerase chain reaction (PCR) and nonradioactive based method of RNA analysis that resembles the well established enzyme-linked immunosorbent assay (ELISA); the design of the bDNA signal amplification assay is shown in Fig. 1. It is evident from this figure that the bDNA assay is designed as a multioignonucleotide approach, wherein three types of gene transcript-specific oligonucleotides are designed against each mRNA target of interest. These three oligonucleotides are termed capture extender, label extender, and blocker probes. Capture extender oligonucleotide probes (Fig. 1, dark blue) consist of a mRNA-specific
sequence, and have an additional 3’ concatenated nucleotide sequence that is complementary to a nucleotide sequence that is fixed to the solid support phase (Fig. 1, short black lines). Thus, the extension on the capture probe functions to anchor the capture probe-mRNA target hybrid to the solid support phase. Label extender oligonucleotide probes (Fig. 1, red) are also specific to a gene product of interest, but have an additional 3’ concatenated nucleotide sequence extension that facilitates hybridization to a cognate sequence on the bDNA molecule. Blocker oligonucleotide probes (Fig. 1, green) are used to span gaps between capture and label extender probes in the probe set. Blocker probes function to minimize RNase-mediated sample degradation in the mRNA target region as well as to stabilize the secondary structure of the mRNA target region. In practice, the bDNA assay uses this novel oligonucleotide chemistry and solution hybridization that functions to both capture the RNA molecule of interest to the solid support phase, and label the RNA with “amplifier” bDNA molecules (Fig. 1, light blue). Detection of the RNA molecule is similar to an indirect ELISA, where an enzyme (e.g., alkaline phosphatase; Fig. 1, red diamonds) is conjugated to an oligonucleotide (label probe; Fig. 1, orange), which hybridizes to the branches of the bDNA molecules, and on addition of substrate, dioxetane, a chemiluminescent signal is produced and measured. So, unlike PCR-based methodologies that amplify the target RNA molecule, this methodology amplifies the hybridization of a single RNA molecule such that the signal generated is proportional to the amount of RNA input into the system. The ELISA-like format and the ability to measure signal from the transcript itself are two major advantages of the bDNA assay, and lend to the increased efficiency of mRNA analysis and reproducibility of results.

The bDNA signal amplification system was initially developed, and has been used extensively, in clinical settings as a tool to monitor HIV and hepatitis (B and C) viral load in patients (Hendricks et al., 1995; Pachl et al., 1995; Detmer et al., 1996). For these applications, bDNA analysis has proven to be an efficient, sensitive, and reliable tool for clinicians. The assay is beginning to find an audience among basic science researchers, where it has been used successfully to monitor insulin RNA processing (Wang et al., 1997), and in the measurement of cytokine mRNA from peripheral blood cells (Shen et al., 1998). In these studies as well as the present study, the bDNA assay had a wide dynamic range of response (more than three orders of magnitude).

Comparisons of the bDNA system with quantitative reverse transcription-polymerase chain reaction (RT-PCR) have demonstrated that the bDNA system is comparable with PCR-based strategies with regard to sensitivity (Collins et al., 1997; Guenthner and Hart, 1998).

The enzymatic assays used to measure changes in chemically inducible forms of P450 enzymes (Table 1) are well characterized, and are still the standard for assessing chemical-mediated changes in CYP activity (Parkinson, 1996). However, the capacity to rapidly monitor changes in the transcriptional regulation of specific gene products, including the CYPs, could benefit a wide array of researchers, clinicians, and commercial organizations. From the knowledge of the nucleotide sequence for any given gene/mRNA, the specificity inherent with oligonucleotide-based techniques such as Northern-blot analysis, RT-PCR, and now bDNA signal amplification can be used to differentiate between highly related molecules at the level of mRNA transcript expression. In an attempt to increase the efficiency, reliability, and reproducibility of analyzing multiple mRNA transcripts within homologous enzyme families, the bDNA signal amplification technology was evaluated in the present study with the major inducible CYP enzymes.

