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INVESTIGATIONS

by

Nancy Beth Beck

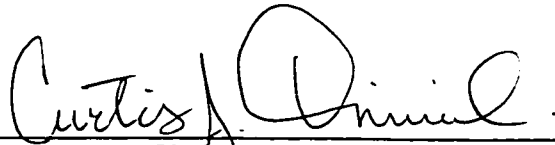
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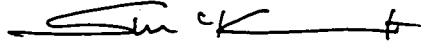
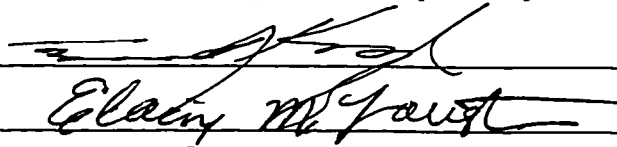
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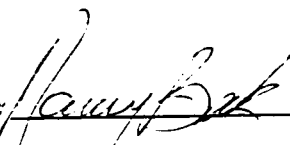
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Abstract

PHENOBARBITAL MEDIATED INDUCTION
OF THE CYTOCHROME P450 2B GENES:
MECHANISTIC INVESTIGATIONS

by Nancy Beth Beck

Chairperson of the Supervisory Committee: Professor Curtis J. Omiecinski
Department of Environmental Health

The cytochrome P450 monooxygenases play a fascinating role as determinants of toxicity stemming from endogenous and foreign chemical exposure. In addition, certain natural and anthropogenic compounds, such as those present in plants, foods, or combustion by-products can dramatically alter the levels of expression of these genes, thereby modulating the metabolism of agents we are subsequently exposed to. The dramatic upregulation of the CYP2B genes upon exposure to phenobarbital (PB) is one such example. This effect is potentially quite important and can be elicited by many dissimilar compounds that also produce a 'PB-like' response.

PB-like inducers can up-regulate the expression of many genes, including the CYP2B subfamily members in mammalian liver. The mechanisms controlling the PB induction response are poorly understood. The aim of this dissertation research was to further characterize the molecular mechanisms underlying the PB induction process through studies involving the examination of post-translational modifications, functional characterization, and protein-DNA binding analysis. The utility of a baculovirus-mediated transfection technique to study CYP gene regulation also was examined.

The results demonstrate that despite the ability of cAMP to dramatically inhibit the PB induction process, PB exposures themselves do not alter intracellular cAMP levels or PKA activity. Exposure of primary cultured rat hepatocytes to baculovirus, an agent tested for its gene delivery potential, also resulted in a marked inhibition of the PB induction response. The mechanisms of this effect were examined and appear to involve baculovirus-induced alterations in cytokine expression. Further examination of post-

translational modifications showed that the global histone deacetylase inhibitor, Trichostatin A (TSA), was without effect on PB mediated CYP2B gene activation.

Affinity purification studies were performed to examine protein-DNA interactions, as well as cooperative interactions between proteins, at the PB responsive unit of the CYP2B genes. Together with transgenic mice approaches, the results demonstrated that NF-1, an avidly bound factor in this region, is not a key determinant of the induction process.

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LIST OF ABBREVIATIONS

- β-gal.** β-galactosidase
- BNF.** β-naphthoflavone
- bp.** base pair(s)
- BROD.** 7-benzyloxyresorufin-o-dealkylase
- cAMP.** cyclic AMP
- CAR.** constitutive androstane receptor
- CAT.** chloramphenicol acetyltransferase
- C/EBP.** CCAAT enhancer binding protein
- CYP.** cytochrome P450
- dBcAMP.** dibutyryl cAMP
- DMSO.** dimethyl sulfoxide
- DTT.** dithiothreitol
- ECM.** extracellular matrix
- EROD.** ethoxyresorufin-o-dealkylase
- GRE.** glucocorticoid response element
- HNF.** hepatocyte nuclear factor
- IL.** interleukin
- ITS.** insulin transferrin selenium
- kb.** kilobase pair(s)
- kDA.** kilodalton(s)
- MOI.** multiplicity of infection

NF-1. nuclear factor 1

PB. phenobarbital

PBRU. phenobarbital responsive unit

PKA. Protein Kinase A

PKC. Protein Kinase C

PKG. Protein Kinase G

RT-PCR. reverse transcriptase polymerase chain reaction

RXR. retinoid X receptor

TCDD. 2,3,7,8-tetrachlorodibenzo-p-dioxin

TE. tris edta

TNF. tumor necrosis factor

TSA. trichostatin A

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DEDICATION

I wish to dedicate this dissertation to my parents, Susan and Robert Beck. Their extraordinary patience, support, and friendship has been far greater than I could have ever imagined. I am quite lucky to have them in my life.

INTRODUCTION

Background and Significance

Each day the human body is confronted with many potentially toxic substances in the form of food items, medicinal products, and environmental agents. Fortunately, the majority of compounds are converted to less hazardous, more excretable substances. However, some substances may be converted from unreactive parent compounds into chemically reactive intermediates that can be toxic and/or carcinogenic. The cytochrome P450s play a fascinating role as determinants of the toxicity of foreign compounds. In addition to their involvement in the metabolism of endogenous compounds, the P450 supergene family plays a critical role in the oxidative metabolism of many exogenous substances. Approximately 65 P450 gene families have been identified with 14 of these families existing in mammals (Nelson *et al.*, 1996). Most of these genes exhibit developmental as well as tissue specific expression. They are key participants in the maintenance of steady-state levels of endogenous ligands which are involved in regulating homeostasis, growth, differentiation and neuroendocrine functions (Denison and Whitlock, 1995; Gonzalez, 1989). Exposure to certain foods, drugs, or compounds present in the environment can dramatically alter the levels of expression of these genes thus changing the way in which compounds we are simultaneously or later exposed to are metabolized. One such example is the dramatic upregulation of the rat CYP2B1 and CYP2B2 genes upon exposure to phenobarbital (PB). The PB induction response is conserved across most animal species, and is even manifested in certain procaryotes. This effect is quite interesting as it can be elicited by many other dissimilar compounds which produce what has been termed a 'PB-like' response.

The mechanism regulating this 'PB-like' response, initiated by other barbiturates and compounds such as organochlorine pesticides, halogenated biphenyls, isosafrole, and known carcinogens such as 2-acetylaminofluorene, is not well understood (Honkakoski and Negishi, 1998b; Waxman and Azaroff, 1992). Interestingly, these 'PB-like' inducers

can also elevate the expression of other P450 subfamily members, including members of the CYP 2A, 3A and 2C subfamilies (Honkakoski and Negishi, 1998b; Waxman and Azaroff, 1992). This induction is not limited to the P450's as other enzyme groups including epoxide hydrolase, aldehyde dehydrogenase, NADPH: P450 reductase, UDP-glucuronyl transferase and some of the glutathione S-transferases are similarly induced (Cresteil *et al.*, 1980; Lubet *et al.*, 1992; Waxman and Azaroff, 1992). The research proposed here will focus on the CYP2B1 and CYP2B2 genes, the major PB-inducible P450 enzymes in the rat liver.

Cytochrome P450 2B Genes

CYP2B1 and CYP2B2 metabolize a broad range of endogenous and exogenous lipophilic compounds including drugs, steroids and polyaromatic hydrocarbons. Both metabolize similar substrates but with distinguishable specificity profiles (CYP2B1 often exhibits higher catalytic activity) and in some cases, as with 7,12.-dimethylbenz[a]-anthracene hydroxylation, they differ in regioselectivity (Gonzalez, 1989). Although 97.4% identical, differing only by 14 of 491 amino acids (Suwa *et al.*, 1985), the CYP2B1 and CYP2B2 proteins are the products of two distinct genes. They are closely linked on rat chromosome 1 (Rampersaud and Walz, 1987). The human CYP2B6, located on chromosome 19q12-q13.2 (Miles *et al.*, 1988; Santisteban *et al.*, 1988; Yamano *et al.*, 1989), has 74% amino acid identity to the rat CYP2B1 protein (Miles, et al., 1988).

The CYP2B1 gene is approximately 25kb in length and contains 9 exons with 8 introns. The corresponding CYP2B2 gene is approximately 15kb in length, and differs from the CYP2B1 gene in the length of intron 1. In the coding region, CYP2B2 shows only a 40bp difference, with CYP2B1, out of about 1800bp. These differences are concentrated in exons 6-9 (Suwa, et al., 1985). CYP2B1 and CYP2B2 differ only slightly in the 5'-upstream region where 95% sequence similarity is seen. Both genes contain a modified TATA box, CATAAA, 20bp upstream from the transcriptional start site. There is an alternating purine/pyrimidine sequence (CA) at about -255bp which is only 5 repeats long in CYP2B1 but consists of 19 repeats in CYP2B2. Other base pair substitutions exist, but with no striking differences. In the 5' region homologies to many known regulatory

elements exist. Some of these are shown in Figure 1. Among these are binding sites for GRE, AP1, C/EBP, ETS, HNF, NF1, and TF elements. The importance of these sites are discussed in a following section. Chromatin sites hypersensitive to the action of DNase have also been identified in this region (Ramsden, unpublished results).

Although extrahepatic gene expression is seen, PB induces the strongest expression of the CYP2B1/2 proteins in the liver. Both genes are regulated similarly during rat liver development but some differences in tissue specificity and induction capacity are seen. A thorough review of these differences is provided by Waxman and Azaroff, 1992. In the uninduced rat liver, the CYP2B2 protein is present but its expression is low; the CYP2B1 protein is 5-10 fold lower and often undetectable. Upon exposure to PB, the quantity of both proteins is significantly increased, with the mRNA levels of CYP2B1 increasing 4-5 fold more than CYP2B2 (Omiecinski *et al.*, 1985). The effects of PB are principally liver-specific but quite pleiotropic as liver weight gain, proliferation of the smooth endoplasmic reticulum and liver tumor promotion are also seen in induced rats. This dissertation will focus upon mechanisms regulating the expression of the PB-induced CYP2B1/2 genes in the rat liver.

Unlike the CYP1A1 gene, where ligand binding to the Ah receptor is crucial for gene activation, no receptor has been identified for PB-inducible genes. Experiments using radiolabeled PB as a ligand have been unable to detect a specific PB binding protein (Denison and Whitlock Jr., 1995). PB is an effective inducer at only relatively high concentration (~ 0.1mM), a property shared by most PB-like agonists. Other recent work, finding a lack of enantioselectivity among ligands affecting PB responsiveness, has provided more evidence against the existence of a specific high affinity PB receptor (Nims *et al.*, 1994), although it remains possible that a loosely selective receptor protein may exist and may mediate the response.

Induction of the CYP2B1/2 genes is due primarily to transcriptional activation leading to an increase in the steady state levels of CYP2B1/2 mRNA. Protein and mRNA stabilization, as well as transcription elongation, gene amplification and gene rearrangement have been ruled out as likely mechanisms (Waxman and Azaroff, 1992). Rapid transcriptional activation is detectable 30-60 minutes after induction (Atchison and Adesnik, 1983;

Hardwick *et al.*, 1983). Cycloheximide, used in *in vitro* studies to block protein synthesis, blocked PB induced expression of the CYP2B1/2 genes (Waxman and Azaroff, 1992). However, a more recent study using a negative analog of puromycin evaluated the specificity of protein synthesis inhibitors on the PB induction process found that *de novo* protein synthesis was not a requirement for PB mediated induction (Sidhu and Omiecinski, 1998).

Examining PB Induction *In Vitro*

Historically, the PB induction response has been extremely difficult to reproduce *in vitro*. Most transformed cell lines have been refractive to PB. However, recent advances in the culturing of primary rat hepatocytes now allow preservation of the PB response as well as maintenance of other differentiated hepatocyte functions. This was accomplished by culturing cells on plates coated with collagen, Matrigel, sandwiched between the two, or using an overlay of Matrigel (Schuetz *et al.*, 1988; Sidhu *et al.*, 1993; Sidhu and Omiecinski, 1995b; Sinclair *et al.*, 1990). In addition, studies have demonstrated the necessity of using specifically defined medias, cofactors, and hormonal supplements, e.g. the presence of low concentrations of dexamethasone, to achieve an induction response that mimics the *in vivo* situation. The availability of optimized hepatocyte culture conditions now allows us to pursue various *in vitro* assays to further study the mechanisms behind the PB response. For instance, the first of many studies that can be conducted to study functional expression in these systems was published recently (Trottier *et al.*, 1995) and will be discussed further in future sections of this dissertation.

Identification of the PB Responsive Elements

Until quite recently, there has been little consensus regarding the identity of a PB responsive element in the CYP2B1/2 genes (Figure 2). Even proposed theories of PB action are at odds. First, as a PB receptor has not been identified, it has been suggested that the common site of binding is the P450 itself (Fonne and Meyer, 1987). If PB was to

bind the P450 that metabolizes endogenous inducer, thus blocking inducer metabolism, the inducer would then accumulate to levels high enough to bind a receptor on the CYP2B1/2 gene and transcriptional activation would occur. The inducer may normally be present in the cell at low levels but consistent metabolism may render it inactive. If its metabolism was blocked, the steady state levels would increase and receptor binding would occur (Shaw *et al.*, 1993). Similarly, it has been suggested that such an endogenous compound could be a repressor (Burnet *et al.*, 1986). In this scenario the repressor molecule may normally be metabolized to its active state, thus blocking constitutive gene activation. If PB binds the P450 which blocks repressor metabolism, the repressor would remain inactive and gene transcription would occur. It has also been suggested that the presence of PB acts to create general changes in membrane fluidity and structure. These changes at the membrane level might then lead to changes in the signal transduction cascade that begins at the membrane level. According to the latter hypothesis, the changes in released second messengers, such as cAMP, would then account for the changes in gene activation.