Although there are now at least 53 rat CYP genes in the Unigene database (Schuler et al., 1996), there are four CYP gene families (i.e., CYP1, CYP2, CYP3, and CYP4) that encompass eleven CYPs commonly used to characterize drug-drug interactions. These eleven enzymes (CYP1A1, CYP1A2, CYP2B1, CYP2B2, CYP2E1, CYP3A1, CYP3A2, CYP3A23, CYP4A1, CYP4A2, and CYP4A3) span four CYP gene subfamilies (i.e., CYP1A1, CYP2B1, CYP2E1, CYP3A, and CYP4A). As a general rule, within a CYP subfamily, CYP enzymes respond similarly to a class of compounds (i.e., CYP1A1 and CYP1A2 gene expression is induced by polychlorinated hydrocarbons). However, between CYP subfamilies, each subfamily of P450s are differentially up-regulated by chemicals such as dioxin, phenobarbital (PB), rifampicin, ethanol, and clofibric acid (CLO; Porter and Coon, 1991; Parkinson, 1996; Dogra et al., 1998). In this report, the well characterized response of a particular CYP to a specific chemical was used to evaluate the use of the bDNA assay as a high-throughput technique for assessing xenobiotics as CYP inducers.

Our rationale for assessing the bDNA assay as a technique for monitoring xenobiotic-mediated enzyme induction originated from the specificity, sensitivity, reliability, and efficiency of the bDNA assay. These characteristics were indicative of a technology that could serve as a primary screening tool for xenobiotic induction of the CYP enzymes. Therefore, in this report the bDNA signal amplification assay was used to monitor the expression of CYP1A1, CYP1A2, CYP2B1/2, CYP2E1, CYP3A1/2, and CYP4A2/3 in rats treated with classical enzyme-inducing chemicals. From this study, the validity of the bDNA system in assessing CYP induction was determined to use this system to predict potential drug-drug interactions. Thus, the intention of this report is to provide the research community with a data set derived from applying the bDNA system to a well known chemical response paradigm, chemical induction of specific CYPs.
Materials and Methods

Animals. Male Sprague-Dawley rats (200 g; Harlan Sprague-Dawley, Inc., Indianapolis, IN) were acclimated to the housing facility (2–3 rats/cage, 50% relative humidity, 12-h light/dark cycle) for 1 week before the initiation of the study. Animals were given free access to water and rat chow (Teklad; Harlan Sprague-Dawley, Inc.).

Chemical Treatment of Animals. Rats were randomly grouped into seven treatment groups (4 rats/chemical treatment). Animals were injected (1 injection/day for 4 days; i.p.; 5 ml/kg) with PB (80 mg/kg), 3-methylcholanthrene (3MC; 27 mg/kg), pregnenolone-16α-carbonitrile (PCN; 50 mg/kg), isoniazid (ISO; 200 mg/kg), or CLO (200 mg/kg). Control animals were given the appropriate vehicle (corn oil for 3MC and PCN, or saline for PB, ISO, and CLO).

Isolation of RNA. Total RNA was isolated using RNAzol B reagent (Tel-Test Inc., Friendswood, TX) as per the manufacturer protocol. Each RNA pellet was resuspended in 0.2 ml of 10 mM Tris-HCl buffer, pH 8.0. The concentration of total RNA in each sample was quantified spectrophotometrically at 260 nm. Each RNA sample was analyzed by formaldehyde-agarose gel electrophoresis. The quality of each RNA sample was visualized under ultraviolet light by fluorescence of ethidium bromide intercalated into 18S and 28S rRNA. The presence of these bands indicated the RNA was of sufficient quality for use in subsequent analyses.

Development of Specific Oligonucleotide Probe Sets for bDNA Analysis of CYPs. The CYP gene sequences of interest accessed from GenBank are listed in Table 1. Each of these CYPs has multiple GenBank accession numbers, where an accession number corresponding to the complete coding sequence was used for probe development; the Unigene accession numbers are given for simplicity. Before development of each probe set, the nucleotide sequences were aligned using CLUSTALW (Thompson et al., 1994) with software provided by OMIGA (Oxford Molecular Group, Inc., Oxford, UK) to determine specific target regions (i.e., nucleotide regions of dissimilarity between CYP sequences) for oligonucleotide probe development (Table 1). These target sequences were analyzed by ProbeDesigner Software Version 1.0 (Bayer Diagnostics, formerly Chiron Diagnostics Corp., Emeryville, CA and East Walpole, MA). Multiple and specific probes were developed to each CYP mRNA transcript. Oligonucleotide probes designed in this manner were either specific to a single mRNA transcript (i.e., CYP1A1, CYP1A2, or CYP2E1) or to multiple transcripts within a CYP subfamily (i.e., CYP2B1/2, CYP3A1/23, or CYP4A2/3; Table 2). All oligonucleotide probes were designed with a Tm of approximately 63°C. This feature enables hybridization conditions to be held constant (i.e., 53°C) during each hybridization step and for each oligonucleotide probe set. Every probe developed in ProbeDesigner was submitted to the National Center for Biotechnological Information (NCBI) for nucleotide comparison by the basic logarithmic alignment search tool (BLASTn; Altschul et al., 1997) to ensure minimal cross-reactivity with other rat sequences. Oligonucleotides with a high degree of similarity (≥80%) to other rat gene transcripts were eliminated from the design. All probes were synthesized (i.e., 50-nmol synthesis scale) by Operon Technologies (Palo Alto, CA), and obtained desalted and lyophilized. A total of 107 oligonucleotide probes were generated to the different CYP mRNA transcripts (Table 2). A schematic map of the location and function of all of these probes is graphically illustrated in Fig. 2. Based on the analysis of these probe sets in BLASTn, the probe sets were deemed specific and sufficient to detect differential expression of CYP mRNA. All probes (i.e., blocker probes, capture extenders, and label extenders) were diluted in 1.0 ml of 10 mM Tris-HCl, pH 8.0, with 1 mM EDTA and stored at −20°C. As commonly used in Northern blot analysis, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization of mRNA expression between wells. The probe set to rat GAPDH was directed toward a 400-nucleotide region (basepair 112–512; GenBank accession numbers AF106860 and M17701), and consisted of four capture extender, five label extender, and one blocker oligonucleotide probes. The GAPDH probe set information was generously donated by Jeff Donahue of Bayer Diagnostics (East Walpole, MA).

bDNA Assay. Specific CYP oligonucleotide probe sets (i.e., blocker probes, capture probes, and label probes) were combined and diluted to 50 fmol/µl in the lysis buffer supplied in the Quantigene bDNA Signal Amplification Kit (Bayer Diagnostics, East Walpole, MA). The GAPDH probe set was used at 50, 100, and 200 fmol/µl for capture probes, blocker probes, and label probes, respectively. All reagents for analysis (i.e., lysis buffer, capture hybridization buffer, amplifier/label probe buffer, washes A and D, and substrate solution) were supplied in the Quantigene bDNA Signal Amplification Kit; the components of these reagents were published previously (Wang et al., 1997). Total RNA (1 µg/µl; 10 µl) was added to each well of a 96-well plate containing capture hybridization buffer and 100 µl of each diluted probe set. Total RNA was allowed to hybridize to each probe set containing all probes for a given transcript (blocker probes, capture probes, and label probes) overnight at 53°C in a Quantiplex bDNA Heater (Bayer Diagnostics). Subsequently, the plate was removed from the heater, cooled to room temperature, and rinsed with wash A. Samples were hybridized with a solution containing the bDNA amplifier molecules (50 µl/well) diluted in amplifier/label probe buffer and incubated for 30 min at 53°C. The plate was again cooled to room temperature. The amplifier solution was aspirated and wells were washed with wash A (3×). Label probe, diluted in amplifier/label (same as above) probe buffer, was added to each well (50 µl/well), and hybridized to the bDNA-RNA complex for 15 min at 53°C. The plate was cooled to room temperature, and each well was rinsed with wash A (2×), followed by wash D (3×). Alkaline phosphatase-mediated luminescence was triggered by the addition of a dioxetane substrate solution (50 µl/well). The enzymatic reaction was allowed to proceed for 30 min at 37°C, and luminescence was measured with the Quantiplex 320 bDNA Luminometer (Bayer Diagnostics) interfaced with Quantiplex Data Management Software Version 5.02 (Bayer Diagnostics) for analysis of luminescence from 96-well plates.