Dr. Fulco and colleagues from UCLA have been studying the PB inducible P450_{BM-3} protein from *Bacillus megaterium*. This group has cloned and characterized a 119Kd protein which, unlike the rat CYP2B2, is not constitutively expressed but is induced upon exposure to barbiturates (Narhi and Fulco, 1986; Wen and Fulco, 1987). It was first believed that this response was triggered by the presence of an endogenous repressor, which when bound by PB, was unable to block gene transcription. After characterizing the 5'-upstream region of the BM-3 gene, a 17bp element located at -73 to -89 (from the transcription start site) was identified as being a repressor binding site. A second repressor site was also found at > 1kb upstream of the transcription start site. Interestingly, this 17bp element, termed the Barbie Box, was demonstrated to show high homology with a 17bp sequence in the rat CYP2B1/2 genes (8/17 nucleotides), the PB inducible P450_{BM-1} gene in *B. megaterium*, and the PB-inducible rabbit CYP2C1 gene (He and Fulco, 1991). Further investigation has found that homology to this 17bp sequence exists in other PB inducible genes including glutathione S-transferases, aldehyde dehydrogenase, and epoxide hydrolase (Lubet, et al., 1992). Thus, detection of these homologies has made the Barbie Box a region of great interest.

Using electrophoretic-mobility shift assays, Fulco and his group have found that this 17bp region in the P450_{BM-3} gene binds control liver nuclear extracts more avidly than extracts from PB treated cells. They also determined that no *de novo* protein synthesis was required for this change in binding; thus further supporting the theory that an endogenous repressor exists (He and Fulco, 1991). Further work has identified a repressor protein, bm3R1, and a promotor protein, bm3P1, that are believed to compete for binding at the Barbie Box element as well as at an operator site (Liang and Fulco, 1995; Liang *et al.*, 1995; Shaw and Fulco, 1992; Shaw and Fulco, 1993). The mechanism is not quite as clear as one would hope as other proteins and binding sites are also believed to be involved in the P450_{BM-3} gene regulation. In summary, it is believed that in the absence of inducer, the repressor protein binds the Barbie Box and operator sequence causing DNA looping which blocks binding of transcription factors and polymerases to the gene. In the presence of barbiturate, the promotor protein binds the gene causing dissociation of the repressor protein, reversing the DNA looping and rendering the promotor accessible to transcription (Liang and Fulco, 1995). Refuting the work from Dr. Fulco's laboratory, a more recent study has found evidence that the Barbie Box is unlikely to be the key element in barbiturate mediated induction of the P450_{BM-1} gene (Shaw *et al.*, 1998). Although the studies by these groups are quite comprehensive, working with a procaryotic system may not directly apply to the eucaryotic regulation of the genes. It is perhaps noteworthy that the bacterial induction phenomenon requires doses of barbiturate approximately one order of magnitude higher than for the counterpart mammalian systems, and displays a much more rapid kinetic induction response (minutes vs. hours in bacteria and rat, respectively). Nevertheless, the results are intriguing and may serve as a potential framework for understanding the PB induction response in mammalian organisms.

In 1990, a group studying the rat CYP2B1/2 genes, reported that the presence of PB led to the transcription of a novel heme-modulated binding factor which could reverse the effects of a repressor that was believed to be binding within 179bp upstream of the transcription start site of the CYP2B1/2 genes (Rangarajan and Padmanaban, 1989; Rao *et al.*, 1990; Upadhy *et al.*, 1992). It was further determined, through DNA footprinting, that a negative element was binding at -127 to -160, an area which contains a core GRE binding site; and a positive element was binding at -69 to -98, the area which contains the 17bp homology sequence which is believed to bind a negative element in procaryotes (Ram *et*

al., 1995; Upadhyaya, et al., 1992). Additionally, gel mobility shift analysis of the putative positive element indicated that a new protein-DNA complex was formed when nuclear extracts from PB treated rats were used (Ram, et al., 1995). Protein purification studies provided evidence that the same 26-28Kd protein was binding to both the negative and positive binding sites, and could give rise to three different size protein-DNA complexes depending on the induction state. Alkaline phosphatase and protein kinase treatments were used and it was implied that the phosphorylation state of the protein differed between control and PB extracts (Prabhu *et al.*, 1995). The investigators hypothesize that binding of the dephosphorylated protein to the negative element is responsible for the basal CYP2B1/2 transcription and PB caused a phosphorylation of the protein; the phosphorylated protein is believed to have a stronger binding affinity for the positive element thus leading to an upregulation of gene transcription. Overall, this model is suggestive of PB causing a derepression of the CYP2B1/2 genes. Unfortunately, other investigators have been unsuccessful in duplicating the above mentioned 1989 and 1990 studies and the results implied by Ram et al., (1995), are not in agreement with other laboratories. Although the suggested model for PB induction may be prove to be valid, the lack of reproducibility of studies from this group make interpretations of their research difficult.

Other investigators also suggest that derepression is the mechanism of PB action. Hoffman et. al., (1992), have suggested this mechanism although their gel shift studies do not show any qualitative differences between control and PB induced extracts when binding the area covering -71 to -104 of the CYP2B2 gene (Hoffman *et al.*, 1992). Similarly, Shephard et. al., (1994), using gel mobility shift analysis, reported that PB extracts bind to an area covering -31 to -72 with greater affinity than control extracts, however, no evidence of interaction at the Barbie Box region spanning -73 to -89 was observed (Shephard *et al.*, 1994). Footprinting studies also failed to evidence any binding in this area although protected regions were seen at -31 to -72 and -199 to -183 with both PB and control extracts (Shephard, et al., 1994). Unlike the Shephard and Hoffman groups, unpublished gel shift results from our laboratory do show comparatively increased binding (~2-3 fold) to the Barbie Box region when nuclear extracts from PB treated rats are used (Sommer, Beck unpublished results). Although our own footprinting results indicated a protected region spanning -42 to -66 and -307 to -337, the area covering the Barbie Box region did

not show any protein binding in these studies (similar to Shephard et al., 1994) (Sommer *et al.*, 1996). As seen in Figure 2, no group of investigators has been able to exactly reproduce the results found by other investigators in this region. The only agreement about the area between +1 and -179 is that it contains the minimal promoter for basal CYP2B1/2 expression (Hoffman, et al., 1992; Ram, et al., 1995; Ramsden *et al.*, 1993; Shephard, et al., 1994).

Work from our laboratory by Ramsden et. al., (1993), was the first to point elsewhere in the search for a PB responsive genetic element. These studies used transgenic mice approaches to identify a region between -0.8 and -20kb of the CYP2B2 gene that was critical for PB induction and tissue specific expression. Constructs which included the complete CYP2B2 gene with 0.8kb of 5' flanking sequence showed high constitutive expression of CYP2B2 in the absence of PB challenge. Furthermore, this ordinarily liver specific gene exhibited high levels of expression in the kidneys of the transgenic animals. However, in mice containing the 20kb of 5'-upstream sequence, the pattern of CYP2B2 expression was rat-like. These animals exhibited low constitutive CYP2B2 expression which was greatly increased following PB treatment. As shown by this study, *in vivo*, a PB responsive region exists upstream of -0.8kb but within -20kb of 5'-upstream flanking sequence (Ramsden, et al., 1993). DNase hypersensitivity studies from our laboratory and others (Luc *et al.*, 1996) (Ramsden et. al., submitted), also suggest that elements involved in the PB response may be further upstream as hypersensitive sites are seen at -1400 and -2200bp. In a recent report by another laboratory, transfection studies in a PB inducible primary rat hepatocyte system identified a 163bp fragment between -2155 and -2318bp responsible for a 6 fold induction in the presence of PB. These studies failed to identify any PB responsiveness in the first 200 bases of 5' sequence (Trottier, et al., 1995). Similar transfection studies in our laboratory have also been unable to identify a PB response element in the first 1400 bases of 5'-upstream sequence (Beck, unpublished results). Furthermore, transient transfections in primary chick hepatocyte cultures show that a PB response element for the barbiturate inducible CYP2H1 gene exists between -1.1 to -5.9kb of 5'-upstream sequence (Hahn *et al.*, 1991).

Identifying the PBRU

In recent years, significant progress has been made in identifying the elements critical for the PB induction response (Trottier, et al., 1995). Although the increase in PB mediated expression was only 2-3 fold, using *in situ* transfections, the functionality of the 163 bp phenobarbital responsive unit (PBRU), located at -2155 through -2317 in the 5' region of the CYP2B2 gene, was duplicated (Park *et al.*, 1996). Unfortunately the transfection efficiencies were low and dexamethasone was required for optimal expression of transgenes. These studies also tested constructs that contained the sequences of the Barbie Box fragment and determined that it was not involved in the PB induction process.

In the mouse model, a region termed the PBREM of the mouse CYP2B10 gene was found to show 80% sequence similarity to the rat PBRU (Honkakoski *et al.*, 1996). Using deletion analysis and transfection studies in primary mouse hepatocytes, this fragment (-971 to -1401) was shown to be responsible for the PB mediated induction of the CYP2B10 gene (10 fold maximal induction) and again the involvement of Barbie Box sequences was disproved (Honkakoski, et al., 1996). Using *in vitro* DNA-protein binding studies, five major protein binding sites were delineated, although the binding was similar between control and PB induced nuclear extracts. A core AGGTCA sequence belonging to the ligand dependent transcription factor superfamily was identified. Further characterization of the PBREM led to the delineation of a 33bp core which was found to be essential, as well as sufficient, for induction (Honkakoski and Negishi, 1997). With this core element, the PB induction was only increased 2-3 fold over uninduced. This core sequence, containing binding sites for NF-1 and a member of the nuclear receptor superfamily (NR), was able to act as an enhancer when placed in front of a heterologous promoter. Extension of this core to a 51bp element was found to be fully inducible by PB as well as other xenobiotics that are able to mimic the PB induction response (Honkakoski and Negishi, 1998a). Further examination of this core region, has shown that a CAR-RXR heterodimer can bind at the NR sites in the CYP2B genes and regulate transcription of the CYP2B2 gene in HepG2 and HEK29 cell lines (Honkakoski *et al.*, 1998b). The effects of PB on the binding of CAR-RXR has not yet been determined. As the RXR can heterodimerize with multiple nuclear orphan receptors, other orphan receptors, besides CAR, may be involved in modulating CYP gene expression.

Interestingly, further deletion analysis studies with the rat PBRU, found that the minimal sequence required for induction was -2170 to -2258. This 38bp sequence was found to be necessary but not sufficient for PB induction. Mutations of the NF-1 site reduced the induction response, but did not eliminate it (Liu *et al.*, 1998). In the PBRU, using site directed mutagenesis, a GRE, NF-1, and a nuclear receptor hexamer half site were found to be essential for maximal induction (Stoltz *et al.*, 1998). From our own laboratory, further studies with transgenic mice have confirmed that the PB responsive elements lie within the upstream -1700 and -2500 bases of the CYP2B2 gene (Ramsden *et al.*, submitted). When the NF-1 site was mutated, the transgenic mice remained highly inducible. In our laboratory protein binding studies using the PBRU have shown that in addition to NF-1, there is also binding of HNF-4, and Histone H1. Although all *in vitro* binding studies have been unable to detect differences in protein binding between control and PB treated nuclear extracts (Ramsden *et al.*, submitted) (Honkakoski, *et al.*, 1996; Trottier, *et al.*, 1995), a recent study using *in vivo* footprinting was able to discern a difference. A 25bp protected area, centered around an NF-1 site, was detected with the control samples and upon PB treatment the footprint was extended to a 60bp protected region (Kim and Kemper, 1997). These results show that PB may not only mediate its effects through alterations of chromatin structure, but it alters the architecture of proteins binding to the PBRU. It is clear from all the above mentioned studies that the PBRU is a multicomponent element containing functional binding sites for multiple regulatory proteins. Exactly which proteins are binding and how they are interacting to control CYP2B transcription still needs to be determined.

PB and Second Messenger Systems

Other studies have focused on the effect of PB on second messengers. Early whole animal experiments and studies with hepatocytes in suspension, concluded that PB activated a cAMP dependent protein kinase that in turn increased the levels of P450 gene expression (Byus *et al.*, 1976; Canepa *et al.*, 1985; Hutterer *et al.*, 1975). These studies were quite crude however, as imprecise endpoints were measured and often super-physiologic doses of cAMP were used. Of interest from the tumor promotion standpoint, PB was reported to

inhibit the phorbol ester induced translocation of PKC to the plasma membrane in hepatocytes from rats that ingested 0.1% PB in their water (Brockenbrough *et al.*, 1991). In addition, it has been suggested that PB inhibits PKC by occupying the diacylglycerol binding site (Chauhan and Brockerhoff, 1987). Recent work from our own laboratory yielded a different spectrum of conclusions. In rat hepatocyte cultures, cAMP analogs and adenylate cyclase activators, such as forskolin, dramatically inhibited the PB induction response of CYP2B genes (Sidhu and Omiecinski, 1995a). Evidence was provided that the second messenger pathway was PKA rather than PKC or PKG. Additionally, it was determined that okadaic acid markedly potentiated the repressive effects of cAMP on the PB induction process (Sidhu and Omiecinski, 1997). Similar repression was also seen in mouse hepatocytes with the CYP2B10 gene (Honkakoski and Negishi, 1998a). *In vivo*, CYP2B induction after PB injections in rats was antagonized by simultaneous injection of okadaic acid (Nirodi *et al.*, 1996). In the same study, injections of the protein kinase inhibitor 2-aminopurine, in conjunction with PB, also inhibited CYP induction, a result that appears contradictory. In chicken hepatocytes, PB mediated activation of CYP2H1 was inhibited by 2-aminopurine but again a specific kinase pathway was not identified (Dogra and May, 1996). In another set of primary rat hepatocyte experiments, PKA inhibitors weakened the PB induced CYP2B induction (Brown *et al.*, 1997). The available data are strongly suggestive of the involvement of cAMP and PKA in modulating PB responsiveness. Although repression of PB mediated CYP2B induction by okadaic acid has been seen in multiple laboratories (Honkakoski and Negishi, 1998a; Nirodi, *et al.*, 1996; Sidhu and Omiecinski, 1997), further studies are needed to determine not only the specific pathways involved but also how they are mediating their effects.