Statistical Analysis. All values are either relative luminescence units (RLU) or the ratio of expression for a specific CYP isofrom to that of GAPDH. Final determinations are the mean ± S.E. for an n of 4 animals. One-way ANOVA was used (P < .05). Post hoc comparisons were made using Dunnett’s multiple range analysis.

TABLE 1
Genes, GenBank accession numbers, Unigene accession numbers, and target sequences used for oligonucleotide probe design

<table>
<thead>
<tr>
<th>CYP Gene</th>
<th>GenBank No.*</th>
<th>Unigene No.</th>
<th>References</th>
<th>Target*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A1</td>
<td>I00372</td>
<td>Rn. 10352</td>
<td>Oeda et al., 1988</td>
<td>570–972</td>
</tr>
<tr>
<td>1A2</td>
<td>X01031</td>
<td>Rn. 5563</td>
<td>Yabuysaki et al., 1984</td>
<td>501–998</td>
</tr>
<tr>
<td>2B1, 2B2</td>
<td>J00719</td>
<td>Rn. 2267</td>
<td>Fujiy-Kuriyama et al., 1982</td>
<td>566–962</td>
</tr>
<tr>
<td>2E1</td>
<td>S88325</td>
<td>Rn. 13772</td>
<td>Richardson et al., 1992</td>
<td>501–963</td>
</tr>
<tr>
<td>3A1, 3A2</td>
<td>D13912, X0672</td>
<td>Rn. 11291</td>
<td>Kiriti and Matsubara, 1993; Komori and Oda, 1994</td>
<td>616–914</td>
</tr>
<tr>
<td>4A2, 4A3</td>
<td>M57719, M35936</td>
<td>Rn. 33492</td>
<td>Kimura et al., 1989a,b</td>
<td>501–865</td>
</tr>
</tbody>
</table>

* Accession numbers were downloaded from GenBank into ProbeDesigner Software Version 1.0.

1 Target sequences were selected from nonhomologous sequences after nucleotide sequence alignments were performed in OMIGA (Cambridge, UK).
<table>
<thead>
<tr>
<th>Probe ID</th>
<th>GenBank ID</th>
<th>Target</th>
<th>Function</th>
<th>Probe Sequence</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A1</td>
<td>I00732</td>
<td>570–591</td>
<td>CE</td>
<td>gatcctggcctcctgtgcctgTTTTTctcttggaaagaaagt</td>
<td>CYP1A1</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>X01031</td>
<td>501–522</td>
<td>LE</td>
<td>cttgtaaccacgctcctgTTTTTctcttggaaagaaagt</td>
<td>CYP1A2</td>
</tr>
<tr>
<td>CYP2B1</td>
<td>I00732</td>
<td>570–591</td>
<td>BL</td>
<td>ggttcatggtctactctgTGTTTTTctcttggaaagaaagt</td>
<td>CYP2B1</td>
</tr>
<tr>
<td>CYP2B1</td>
<td>I00732</td>
<td>570–591</td>
<td>BL</td>
<td>ggttacctgcctctgTTTTTctcttggaaagaaagt</td>
<td>CYP2B1</td>
</tr>
<tr>
<td>CYP2B1</td>
<td>J00719</td>
<td>959–982</td>
<td>LE</td>
<td>ctgatctgacccattcTTTTTctcttggaaagaaagt</td>
<td>CYP2B1/2</td>
</tr>
<tr>
<td>CYP2B1</td>
<td>J00719</td>
<td>938–955</td>
<td>CE</td>
<td>actgtgactggaggtgTTTTTctcttggaaagaaagt</td>
<td>CYP2B1/2</td>
</tr>
</tbody>
</table>

**TABLE 2**

List of oligonucleotide probes generated for analysis of CYP expression by bDNA signal amplification.
Results

Linearity of Response. To determine the RNA concentration range in which bDNA analysis could be used in this study, various concentrations of total RNA were analyzed with the CYP2B1/2 probe set to generate a standard curve of luminescence versus amount of RNA (Table 3). When total RNA was increased, the bDNA analysis could be used in this study, various concentrations (i.e., different plates on different days). When a single sample from a control and a PB-treated rat was analyzed repeatedly within a day, the resulting data was reproducible between sample wells on a single plate and between days (Table 3). Replicates within an experiment were very reproducible (c.v. 8–15%). Experimental reproducibility was also reliable between days where the c.v. for raw luminescence values for both control and treated samples were 25% (Table 3).

Chemical Responsiveness. The chemical responsiveness of the CYP family of drug-metabolizing enzymes is the standard measurement for assessing drug/chemical CYP interactions, where specific chemicals are known to induce specific CYP genes (Table 4). In this study, we used this classic response paradigm to evaluate the bDNA system. The detection of the chemical effects on specific CYP enzymes indicates that the oligonucleotide probe sets and the bDNA system detect the classical differential response of specific CYPs to various microsomal enzyme-inducing chemicals (Fig. 4).

The CYP1A CYP gene family is largely inducible by polyaromatic hydrocarbons, like 3MC (Table 4). From bDNA analysis for CYP1A1 and CYP1A2 mRNA transcripts, results demonstrated that in the absence of a chemical stimulus, constitutive levels of hepatic CYP1A1 mRNA were very low (2-fold above background), whereas a much greater level of constitutive CYP1A2 mRNA expression was detected (Fig. 4). In stark contrast, in rats treated with 3MC, hepatic expression of CYP1A1 and CYP1A2 was increased 670- and 11-fold, respectively (Fig. 4 and Table 5). In general, results obtained from rats treated with 3MC were consistent with previous reports for CYP1A1 and CYP1A2, respectively (Fig. 4 and Table 5). In general, results obtained from rats treated with 3MC were consistent with previous reports for CYP1A1 and CYP1A2, respectively (Fig. 4). In stark contrast, in rats treated with 3MC, hepatic expression of CYP1A1 and CYP1A2 was increased 670- and 11-fold, respectively (Fig. 4 and Table 5). In general, results obtained from rats treated with 3MC were consistent with previous reports for CYP1A1 and CYP1A2 mRNA expression.

The CYP2B CYP family is classically known to be inducible by PB (Table 4). The oligonucleotide probes used to detect CYP2B1/2
mRNA levels were specific for this subfamily of CYP mRNA transcripts, demonstrating that PB caused a significant increase in the levels of CYP2B1/2 (Fig. 4). PCN also significantly increased CYP2B1/2 mRNA levels. Overall, PB and PCN induced a 70- and 5.7-fold increase in hepatic CYP2B1/2 mRNA, respectively (Table 5).