Transcription Factor Involvement in CYP2B Regulation

The 5'-upstream region of the CYP2B1/2 genes appears to regulate tissue-specificity, PB inducibility, and harbors several consensus binding sites for proteins involved in the transcriptional regulation of many genes. Results from recent studies suggest that post-translational modifications of proteins are involved in the PB induction process. Thus it is necessary to further characterize the putative proteins that participate in this regulatory

scheme (as diagrammed in Figure 1). It is noteworthy that footprinting studies have detected protected regions which correspond to sites that not only demonstrate DNase hypersensitivity, but also possess consensus binding motifs for known transcription factors (Ramsden and Sommer, unpublished results). Within the -1200bp and -1500bp region, a DNase I hypersensitive site exists at approximately -1300bp, and consensus binding sites for HNF4, ETS, C/EBP, AP-1, a transferrin gene element, and a GRE have been identified. Similarly, a DNase hypersensitive site at -2200bp maps in close proximity to an NF-1 element.

AP-1

In various genes, the AP-1 site is important. As elegantly stated by Karin et. al. (1992), the AP-1 element... "should be regarded as a nuclear messenger that mediates the actions of signal transduction pathways stimulated by hormones, growth factors, cytokines and neurotransmitters, most of which are often initiated by the activation of phospholipid turnover or tyrosine kinases" (Karin and Smeal, 1992). Interestingly, CREB can form dimers with fos and jun and bind to AP-1 sites (Condie *et al.*, 1996). This is intriguing as cAMP, which activates CREB, represses the CYP2B induction response (Sidhu and Omiecinski, 1995a). Although the 5' region of the CYP2B genes contain no CRE, it is possible that cAMP is mediating its effects through an AP-1 site. Roe et. al. (Roe *et al.*, 1996), have shown that, after chronic exposure in rats, PB increases binding to a synthetic AP-1 consensus oligomer. AP-1 has also been shown to interact with the glucocorticoid response element (GRE) and the glucocorticoid receptor (GR). *In vitro*, AP-1 activation is repressed by the induction of GR in the presence of dexamethasone (Pfahl, 1993). This suggests a direct interaction between GR and the AP-1 components fos and jun; *in vitro*, fos and GR can form stable complexes (Pfahl, 1993). Interestingly, *in vivo* footprinting of AP-1 doesn't change in the presence of dexamethasone, suggesting that the interaction *in vitro* may be due to high concentrations of receptor which may not reflect the mechanism by which the receptors antagonize AP-1 *in vivo*. The two regions that most strongly affect the inhibition of AP-1 by GR contain domains that can serve for protein-protein or protein-DNA interaction. GR and AP-1 do not compete for their specific response elements with each other but mutually inhibit their activities by a mechanism which probably involves direct protein-protein interaction. In a suggested model AP-1 components may act as direct

bridging proteins between AP-1 and the basic transcription complex (Pfahl, 1993). This is consistent with footprinting results and would allow for crosstalk between different classes of proteins.

NF-1

NF-1 proteins belong to a diverse family of DNA-binding proteins with functions that include transcriptional promotion (Jones *et al.*, 1987), enhancement (Roy and Gu'erin, 1994), repression (Reifel *et al.*, 1991; Roy and Gu'erin, 1994), and anti-repression (Dusserre and Mermod, 1992). Individual NF-1 forms may have unique activation properties (Altmann *et al.*, 1994; Apt *et al.*, 1993; Reifel, *et al.*, 1991) which can be influenced by the nucleotides surrounding the NF-1 site (Gronostajski, 1987). NF-1 sites may be important in gene activation as they act to alleviate the histone H1 mediated inhibition of the transcription initiation complex (Dusserre and Mermod, 1992). NF-1 has also been implicated in the ligand mediated regulation of a glucocorticoid responsive mouse mammary tumor virus promoter (Truss *et al.*, 1995; Zaret and Yamamoto, 1984) as well as in the regulation of the CYP 1A1 gene (Wu and Whitlock, 1992). As discussed in the previous section, the NF-1 site is believed to be located in the centers of the PBRU and PBREM of the CYP2B2 and CYP2B10 genes, respectively.

HNF

Although the exact nature is unknown, the HNF, hepatocyte nuclear factor, family of proteins play important roles in the liver specific expression of many genes. The HNF-3 proteins, which exhibit homology to the *Drosophila* forkhead gene family, mediate the coordinate expression of a number of hepatocyte specific genes and also play a role in hepatocyte differentiation (Clevidence *et al.*, 1993). HNF-3 was first discovered during studies on the functionality of the transthyretin (TTR) gene promoter. Unlike the other HNF proteins, HNF-3 binds to DNA as a monomer, but can regulate expression of other HNF proteins e.g., expression of HNF-1 can be regulated by HNF-3. Likewise, HNF-4 can control the regulation of HNF-1 (Griffo *et al.*, 1993). Both HNF-3 and HNF-4 have been identified as playing a role in the regulation of CYP2C2 and CYP2C6 (Chen *et al.*, 1994; Shaw *et al.*, 1994). HNF-4 also plays a role in the regulation of liver specific genes.

For instance, it can cooperate with C/EBP β in the regulation of the ornithine transcarbamylase gene (Nishiyori *et al.*, 1994). The transferrin promoter element, TF, has been identified as being a positive regulator of the human transferrin gene and can cooperate with C/EBP α and HNF-3 (Schaeffer *et al.*, 1989). The TF element is homologous to the HNF-4 binding site (Zakin, 1992). HNF-4 has been demonstrated to bind DNA sequences which are similar to COUP-TF in the apolipoprotein CIII gene (Mietus *et al.*, 1992). Interestingly, in this gene, as well as in the ornithine transcarbamylase promoter, HNF-4 activates the gene and COUP-TF represses it (Kimura *et al.*, 1993; Mietus, *et al.*, 1992). It was also established that the RXR- α and COUP-TF recognize indistinguishable sites and COUP-TF can repress RXR- α mediated transcriptional activation (Kliwer *et al.*, 1992). HNF-4 DNA binding activity has been determined to be modulated by phosphorylation *in vivo*, *ex vivo*, and *in vitro* (Viollet *et al.*, 1997). The *in vivo* studies showed that the DNA binding activity of HNF-4 was reduced by inducers of cAMP. This suggests that HNF-4 is a target of the cAMP/PKA pathways that might be involved in the inhibition of liver specific genes by cAMP inducers, such as the CYP2B genes.

GRE

The GRE located at about -1300 in the CYP2B2 gene has been demonstrated to be functionally active (Jaiswal *et al.*, 1990). In addition to synergizing with HNF-3, the GRE in the tyrosine aminotransferase gene interacts with C/EBP. The glucocorticoid receptor selectively regulates transcription by binding to specific partially palindromic 15bp sequences on the DNA and subsequently activates many genes. It has been suggested that the glucocorticoid receptor may modulate gene expression through protein-protein interactions (Caldenhoven *et al.*, 1995). The glucocorticoid receptor can be altered by cell cycle regulated kinase and phosphatases, which are believed to selectively impact its functions (Hsu and DeFranco, 1995). It is noteworthy that results from our own laboratory indicate that low levels of glucocorticoids are required for, and synergize with, the PB induction response (Sidhu and Omiecinski, 1995b).

ETS

The Ets family of transcription factors, which have no structural homology to other known DNA-binding motifs, play a pivotal role in cell growth, differentiation, and transformation. They have a conserved DNA binding domain and regulate transcription from a myriad of viral and cellular gene promoters (Macleod *et al.*, 1992). Ets-1 and Ets-2 interact with AP-1 to coordinately regulate gene expression, as was seen in studies of the polyoma virus enhancer. Similarly, other Ets family members, like ELK-1, form ternary complexes with the serum response factor (SRF) present on many genes (Janknecht and Hunter, 1996; Janknecht and Nordhein, 1993). In participation with glucocorticoids, Ets proteins are capable of participating in the signal transduction pathways triggered by other nonsteroidal stimuli to regulate expression of the liver specific tyrosine aminotransferase gene (Espinosa *et al.*, 1994). The Ets proteins are induced by protein kinase C activators and MAP kinases. Additionally they are phosphorylated by other unidentified kinases (Janknecht and Hunter, 1996; Janknecht and Nordhein, 1993).

C/EBP

Several members of the CCAAT enhancer binding protein family, C/EBP, have been identified. The C/EBP proteins contain a bipartite DNA binding domain which consists of a basic region next to a leucine zipper motif (Landschultz *et al.*, 1988). The three isoforms of C/EBP are differentially regulated throughout development with C/EBP α dominating in the quiescent state and in terminally differentiated liver cells. Along with C/EBP β , C/EBP α regulates the balance of growth and differentiation in hepatocytes, C/EBP δ is expressed at low levels in the liver (Johnson, 1990). The C/EBP proteins are ubiquitously expressed, but have been shown to be important regulators of hepatic gene expression. C/EBP is essential for liver specific expression of the transferrin gene but does not appear to be important in its expression in the brain (Theisen *et al.*, 1993). C/EBP can interact with HNF-3 to control the expression of the liver specific vitellogenin gene (Cardinaux *et al.*, 1994). C/EBP β was first identified as a nuclear factor binding to the IL-1 responsive element in the IL-6 gene (Akira *et al.*, 1990). It also activates several acute phase protein genes through this IL-6 element. Interestingly, it was reported that IL-6 confers a dose-dependent decrease in the PB induction of the CYP2B1/2 genes (Williams *et al.*, 1991).

C/EBP β can be regulated by phosphorylation changes through the MAP kinase and calcium-calmodulin signaling pathways (Metz and Ziff, 1991; Nakajima *et al.*, 1993; Wegner *et al.*, 1992). These two pathways appear to act antagonistically. In the CYP2B genes, it has recently been shown that the promoter contains a functional C/EBP site (Luc, *et al.*, 1996; Park and Kemper, 1996). In the chicken, HNF-1, HNF-3, C/EBP, and the ubiquitous factor USF were all necessary to drive expression of the CYP2H1 gene in chick hepatocytes (Dogra and May, 1997). This study, as well as others, suggest that C/EBP often acts in a combinatorial fashion with other liver-specific transcription factors to drive the basal transcriptional apparatus.

Summary

In summary, based upon the available data and structural analyses of the CYP2B1/2 genes, there are many proteins that may participate in the PB induction response. These proteins are potentially modulated by pathways believed to be involved in PB's activation of the CYP2B1/2 genes. In light of the lack of agreement regarding the specific PB response element, one aim of the proposed research was to address the controversy that remains regarding the DNA elements necessary for induction. Additionally, it was my goal to take this research further by delineating specific proteins and cytokines that may play a role in CYP2B1/2 regulation. These studies will dovetail with signal transduction work being conducted in parallel in our laboratory. The involvement of the cAMP and PKA signaling pathways, as well as the role histone acetylation may play in modulating CYP2B gene regulation were examined. The results of these studies should better enable us to map the path of PB as it strikes the cell membrane and causes changes in signal transduction pathways which culminate in the modulation of nuclear proteins responsible for regulating the CYP2B1/2 genes.

Hypothesis

Based on the available scientific data, it is hypothesized that cooperative protein-DNA and protein-protein interactions between several arrayed nuclear factors in the -800bp to -20kB 5'-upstream region of the CYP2B1/2 genes dictate the overall regulatory potential of PB induction. Furthermore, it is hypothesized that the PB induction response is the culmination of several interacting pathways as chromatin structure and the phosphorylation status of *cis*-acting factors play important roles in the PB induction response.

Specific Aims

- 1) To identify regions of the 5'-upstream sequence of the CYP2B1/2 genes that are critical for regulation the PB-induction response.** This will be accomplished using gene transfection techniques in a primary rat hepatocyte culture system. Fusion constructs, which contain various portions of the 5'-upstream region of the CYP2B1/2 genes linked to a reporter gene, will allow systematic appraisal of the transcriptional strength of various 5' flanking regions of the CYP2B1/2 genes in both the presence and absence of PB.
- 2) To study cooperative interactions of nuclear proteins with the 5'-upstream region of the rat CYP2B1/2 genes.** Sequence analysis of the 5'-upstream region of the CYP2B1/2 genes indicates homologies to known binding motifs for several nuclear binding factors, including members of the C/EBP, ETS, GRE, and NF-1 gene families. Employing gel mobility shift and supershift assays using antibodies corresponding to specific transcription factors will allow identification of specific protein-DNA interactions that exist either in control or PB treated rat liver nuclear extracts. Additional studies will be conducted using protein affinity purification to pull out and examine specific proteins that may be interacting with the putative PBRU.
- 3) To asses the phosphorylation status of nuclear proteins that are identified as either interacting differentially with the CYP2B1/2 5'-**

upstream regions depending on PB induction state or are located in areas that are critical to the PB induction response. As PB exposure effects signal transduction pathways, and protein phosphorylation has been shown to modulate the activity of many transcription factors, including those mentioned in the above section. I plan to examine the role of phosphorylation in determining PB inducibility. This portion of the project will be accomplished using specific antibodies to examine the phosphorylation status of transcription factors which may play a role in PB induction.

4) To examine the effects of PB on cAMP expression and PKA activity levels in rat primary hepatocyte cultures. Although recent reports have suggested a requirement for phosphorylation and dephosphorylation events in controlling PB mediated CYP expression, these studies induced cells in the presence of agents which modulate signal transduction processes. Alterations in signaling pathways immediately following cellular exposure to PB have not been investigated. It is my goal to examine cAMP levels and PKA activities in time course experiments, subsequent to the exposure of primary rat hepatocytes to PB, and demonstrate the role that this signaling pathway may play in the early phases of the PB induction response.