The CYP2E1 enzyme is induced in chronic disease states like that observed in diabetes and chronic alcoholism. This enzyme is also up-regulated by acetone, pyrazole, and ISO (Table 4). However, the mechanism by which CYP2E1 is up-regulated is primarily due to stabilization of the protein and, secondarily, to transcriptional induction. Therefore, this gene is generally less responsive to chemical stimuli. An oligonucleotide probe set to CYP2E1 was developed and used to monitor chemical modulation of hepatic CYP2E1 mRNA by bDNA analysis. Our results for CYP2E1 mRNA levels indicated that this CYP maintains a high level of constitutive expression (i.e., control levels), and that the CYP2E1 gene is largely refractory to chemical-mediated induction (Fig. 4). The effect of ISO on CYP2E1 was not statistically significant, but was 1.6-fold greater than saline-treated controls; this result agrees with ISO being a weak transcriptional inducer of CYP2E1 (Table 5).

The CYP3A family is known to be inducible by PCN (Table 4) and other steroid-like compounds. Analysis of CYP3A1/23-mRNA expression by bDNA analysis demonstrated that PCN and PB significantly increased the expression of hepatic CYP3A1/23 (Fig. 4). PCN and PB increased CYP3A1/23 expression 34- and 15-fold, respectively (Table 5). These results are consistent with PCN effects on CYP3A expression (Table 4).

The CYP4A family of CYP enzymes is induced at the transcriptional level by a number of plasticizers and antihyperlipidemic drugs. We used the peroxisome proliferator, CLO, to evaluate the CYP4A2/3-oligonucleotide probe set during bDNA analysis. Expression of CYP4A2/3 is very low in control liver and the CYP4A2/3-mRNA levels were specifically increased by CLO (Fig. 4). Compared with control levels of CYP4A2/3, in response to CLO, CYP4A2/3 was induced nearly 5-fold (Table 5).

**Discussion**

The ability to evaluate rapidly and accurately drug/chemical-elicited changes in drug metabolism enzymes would be a valuable commodity to pharmaceutical companies as well as to clinical investigators. The inducible CYP enzymes are markers of drug-mediated gene expression and predictors of potential drug-drug interactions. To date, analysis of P450 activity has been the standard for assessing such interactions (Parkinson, 1996). In this report, bDNA signal amplification technology (Fig. 1) was used to assess the differential expression of CYPs as an alternative approach to enzymatic assays. We developed oligonucleotide probe sets to the inducible CYPs and monitored the mRNA expression of these enzymes in response to five different chemicals known to induce specific CYP enzymes by bDNA signal amplification technology. The present results demonstrate that the bDNA signal amplification assay can be used as a specific, efficient, and reproducible assay to monitor chemical-induced changes in CYP expression.

A critical component of the bDNA signal amplification system is that multiple and highly specific oligonucleotides are generated to a target region of a mRNA transcript. This novel approach is advantageous as it uses the specificity inherent with short oligonucleotide
probes and the sensitivity of longer oligonucleotides or cDNA probes. Designing specific probe sets to individual CYP mRNA transcripts can be challenging as there are multiple and highly identical homologs within the P450 superfamily. As summarized in Table 2, probe sets to CYP1A1, CYP1A2, and CYP2E1 were designed to be specific to each of these individual CYP transcripts. Individual oligonucleotide probe sets for the CYP1A subfamily of CYP transcripts were developed to both CYP1A1 and to CYP1A2, where these two transcripts share 76% nucleic acid identity when analyzed by pair-wise sequence alignment (Smith et al., 1996). The results obtained with these CYP1A probe sets in the bDNA assay were consistent with previous reports for CYP1A1- and CYP1A2-mRNA expression in response to chemical stimuli. In the absence of a chemical stimulus, constitutive levels of hepatic CYP1A1 mRNA were very low, whereas a much greater level of constitutive CYP1A2 mRNA expression was detected (Fig. 4; Table 5). In addition, both CYP1A1 and CYP1A2 mRNA levels were substantially induced by 3MC (Fig. 4; Table 5). These results demonstrate that the bDNA system can be useful to detect differences between highly similar homologs, like CYP1A1 and CYP1A2, which share greater than 75% nucleic acid identity.

Like the CYP1A family, the CYP2E1 probe set was developed to be specific to the CYP2E1 transcript. The bDNA data for hepatic CYP2E1-mRNA expression in control and ISO-treated rats is in accord with previous reports. Results for CYP2E1 gene expression as measured by bDNA analysis demonstrate that this transcript is expressed constitutively at high levels, and that the CYP2E1 gene is relatively insensitive to chemical stimuli. Our data (Fig. 4 and Table 5) suggest that ISO can mediate a mild induction of CYP2E1 gene expression (1.6-fold; Table 5), whereas others have reported a similar range (2–5-fold increase) for ISO induction of CYP2E1 mRNA (Parkinson, 1996).

Unlike the probe sets designed for CYP1A1, CYP1A2, and CYP2E1, which were generated to delineate between these transcripts, other probe sets were designed to be specific to individual CYP subfamilies. This was due to the high nucleotide similarity between the CYP transcripts in the CYP2B, CYP3A, and CYP4A subfamilies. Within each of these subfamilies, there are at least two highly similar transcripts that share greater than 95% nucleotide identity (e.g., CYP2B1 and CYP2B2 are 97% identical). In designing oligonucleotide probes within each of these three CYP subfamilies, a sufficient number of specific capture and label probes could not be developed to differentially measure these transcripts. Therefore, in the probe sets developed to the CYP2B, CYP3A, and CYP4A subfamilies, probes were designed to hybridize to multiple CYP isoforms within each subfamily, where CYP2B1/2, CYP3A1/23, and CYP4A2/3 are detected (Table 2). In each case, it is known that the subfamily members respond to the same chemical stimulus as described below (Table 4).

As alluded to, probes were developed to the CYP2B subfamily because CYP2B1 and CYP2B2 transcripts are 97% identical at the nucleotide level, and both genes are induced by PB (Parkinson, 1996). One label probe in the CYP2B probe set, specifically CYP2B2.2048, also interacts with CYP2B3, CYP2B12, and CYP2B15 (Table 2). However, these transcripts are not targets for any capture probes, and as such, only the signals from CYP2B1 and CYP2B2 were measurable. When the CYP2B1/2 probes were used to analyze chemical-induced differential expression of CYP mRNA, PB was shown to elicit a 70-fold increase in hepatic CYP2B1/2 mRNA as compared with control, and indicates that this probe set was sensitive to PB-induced changes in CYP2B1/2 mRNA levels. These results complement those reported by LeCluyse and colleagues (1999) for PB induction of CYP2B activity, where PB caused a 61-fold increase in CYP2B-mediated 7-epoxyresorufin O-dealkylation.

As with the CYP2B subfamily, probe sets to the CYP3A subfamily encompass both CYP3A1 and CYP3A23. These transcripts are 98.6% identical, and both genes are up-regulated by PCN (Kiritia and Matsumura, 1993; Komori and Oda, 1994). Although CYP3A2 is 90% identical with CYP3A1 and CYP3A23, only three probes, CYP3A.2120, CYP3A.2121, and CYP3A.2122 (Table 2) interact with CYP3A2 in BLAST analyses, and none of these three probes are capture probes. Therefore, in theory CYP3A2 mRNA levels were not measurable in these experiments. The nearly 34-fold increase (compared with control) in CYP3A1/23 signal obtained from samples isolated from rats treated with PCN were consistent with the known effects of this compound on CYP3A expression (Table 4). Additional studies with a probe set developed to CYP3A2 combined with a comparison of male and female CYP3A2 expression could indicate whether our probe set to CYP3A1/23 is specific only for CYP3A1/23, as CYP3A2 is expressed predominately in male rats.