5) To examine the role of histone acetylation in modulating PB inducible CYP2B gene expression. Histone acetylation has now been implicated in controlling the transcription of many genes, including those coding for liver enzymes such as CYP1A1. To understand and address potential avenues of PB mediated gene regulation, I would like to examine the role histone acetylation may play in modulating CYP2B gene transcription. Using global histone deacetylase inhibitors (sodium butyrate and Trichostatin A) to modulate histone acetylation, I should be able to determine whether or not the PB induction response can be turned on or potentiated by changing histone acetylation status and creating a more accessible chromatin structure.

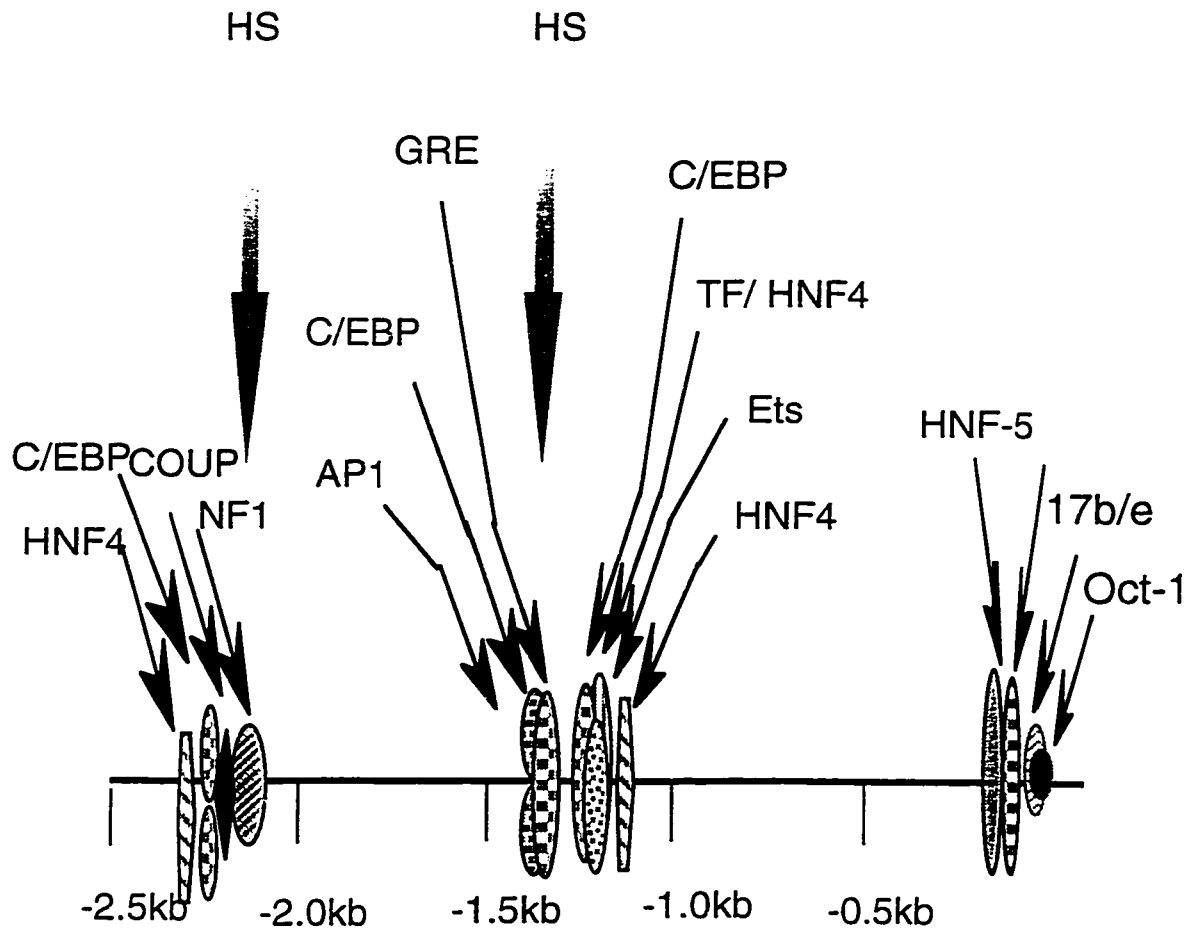
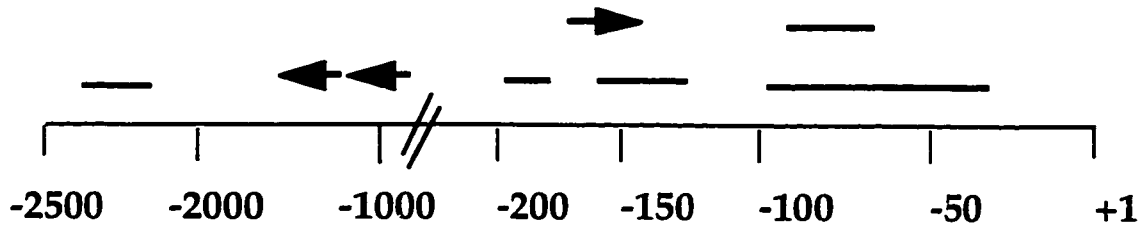


Figure 1. Transcription Factor Binding Sites in the 5'-Upstream Region of the CYP2B Genes.



Omiecinski: >800, HS sites at -1500, -2200

Phillips: -31 to -72, -183 to -199;
no binding at barbie box

Padmanaban: -69 to -98 (pos. element),
-127 to -160 (neg. element)

Fulco: 17bp essential barbie box -73 to -89

Anderson: -2155 to -2318; 6 fold functional increase w/ PB

Park: -2155 to -2318; 2.5 to 5 fold functional increase w/ PB

Negishi: -971 to -1401 in mouse(PBREM), >90% homology to
rat

May: -1100 to -5900 in chicken

➡ : minimal promotor

Figure 2. Suggested PB Response Elements.

CHAPTER 1: THE EARLY YEARS: IDENTIFICATION AND CHARACTERIZATION WORK

Introduction

This chapter will review the work that was conducted during my first years in the Omiecinski laboratory. My efforts were primarily directed towards accomplishing Specific Aims 1 and 2. The primary goal of Aim 1 was to establish a means by which to transfect rat primary hepatocytes while maintaining PB inducibility in the cells. This would allow us to identify regions in the 5'-upstream sequence of the CYP2B1/2 genes that were critical for the PB induction response. Aim 2 involved studying the cooperative interactions of liver nuclear proteins that bind in the 5'-upstream region of the CYP2B genes. I will first address my success with primary cell transfection techniques and then present the results from studies that examined DNA-protein binding interactions. Preliminary studies were also conducted to address Aim 3. These results are also presented in this chapter.

Identification of Regions Critical for the PB Induction Response

OPTIMIZATION STUDIES

A great deal of effort has been put forth in this area. When I first started this project, Dr. Sidhu had just completed optimization work on maintaining PB responsive primary hepatocytes in culture. Maintaining PB responsiveness in these cells required the addition of a substratum or extracellular matrix (Matrigel) overlay which interferes with lipid-mediated transfection of cells. My first project was to conduct many optimization studies to establish a protocol which could be consistently used with high success. Early studies

determined that PB inducibility could be maintained if the cells were transfected before the addition of Matrigel overlay.

The primary hepatocyte isolation procedure is as follows:

Rat hepatocytes are isolated by a modification of the two-step collagenase perfusion *in situ* (Seglen, 1976) and cultured with a modification of a protocol designed by Dr. Sidhu in our laboratory (Sidhu, et al., 1993). Briefly, cells are isolated from non-induced rat liver and plated, day 1, in Williams E medium supplemented with 2 mM L-glutamine, 10mM Hepes, pH 7.4, ITS+ (6.25 µg/ml transferrin, 6.25 ng/ml selenious acid, 1.25 mg/ml bovine serum albumin, 5.35 µg linoleic acid), Pen/Strep (100 units/ml penicillin and 1 mg/ml streptomycin), 100 nM dexamethasone, and 8% NuSerum. Cells are allowed to attach for 3 hours and are then washed with EBSS to remove any debris and unattached cells. Fresh serum-free, fully supplemented (complete) Williams E medium is added. For the subsequent culturing period the dexamethasone concentration is reduced to 25 nM. Fresh supplemented Williams E medium (25 nM dex.) is added on day 2 and transfections are then conducted.

The first optimization study conducted involved determining the best lipid carrier for transporting DNA into the cells. Earlier work in the laboratory had shown that electroporation and calcium-phosphate transfection techniques were toxic to the cells and were also ineffective delivery methods in primary hepatocytes. Multiple studies were then conducted using various lipid carriers including, but not limited to: Lipofectin, Lipofectamine, DOTMA, DOTAP, and DOSPER. Lipofectin was by far the most effective at getting DNA into the cells. Many carriers, including DOSPER and Lipofectamine, were unable to successfully transfect the primary hepatocytes. These studies were conducted using various promoters fused to a CAT (chloramphenicol acetyltransferase) reporter gene. Transfection success was determined by harvesting cells and examining them for CAT activity. Multiple promoters were tested, and it was determined that the strongest one, CMV, yielded the highest levels of reporter gene expression. Table 1 shows the CAT activity levels from cells that were transfected with lipofectin and DOTMA using different promoters and different transfection conditions. These lipids were clearly the best. Further studies involved using these lipids, under differing protocols. The highest transfection efficiencies were obtained when the DNA and lipid were mixed together in the presence of the hepatocytes; creating a lipid/DNA premix significantly reduced CAT expression.

The next step of the optimization was to determine the length of the transfection period. The hepatocytes need to be exposed to the lipid/DNA complex long enough for the complex to be taken up by the cells. If this period is too long, the lipid will have toxic effects and the PB induction potential of the cells will be compromised. Table 2 shows the results of the experiments which examined the transfection period. Even more studies were conducted to determine the best substratum for growing and transfecting hepatocytes, as well as the best media to use for the transfections. Once all these conditions were optimized, we next determined the optimal time for adding PB to the cells. It was determined that PB induction was optimal when the transfections were stopped after 4 hrs by the addition of complete Williams E medium plus Matrigel. For optimal induction, PB was added to the medium when the transfections were stopped and was then maintained in the medium until the cells were harvested.

I was able to develop a protocol to successfully transfect the primary rat hepatocytes, using viral promoters, without losing PB responsiveness. Optimization studies involved: testing of different lipid carriers, testing various transfection protocols, examining varying lipid:DNA ratios, checking for the optimal length of transfection to prevent toxicity while maximizing DNA uptake, testing of various media and supplements, as well as finding the best substratum and overlay concentration. The optimal protocol is described below:

For all transfections, Williams E medium is used, supplemented with 2 mM L-glutamine, 10mM Hepes, pH 7.4, ITS (6.25 μ g/ml transferrin, 6.25 ng/ml selenious acid, 5.35 μ g linoleic acid), and 25 nM dexamethasone. For cells treated with PB, all media are supplemented with 0.1 mM PB. On day 2, cells are washed with the transfection medium. 12.5 μ g of DNA in 3 ml of transfection medium are then added to the hepatocytes with 60 μ g Lipofectin (Gibco), in 3 ml of transfection medium. Cells are then incubated for 4 hours to allow DNA absorption, after which 3ml of transfection medium with ITS+ are added. An overlay of Matrigel (Collaborative Research), 120 μ g/ml final concentration, is then added. On day 3, the transfection medium is replaced with fresh fully supplemented Williams E medium. On day 4 cells are harvested.

LIPID MEDIATED TRANSFECTION RESULTS

During this time Richard Ramsden in the laboratory had designed and built 57 constructs containing various portions of the 5' flanking region of the CYP2B1 or CYP2B2 gene fused to a CAT reporter which were ready to use in transfection studies. Disappointingly, even though the viral promoters were expressing at high levels, after multiple transfections, with many fusion constructs, under many conditions, it was determined that when fused with any portion of the 5'-upstream region, CAT activity levels were too low to quantify. Various methods of detecting CAT levels were employed, including radiometric assays, ELISA techniques and differing solvent extractions, but the sensitivity of detection could not be substantially increased. Interestingly, at the same time, CAT fusion constructs used in transgenic mice studies yielded similar low levels thus making those study results also inconclusive (Ramsden, unpublished results).

I then created additional fusion constructs using the luciferase reporter (PGL2). The luciferase assay is believed to be more sensitive than CAT assays and should allow for over 100 fold greater detection sensitivity. This work, using 2 flasks for each construct, under each treatment (control or PB), indicated that a PB responsive element did not exist in the first 1400bp of 5' sequence. Unfortunately, when the 2500 bp 5' region was examined in transfection studies a PB response element was again not identifiable. Results were obtained by normalizing for protein levels as well as transfection efficiency.

I also worked on transfecting primary rat hepatocytes obtained from Marshall 520 rats using the complete CYP2B2 gene as its own reporter. These rats do not have a CYP2B2 gene, but do have the CYP2B1 gene, which is phenobarbital inducible. The large plasmid, containing over 20kb of the CYP2B2 gene plus either 0.8kb or 20kb of 5'-upstream sequence, was successfully transfected using the lipofection transfection technique. RNA was isolated using Trizol and then examined on slot blots as well as Northern blots. Although, the presence of plasmid was not detected on the Northern blots, it appeared, from further slot blot analysis, that some of the transfected plasmid DNA was contaminating the RNA samples (data not shown). This issue rendered the data uninterpretable. Unfortunately, the only means available to detect the CYP2B2 mRNA after transfection was with RT-PCR. By definition, efficient transfections should not require the use of such a highly sensitive and non-quantitative procedure. Therefore, with advice from my dissertation committee members, studies using this technique were not continued.

Owing to the lack of success in identifying a PB responsive unit, we decided that further optimization studies were necessary. Although strong reporters were expressed well, the CYP2B promoter, in its uninduced state, is relatively weak and it is possible that our system was still not sensitive enough to detect increases in its activity. Therefore, I then created a fusion construct of a thymidine kinase promoter (TK) and a β -galactosidase (β -gal) reporter. Cells could be transfected, fixed, and then stained to examine β -gal expression on a per cell basis. This method would allow for determination of transfection efficiency and thus we would know exactly what percentage of our cell population was being successfully transfected. Multiple optimization studies were again conducted and different lipid carriers were again tested. Lipofectin was again found to be the best carrier for our system and the conditions we had been using were optimal. Unfortunately, these optimal conditions yielded a transfection efficiency that was only 0.04%. A similar low level of transfection efficiency has been reported by other investigators using optimized methods in primary hepatocytes (Ourlin *et al.*, 1997). This level is quite low and would explain why we were unable to detect any reporter gene expression using the CYP2B promoter. It is also possible that the 0.04% of cells that are transfected with lipofectin do not represent cells that are PB responsive. Results obtained from this type of experiment may lead to erroneous conclusions as we may only be transfecting a small subset of cells. As lipid mediated transfection technologies did not seem adequate for the type of studies we wished to conduct, these studies were abandoned.

BACULOVIRUS MEDIATED TRANSFECTIONS

To increase transfection efficiencies in our hepatocyte cultures, we chose to explore the use of baculovirus (*Autographa californica* nuclear polyhedrosis virus) mediated transfections in our rat primary cultures. Baculovirus has historically been used as a vector for protein overexpression in insect cells (King and Possee, 1992; Luckow and Summers, 1988). The risk of infection to humans is very minimal as the baculovirus can only replicate in invertebrates and baculovirus expression in mammals and plants has never been observed (Luckow and Summers, 1988). However, previous studies have reported that baculovirus vectors can be used to shuttle foreign genes into mammalian cells (Boyce and Bucher, 1996; Hofmann *et al.*, 1995; Shoji *et al.*, 1997). Interestingly, the uptake of the baculovirus was reported as being specific for hepatic cell types and the transfection

efficiency in primary rat hepatocytes was shown to be over 70% (Boyce and Bucher, 1996). Highly successful gene delivery into hepatocytes and liver cell lines, presumably by an endosomal uptake pathway, made baculovirus mediated transfection very appealing for the study of CYP gene regulation (Boyce and Bucher, 1996; Hofmann, et al., 1995).

The procedure used to create the recombinant baculovirus is shown in Figure 3. In our hands, a very high level of transfection efficiency was seen with baculovirus mediated transfection as compared to lipofection or calcium phosphate mediated transfection methods. Since the luciferase expression is cytoplasmic, it was difficult to quantify the transfection efficiency on a per cell basis, but clearly we were transfecting over 50% of our cells when the viral titer was increased. Unfortunately, the presence of the baculovirus has multiple effects on our primary hepatocyte cultures, including antagonization of the PB mediated CYP2B induction response. The effects of baculovirus transfections on our primary hepatocytes are discussed in detail in chapter 4 of this dissertation.

Detection of Cooperative Interactions of Nuclear Proteins Binding to the CYP2B 5' Upstream Region

GEL SHIFT/SUPERSHIFT METHODOLOGY

These experiments focused on examining DNA-protein interactions in the 5'-upstream region of the CYP2B1/2 genes. To study these interactions electrophoretic mobility shift assays and supershift assays were performed.

Nuclear extracts from the livers of rats were prepared as described by Gorski et al. with slight modification (Gorski *et al.*, 1986). The first homogenization was conducted in a 0.5M sucrose buffer (Durrin *et al.*, 1984). For PB treated extracts, rats were dosed with 80 mg/kg PB, in saline, 18 hours before sacrifice. Control rats were treated with saline only.

Gel mobility shift and supershift assays were conducted essentially as described (Ausubel *et al.*, 1994). Nuclear protein extracts (5 μ g) were incubated with 1 μ g

Poly(dI•dC) and 1 μ g BSA in a volume of 10 μ l containing 4 mM Tris (pH 7.9), 12 mM Hepes (pH 7.9), 60 mM KCL, 1 mM EDTA, 1 mM DTT, and 4% glycerol for 30 minutes at 30°C. Ion levels were adjusted as necessary for specific reactions. 20,000 cpm of double stranded [γ^{32} P] ATP polynucleotide kinase labeled oligonucleotide was added and further incubated for 30 minutes at 30°C. For competition experiments 100-200 fold molar excesses of unlabeled competitor oligonucleotides were added. For supershift assays, 1 μ g of antibody was added and the binding reaction was incubated for 1 hour at 4°C. DNA-protein complexes were resolved by electrophoresis at 4°C through 5% polyacrylamide gels in 1x Tris glycine buffer. Gels were dried under vacuum and autoradiographed with intensifying screens at -70°C.

The above assays first focused on examination of the -1100 to -1400 region of the CYP2B1/2 genes. In addition to containing a chromatin site which is hypersensitive to the action of DNase 1, this region also contains consensus binding sites for proteins known to be involved in the regulation of liver specific genes. Extracts from PB and control liver nuclei were studied and compared. Cooperative interactions between proteins that bind near-by consensus sites were also examined.

By creating oligonucleotides which span larger portions of the 5' region (100-200bp), the electrophoretic mobility shift and supershift techniques can also be used to scan the 5' sequence and evaluate areas that exhibit differential binding patterns between control and PB extracts.

GEL SHIFT/SUPERSHIFT RESULTS

Despite not having identified a functional PB response element, significant progress was made in this area. I optimized a general gel shift and supershift protocol that allowed for clear detection of banding patterns. As a control, I was able to shift and supershift a C/EBP consensus binding site (Santa Cruz), using nuclear extracts from both control and PB treated rats. Using this site, distinct protein-DNA interactions and obvious supershift bands were seen when using the C/EBP β antibody (data not shown).

I have gel-shifted the 17bp Barbie Box region of the CYP2B2 gene. This gel is shown in Figure 4. Binding is apparent with extracts from both control and PB treatments and the interaction is stronger with the PB extracts.

Protein-DNA binding interactions occurring near the -1300bp DNase I hypersensitive site have been studied. This work focused on the C/EBP and ETS families. Synthetic double-stranded oligonucleotides were built and are shown in Figure 5. Results from these experiments are presented in Figures 6 through 9. Briefly, when using oligonucleotides representing -1369 to -1383 of the CYP2B2 gene, specific protein-DNA interactions were evident with both PB and control extracts. When an antibody directed against C/EBP α was used, a supershift was seen in the presence of PB and control extracts (Figure 6). Similar results were obtained with a -1221 to -1240 fragment (Figure 7). When using a -1195 to -1213 fragment and an antibody for the ETS family of transcription factors, no supershifts were evident although specific protein-DNA interactions were seen with both PB and control extracts (Figure 8). However, despite continued protein-DNA interactions, a fragment spanning the entire -1195 to -1240 region was unable to form a supershift complex with either extract in the presence of antibodies for C/EBP or ETS (Figure 9). In this gel, the blurring of sharp bands may be due to enhanced interactions with other regulatory proteins. Since supershifts with C/EBP α antibody were no longer visible, this factor appears to have been displaced by other nuclear proteins. These data demonstrate that the neighboring sequences may play an important role in determining the nature of protein-DNA binding interactions in this region of the CYP2B2 gene.

PROTEIN AFFINITY PURIFICATION METHODOLOGY

Although my previous research had focused on the DNase I hypersensitive site at -1100 bp, other work had identified a Phenobarbital Responsive Unit (PBRU) around -2200 bp. For this reason, my efforts changed direction and I began working on the affinity purification of proteins binding to the PBRU. The PBRU and known consensus binding sites for transcription factors in this region are shown in Figure 10. Specific attention was focused on the NF-1 site as previous studies conducted by Karen Sommer in our laboratory had shown a very strong binding interaction at this site. Consequently, Richard Ramsden created a PBRU with a mutated NF-1 site for transgenic mice studies. I used this mutated PBRU in affinity purification studies. The specific mutation that was introduced is shown in Figure 11.

Enrichment of nuclear extracts for proteins with specificity for the CYP2B2 PBRU was conducted using an affinity purification method as previously described (Gabielsen

and Huet, 1993). A flow chart diagramming the affinity purification protocol is shown in Figure 12. A Klenow fill-in reaction was performed on 400 μg of *Xba* I and *Pst* I restriction enzyme digested plasmid CYP2B2 -2271/-2186 pBluescript KS- in the presence of 5 nmoles biotin-16-dUTP (Boehringer Mannheim), followed by digestion with *Bsr* XI and *Sac* I. Coupling of the protein binding site to magnetic beads was achieved by incubating the resulting DNA with M280-Streptavidin Dynabeads™ (Dyna) at a ratio of 10 pmoles of insert DNA/mg Dynabeads™ as described (Gabrielsen and Huet, 1993). A CYP2B2 -2271/-2186 fragment containing a mutated NF-1 site was similarly labeled and complexed to magnetic beads. The mutated NF-1 site differed from the wild type at the two nucleotide positions as described in Figure 11. Affinity purification was performed on crude nuclear extracts prepared from whole livers of control or PB treated rats. In each experiment, 1 mg of nuclear extract was incubated at 4°C for 30 minutes with 10 mg of wild type or mutated CYP2B2 -2271/-2186 DNA complexed with 10 mg of Dynabeads™. Incubations were performed in TGED buffer containing 150 mM KCl, 50 mM sodium fluoride, and 0.2 mM sodium orthovanadate (150 mM KCl-TGED). Dynabeads™ were washed 4 times with 150 mM KCl-TGED containing poly dIdC (13 fold excess by weight). Proteins were eluted in 125 μl of 1.5 M KCl-TGED, dialyzed 1 hour at 4°C on 0.025 μm Millipore "V" Series Membranes against 50 mls of 40 mM KCl-TGED, and stored at -80°C.

Equal volumes of affinity purified proteins were separated via SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a Mini-Protean II 10% Tris-HCl precast gel (Bio-Rad) according to the manufacturer's protocol. Proteins were transferred onto Immobilon P membranes (Millipore) and then probed with specific antibodies. NF- κ B antibody, directed against the p50 subunit, was generously provided by Nancy Rice at the NCI Frederick Cancer Research and Development Center. HNF-4 antibody (provided by Dr. Frances Sladek, University of California, Riverside), NF-1 antibody (Santa Cruz Biotechnology) and Histone H3 antibody (Cortex Biochem) were all used at conditions specified by the suppliers. The secondary antibody used was goat-anti-rabbit-HRP (Pierce) or rabbit-anti-sheep-HRP (Zymed) at a 1:5000 dilution. ECL (Amersham) was used for chemiluminescent visualization of specific immunoreactive proteins.

AFFINITY PURIFICATION RESULTS

When 1 mg of proteins were affinity purified, the final amount of protein that adhered to the PBRU binding site and eluted by high salt was approximately 67 μ g. For SDS-PAGE analysis, 5 μ g of affinity purified proteins were run in each lane. As a control, 15 μ g of the total nuclear protein, before extraction, was also run. This procedure enabled us to monitor for any enrichment that had occurred during the affinity purification process.

Figure 13 shows a coomassie blue-stained acrylamide gel. As can be seen, the bead protocol is clearly enriching for certain proteins and other proteins are not adhering at all to the PBRU. We then used specific antibodies, based upon the known consensus sites, to determine which specific proteins were binding. As shown in Figures 14-16, proteins binding to the PBRU include; C/EBP β , ETS, NF-1, HNF-4, and Histone H3. When examining C/EBP β binding, it appears that only 2 of the 3 protein forms in the unpurified proteins bind to the PBRU (Figure 14). Similarly, as seen in Figure 15, with the ETS antibody, there is a clear enrichment of specific isoforms.

The interaction of NF-1 with the -2271/-2186 region of CYP2B2 was confirmed using a specific antibody directed against this protein (Figure 16). Introduction of 2 mutant bases into the NF-1 binding motif sequence completely ablated the NF-1 interaction. Gel shift experiments were also conducted using radiolabeled double-stranded wild type DNA fragments by Karen Sommer in the laboratory. NF-1 interactions that resulted were analogous to those described for the magnetic bead experiments. Gel shifts were noted with wild type but not mutated NF-1 oligomers. Furthermore, NF-1 mutated DNA was ineffective at competing with the wild type DNA in the gel shift experiments, whereas both wild type DNA and NF-1 consensus DNA sequences (Promega) were effective competitors (data not shown).

HNF-4 also interacted with the -2271/-2186 fragment, however this interaction was not modified by the NF-1 mutant residues. Histone H3 was detected in both wild type and mutated DNA-affinity extracts, whereas NF- κ B did not interact with either fragment. The immunoblots from these experiments can be seen in Figure 16. NF- κ B binding was not expected in our affinity purified protein sample as the PBRU does not contain a consensus binding site for NF- κ B. For all antibodies examined, the level of binding to the PBRU was similar in control and PB treated nuclear extracts. From these studies, we concluded

that an NF-1 family member interacts with this region of the PBRU and that the NF-1 mutations introduced were sufficient to completely ablate the interaction. Even with the NF-1 mutation, other proteins, such as HNF-4 and Histone H3, still appear to bind the PBRU. This work tells us that NF-1 is not acting as a core or tether for cooperative transcription factor binding in this region.