Similarly, an oligonucleotide probe set that recognized the CYP4A2 and CYP4A3 isoforms of the CYP4A subfamily was developed. These two distinct transcripts are 96% identical and both genes are up-regulated by peroxisome proliferators like CLO (Kimura et al., 1989a,b). Although a probe set was not developed to CYP4A1 (a.k.a. CYPLA-omega), which is also inducible by peroxisome proliferators, a specific probe set could easily be generated against CYP4A1 as it is only 68% identical with CYP4A2 and CYP4A3. In this study, a 5-fold induction of CYP4A2/3 transcripts was observed (Table 5). This lower level of CYP4A induction in response to CLO treatment is likely related to the specificity of the probe set to the CYP4A2 and CYP4A3 mRNA transcripts. Sundseth and Waxman (1992) reported that the CYP4A2 and CYP4A3 genes are much less sensitive to CLO induction as compared with the CYP4A1 gene.
Additional studies that use a CYP4A1-specific probe set could further expand on this result.

The specificity and sensitivity that result from the probe design is apparent in our ability to detect the differential expression of highly similar CYP transcripts (Fig. 5). For the CYPs analyzed in this study, the bDNA assay was quite specific, to at least the subfamily level. Overall, the results obtained by bDNA analysis of CYP expression were as expected and consistent with previously reported findings from many other investigators in terms of the differential and specific response of individual CYPs to enzyme-inducing chemicals (Porter and Coon, 1991; Parkinson, 1996). Certainly, the robust response elicited by each chemical inducer on specific CYPs (Tables 3 and 5) supports the use of the bDNA technique as an excellent method to detect drug/chemical modulation of drug-metabolizing enzymes.

In terms of obtaining reproducible data in gene expression experiments, the bDNA assay is as good as any other assay we have routinely used in this laboratory, including Northern blot analysis, in situ hybridization, quantitative RT-PCR, RNase protection assays, as well as the newer cDNA or oligonucleotide array technologies. The cumulative data from Table 3 and Fig. 4 support this statement, where Table 3 demonstrates that the bDNA assay generated reproducible data by both repeated measures of a sample over a number of days, and in the same experiment. It is apparent from Table 3 that the c.v. is relatively high (25%) for repeated analysis of samples between days, however, it is important to point out that this c.v. was determined for non-normalized luminescence measurements, and, as such, appears relatively high. Normalization of the luminescence values to GAPDH expression reduces this value as it controls for slight differences in RNA input between days and day-to-day variation in overall luminescence. Analysis of a given CYP transcript between animals resulted in reproducible data as suggested by the S.E.M. shown in Fig. 4.

The bDNA assay is a very sensitive measure of gene expression. From this data (Fig. 3), a measurable and reproducible signal for CYP2B1/2 was detected with 0.1 µg of total RNA. This is impressive as it is generally assumed that mRNA constitutes approximately 1 to 2% of total RNA. Therefore, 0.1 µg of total RNA would be equivalent to 1 to 2 ng of poly A⁺ mRNA. From this calculation, bDNA analysis would be at least 100 times more sensitive than cDNA or oligonucleotide array technologies, which require 1 µg of poly A⁺ RNA. Others have reported that bDNA system is comparable in sensitivity to PCR-based strategies (Collins et al., 1997; Guenthner and Hart, 1998).

The bDNA signal amplification system has many advantages over other contemporary methods of gene expression analysis. One distinct advantage is that the researcher can use total RNA (as in a Northern blot) or cell extracts for the analysis. Use of total RNA in the bDNA system resulted in experimentally reproducible and appropriate (in terms of chemical-induced CYP expression) data. In this report, the analysis of gene expression data was presented in a semiquantitative...
bayer Diagnostics (Emeryville, CA) for providing assistance in obtaining the necessary equipment and reagents for this study.

Acknowledgments. We thank Nicole Dockendorf and Robert Bencher of Bayer Diagnostics (Emeryville, CA) for technical training with the ProbeDesigner Software and the Quantgene bDNA Signal Amplification system, and for donating the information on the GAPDH probe set.

References