EXAMINATION OF PHOSPHORYLATION STATUS

While conducting these protein purification and immunoblotting studies, I also began to examine phosphorylation status of the proteins in hopes of answering questions posed in Aim 3. Anti-phosphotyrosine, phosphothreonine and phosphoserine antibodies were used to look for changes in phosphorylation status. Unfortunately, even with the affinity purification protocol, we still had multiple proteins present in the purified extracts. This made the determination of changes in phosphorylation very difficult. As many transcription factors are in the same size range, changes in expression could not be connected to a specific protein. Until we can isolate and overexpress specific proteins, further studies using these antibodies are not feasible. As the specific protein or proteins responsible for PB induction have not yet been identified, these studies are on hold.

Despite my inability to examine phosphorylation status, I have conducted studies which examine the signaling transduction pathways and the initial signals that affect protein phosphorylation. This work is presented and discussed in Chapter 3 of this dissertation.

Summary

As shown, considerable progress was made towards accomplishing Aims 1, 2 and 3. Although I was unable to use lipid mediated transfection techniques, I was successful in establishing the insect cell culture techniques and the baculovirus expression system in our laboratory. The results of studies conducted using baculovirus mediated transfection are presented and discussed, in detail, in chapter 4 of this dissertation.

Significant progress was also made towards achieving the second objective. Specific protein-DNA interactions were detected in all of the examined 5' flanking regions. Use of the C/EBP α antibody resulted in supershifts of specific protein-DNA complexes, whereas antibody directed against the ETS proteins was ineffective. As the ETS family contains over nine members, different antibodies may be needed to detect interactions occurring at consensus binding sites. Likewise, C/EBP supershifts that were seen did not account for all the observed specific binding, thus other unidentified proteins, possibly members of the C/EBP family, may be responsible for the interactions. Similarly, homodimer and heterodimer formation between C/EBP family members may account for the observed multiple banding patterns. The gel shift patterns that were seen appeared dependent upon the location of the specific binding site and the nature of the surrounding sequences. This is indicative of cooperative or interactive effects among proteins binding in the regulatory region of the CYP2B2 gene.

Although the use of isolated binding sites may allow detection of protein interactions, these results may be deceiving since the presence of nearby protein-DNA complexes may function to alter or even displace nuclear factors from neighboring sites *in vivo*. Thus, it is important to be careful of interpretations that are made when conducting or analyzing these types of experiments. Thorough examination of the cooperative interactions is necessary. By using the 100bp core fragment of the PBRU in affinity purification studies, I was able to further examine the role that cooperative interactions between proteins may play in governing PB responsiveness. The work with the mutated NF-1 site allowed us to rule out the role of NF-1 as the key or central binding protein in this area, as other proteins continued to bind in its absence. Significant work, in this region, including transgenic mice studies and *in vitro* footprinting has been conducted by Richard Ramsden in our laboratory. His studies as well as the work conducted by Karen Sommer and myself are tied together in manuscript format and shown in Appendix 1. At present this paper has been submitted to *Gene* for publication and has been accepted pending some final revisions which are currently in progress.

Table 1. Promoter and Carrier Optimization

PROMOTER	Lipofectin A	DOTMA A	Lipofectin B	DOTMA B
PrcCMVCAT	135,175	30,589	5,797	4,251
CMVCAT	95,943	8,107	3,659	4,469
RSVCAT	18,069	5,193	3,203	3,529
PBLCAT3	3,193	1,992	3,160	1,994

Values represent CAT activity levels. Protocol A added the DNA and Lipid separately to the hepatocytes. Protocol B involved premixing the DNA and Lipid before adding the mixture to the hepatocytes.

Table 2. Timepoint Optimization

<u>Length of Transfection</u>	<u>CAT Activity</u>
2 hrs	93,778
4 hrs	103,178
6 hrs	67,480
8 hrs	50,909
10 hrs	43,229

Hepatocytes were transfected for the time indicated. At the indicated hour, transfection was stopped by the addition of media with ITS+ and Matrigel. Cells were harvested 48 hrs later and CAT activity was analyzed. Values were normalized for protein content.

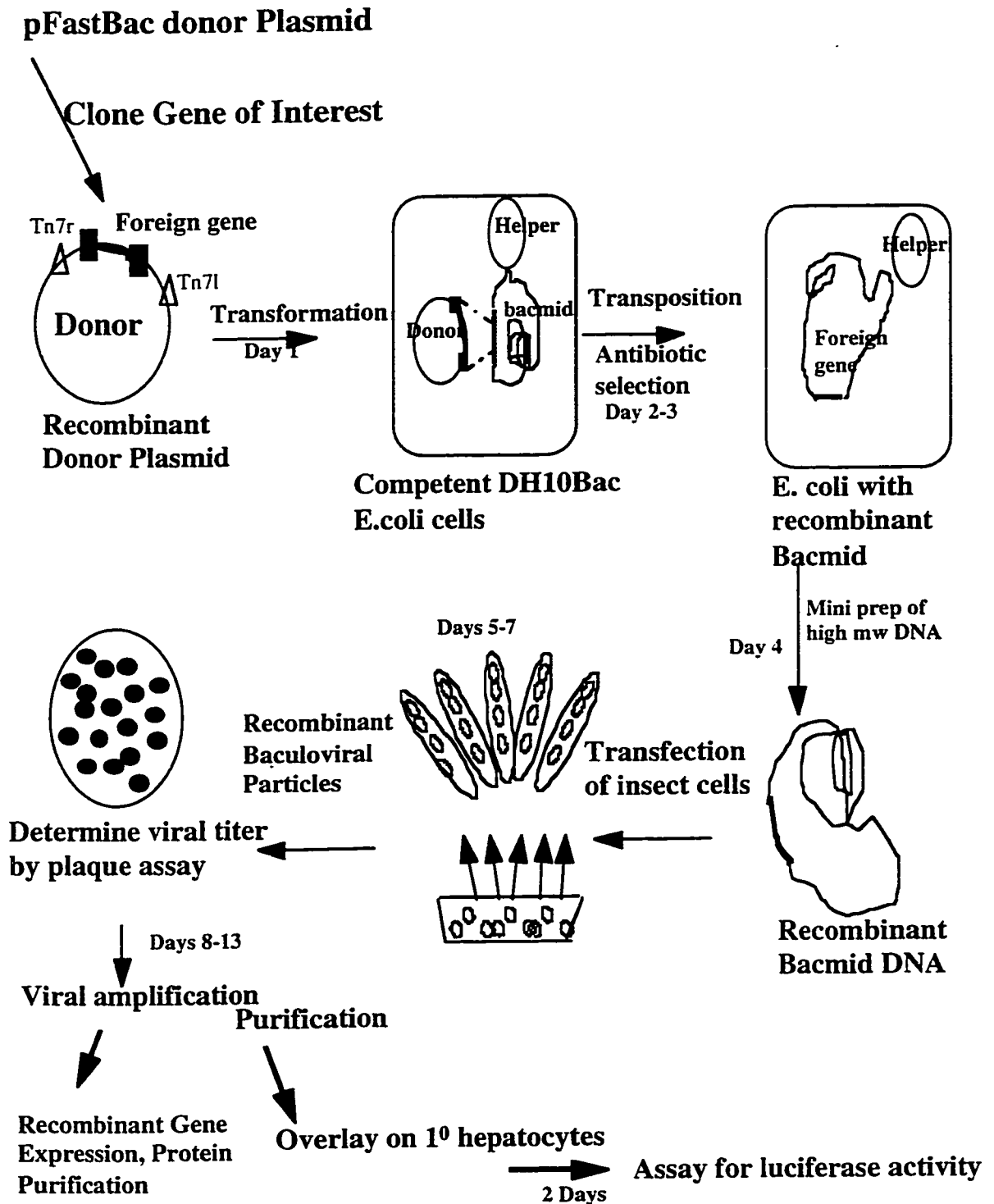


Figure 3. Creating Recombinant Baculovirus.

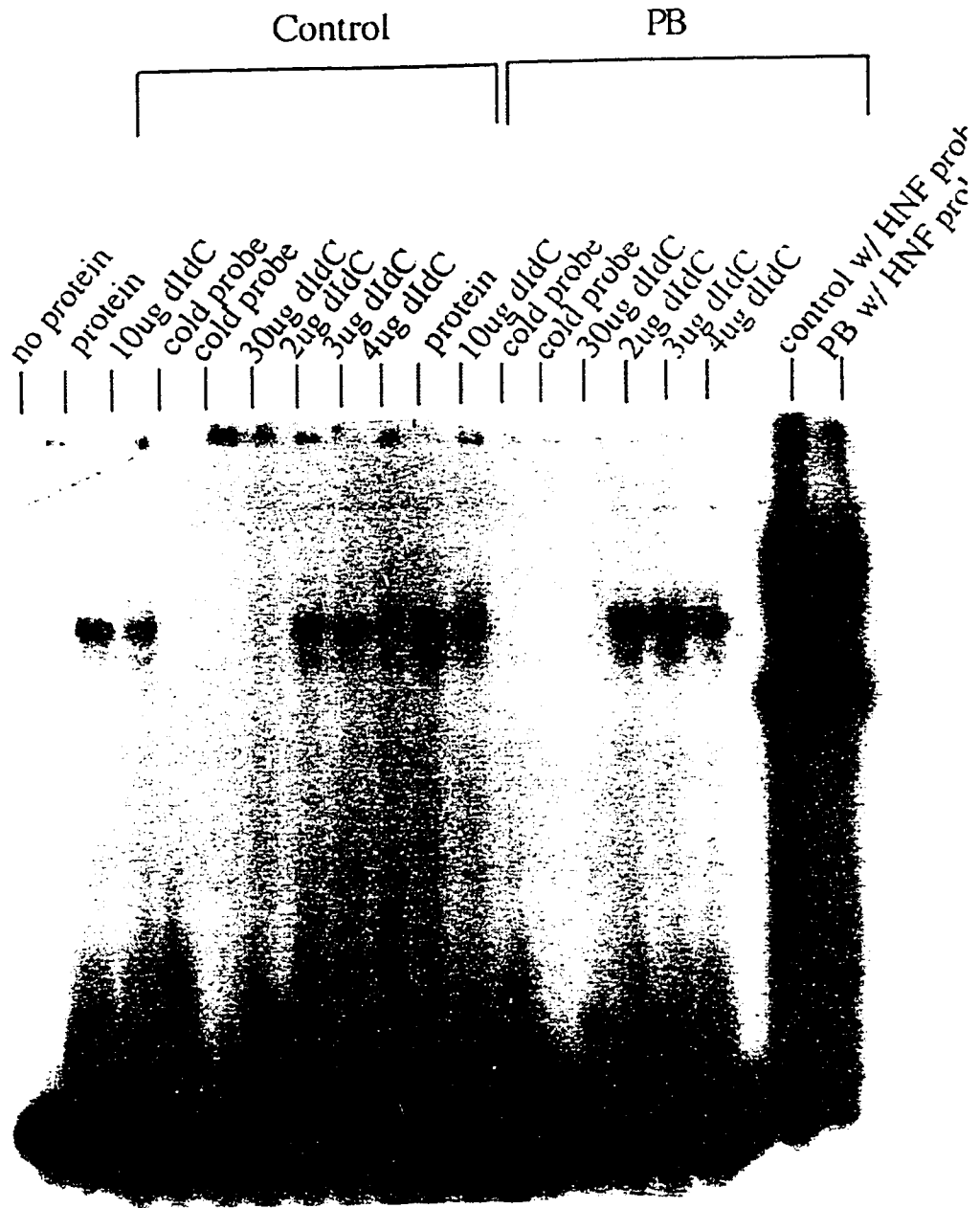


Figure 4. Gel Retardation Analysis of the 17bp Barbie Box Consensus Site in CYP2B2.

Radiolabeled probe was incubated with either Control (C) or PB nuclear extracts. Excess poly dI•dC was used as a non-specific competitor. HNF1 probes were used as a positive control

Combined Consensus Region:

-1240 -1195
 AATTCCTTTTAGCAAGATGGAAGGTCAAAGAACTTCCTGTGCTATG

C/EBP Consensus Site A:

-1240 -1221
 AATTCCTTTTAGCAAGATGGA

ETS Consensus Site:

-1213 -1195
 GAACTTCCTGTGCTATG

C/EBP Consensus Site B: -1383 -1369
 CCTGCCACCCCCCACCCAATAATAT

Figure 5. Mobility Shift Oligonucleotides Used in Gel Retardation and Supershift Studies.

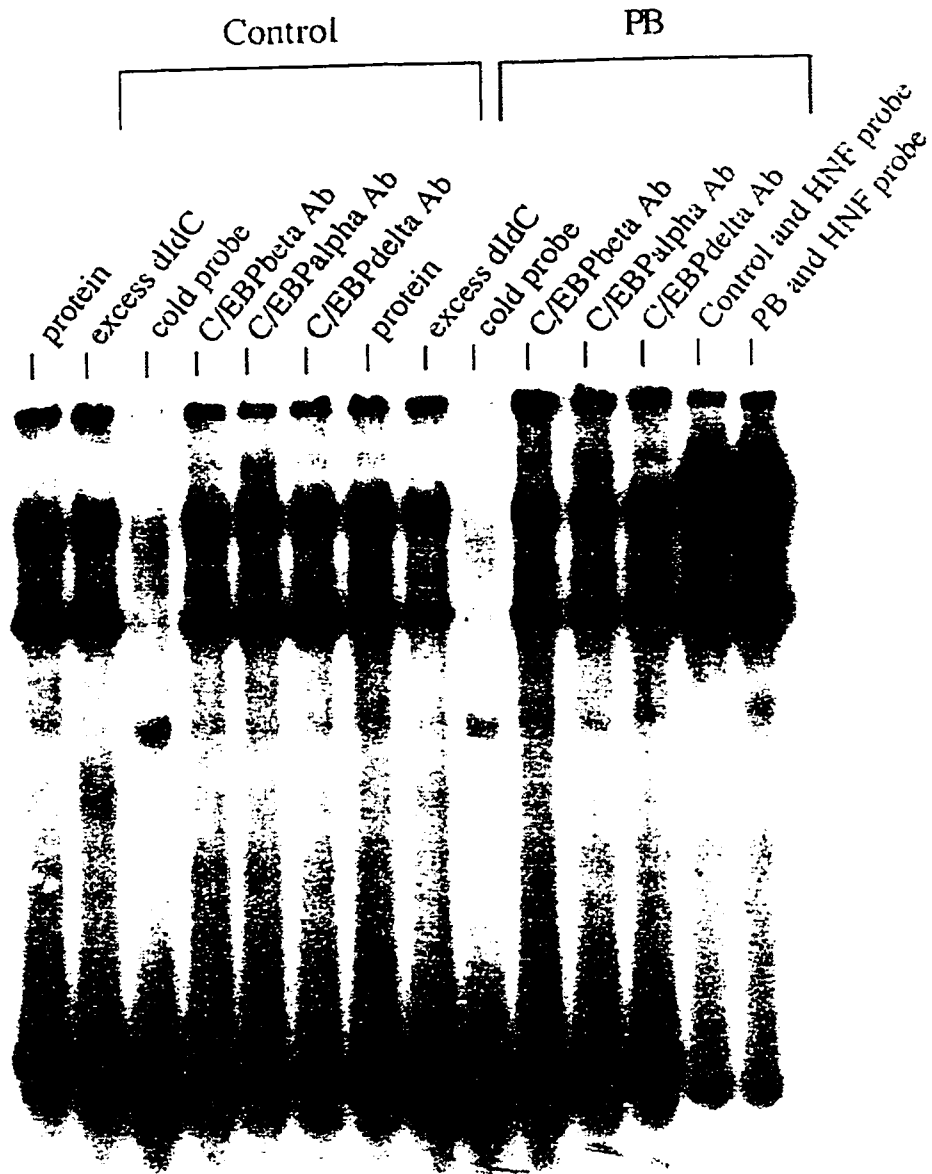


Figure 6. Gel Retardation and Supershift Analysis of the C/EBP Consensus Site B.

This site spans -1369 to -1383 of CYP2B2. Excess poly dI•dC was used in 10x molar excess as a non-specific competitor.

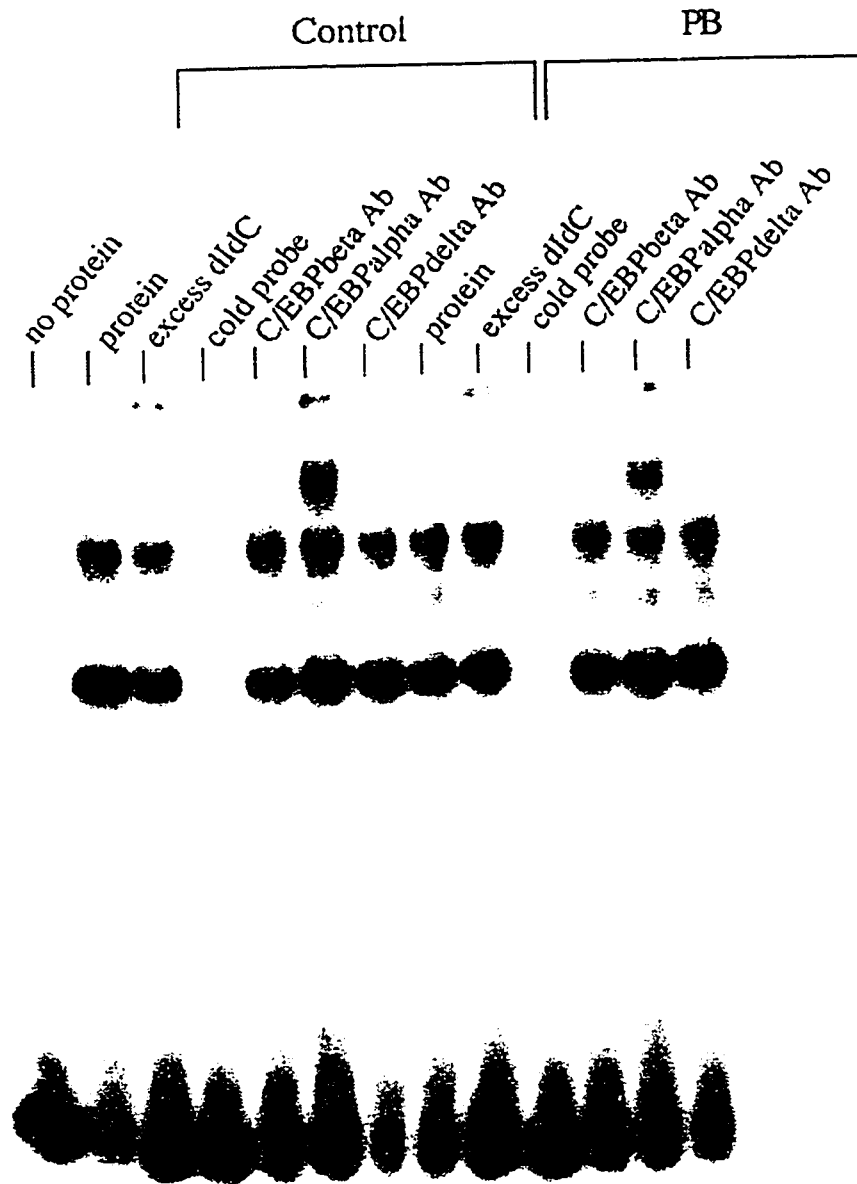


Figure 7. Gel Retardation and Supershift Analysis of the C/EBP Consensus Site A.

This site spans -1221 to -1240 of CYP2B2. Excess poly dI·dC was used in 10x molar excess as a non-specific competitor.

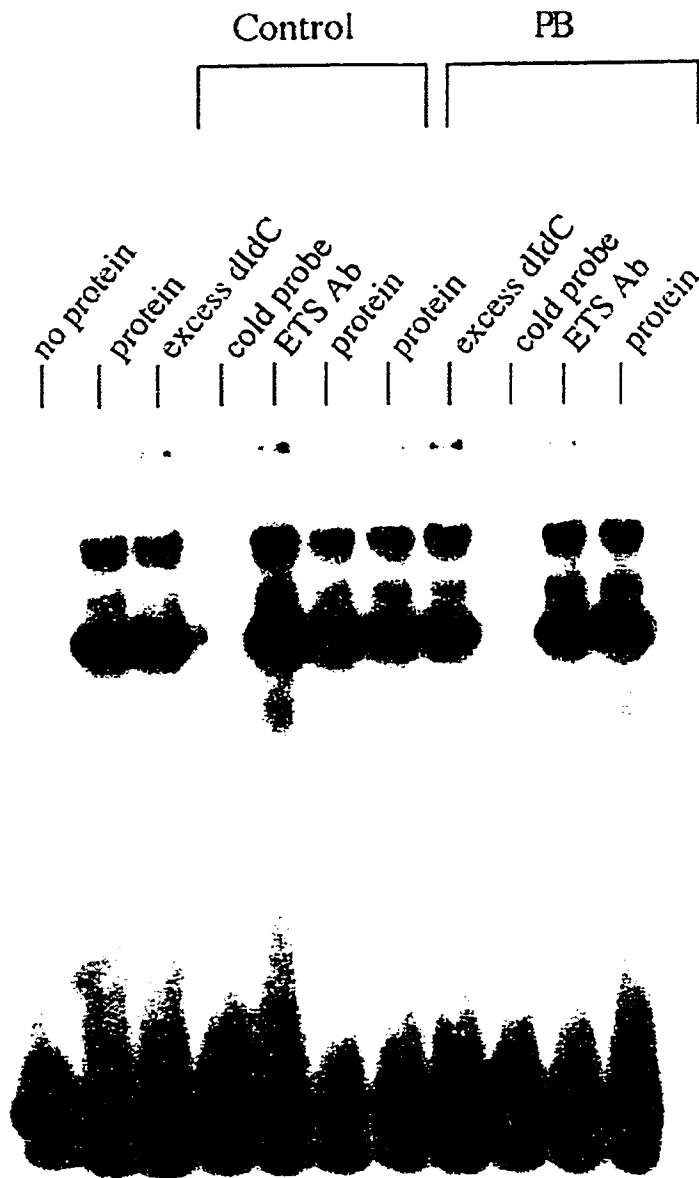


Figure 8. Gel Retardation and Supershift Analysis of the ETS Consensus Site.

This site spans -1195 to -1212 of CYP2B2. Excess poly dI•dC was used in 10x molar excess as a non-specific competitor.

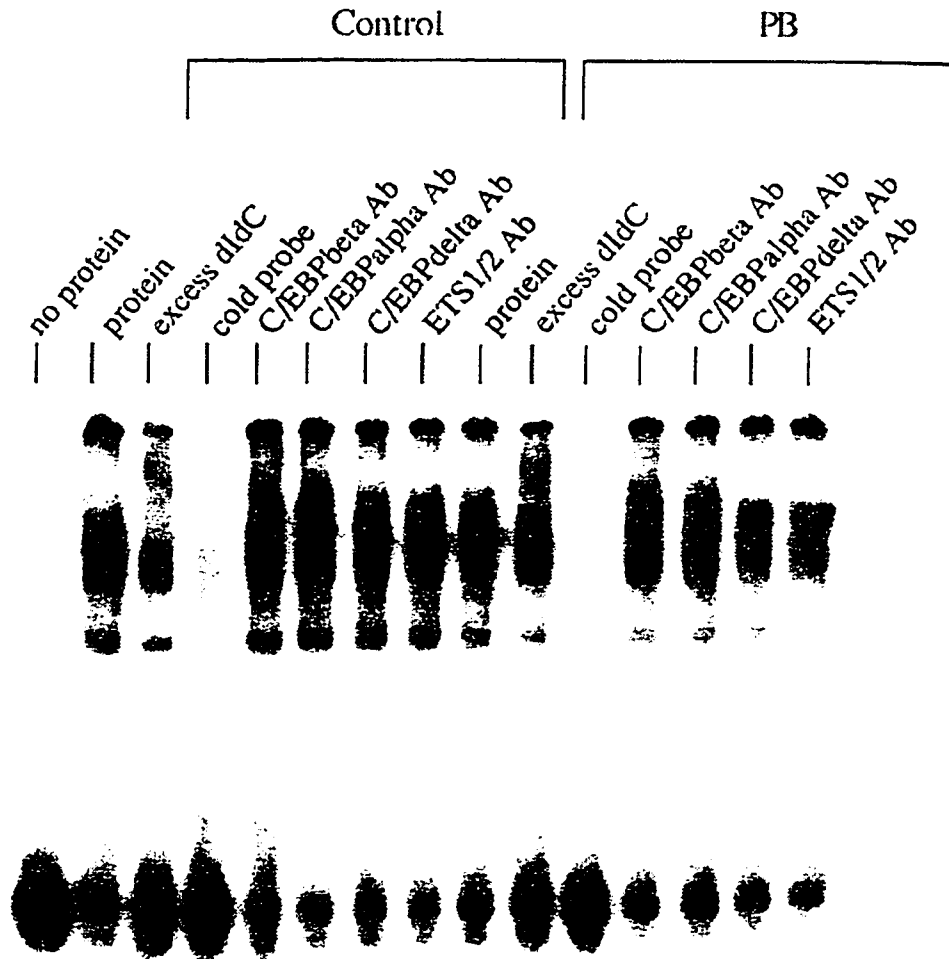


Figure 9. Gel Retardation and Supershift Analysis of the Combined Consensus Region.

This site spans -1195 to -1240 of CYP2B2. Excess poly dI•dC was used in 10x molar excess as a non-specific competitor.

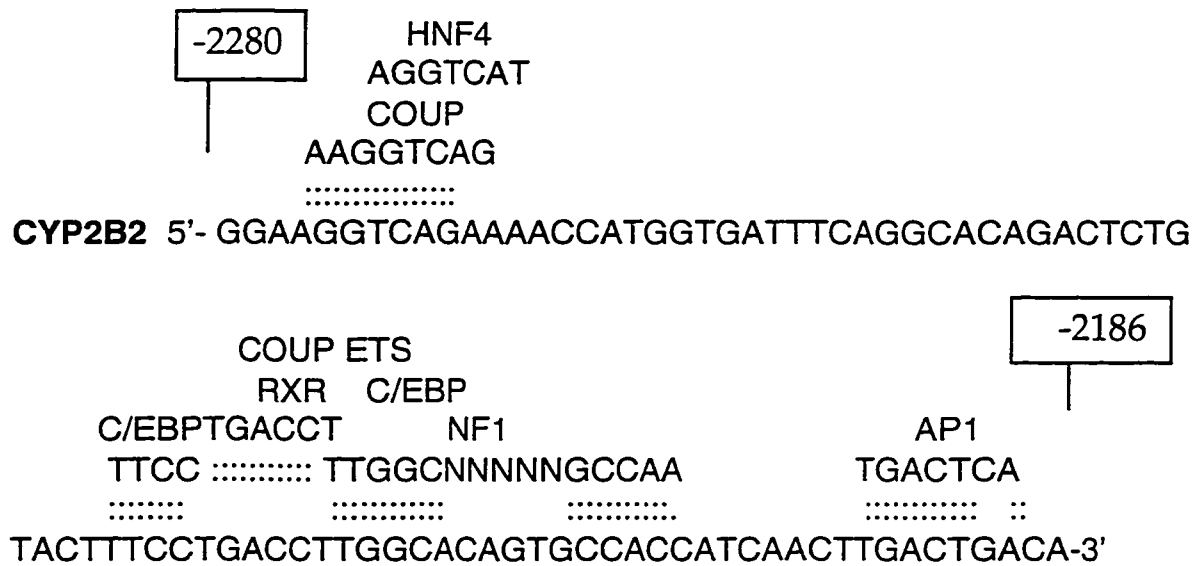


Figure 10. The PBRU Core.

This 100bp fragment was used in the bead affinity purification studies that were conducted.

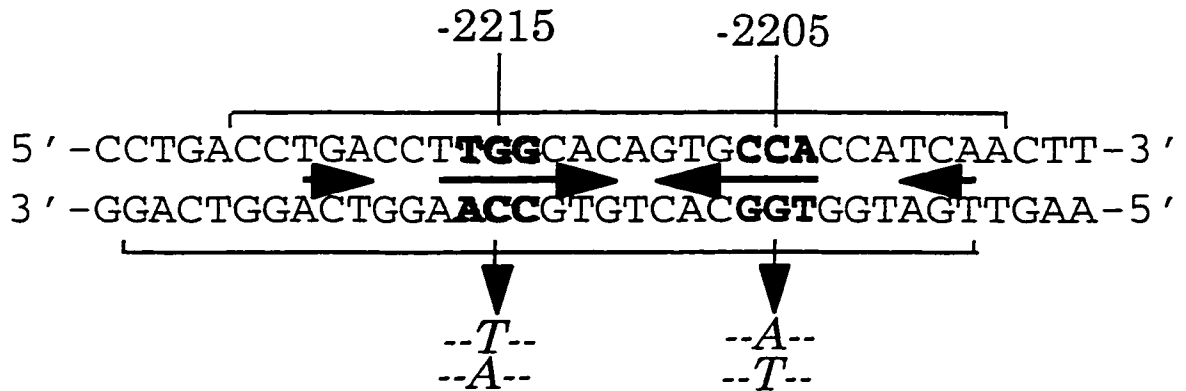


Figure 11. The NF-1 Mutation.

Protected area *in vitro* of the palindromic PBRU NF-1 site. Bracket, protection; bold type, consensus sequence; downward arrows, introduced site mutations.

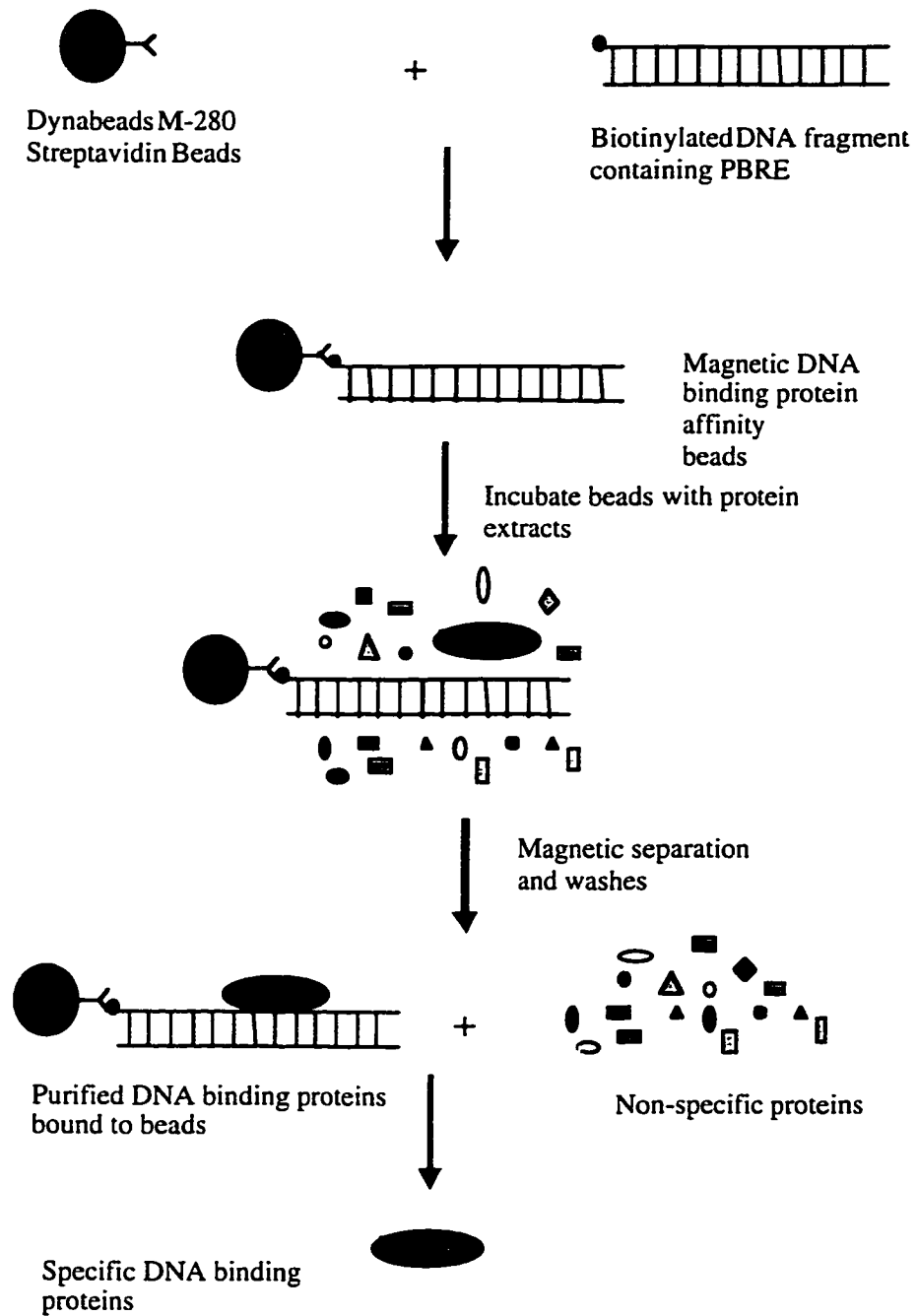


Figure 12. Protein Affinity Purification Protocol.

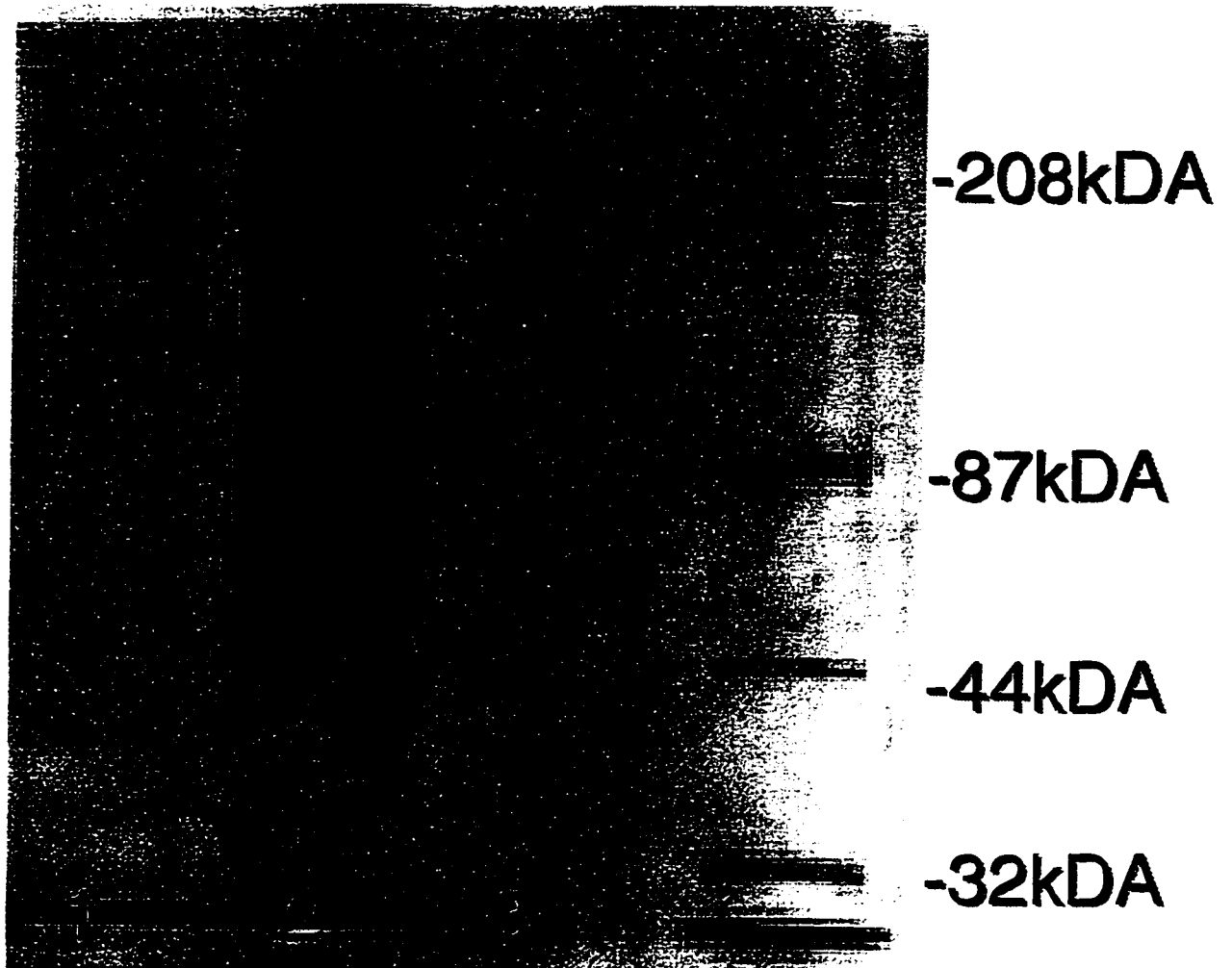


Figure 13. Coomassie Blue Staining.

This figure shows the staining of total protein from SDS-PAGE. lane 1, bead extracted (affinity purified) proteins; lane 2, 18 μ l of total unextracted proteins; lane 3, 1.8 μ l of total unextracted proteins. Molecular weight markers, in kilodaltons, are shown to the right of the immunoblot.

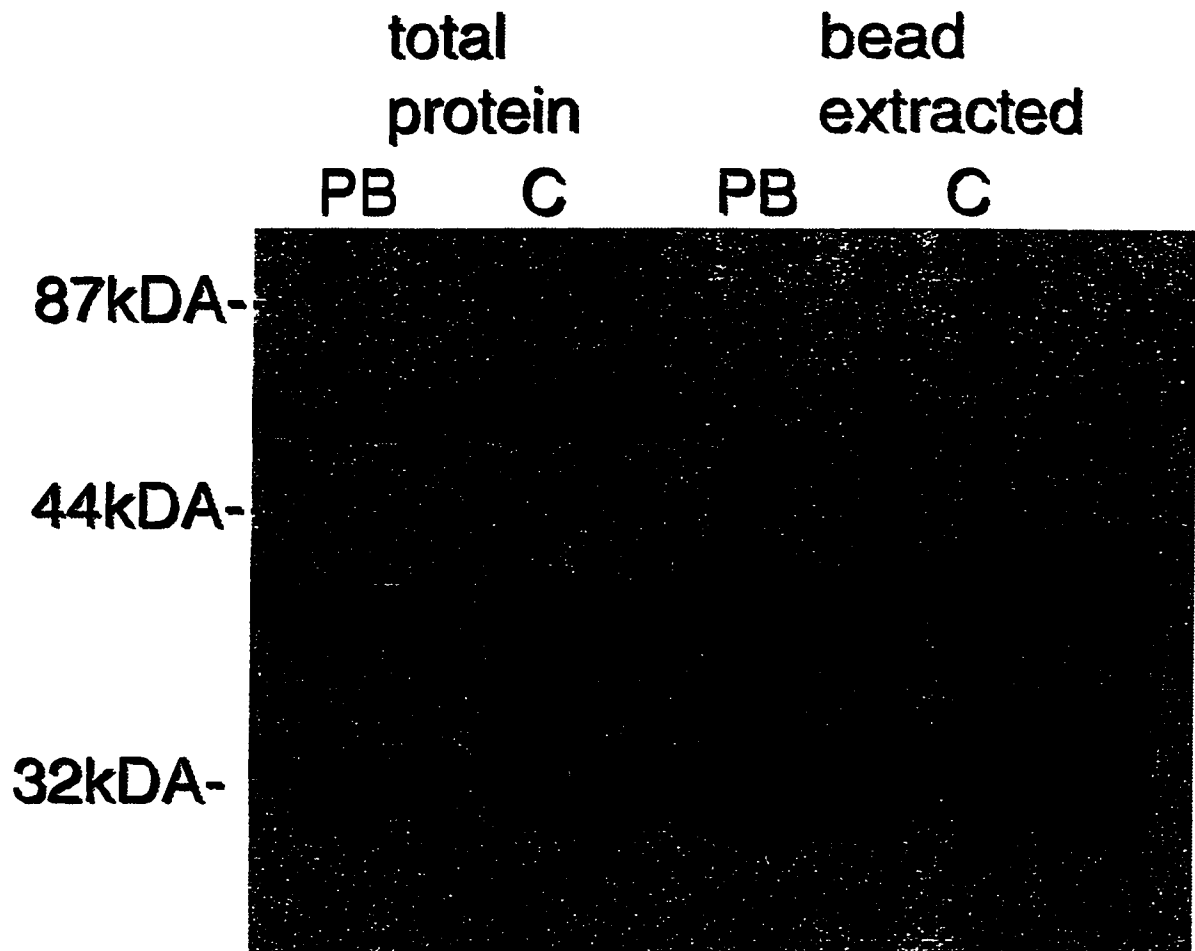


Figure 14. C/EBP Protein Expression.

This figure shows the expression of C/EBP β proteins in total protein and affinity purified (bead extracted) nuclear protein samples from Control and PB treated rat livers. Molecular weight markers, in kilodaltons, are shown to the left of the immunoblot.

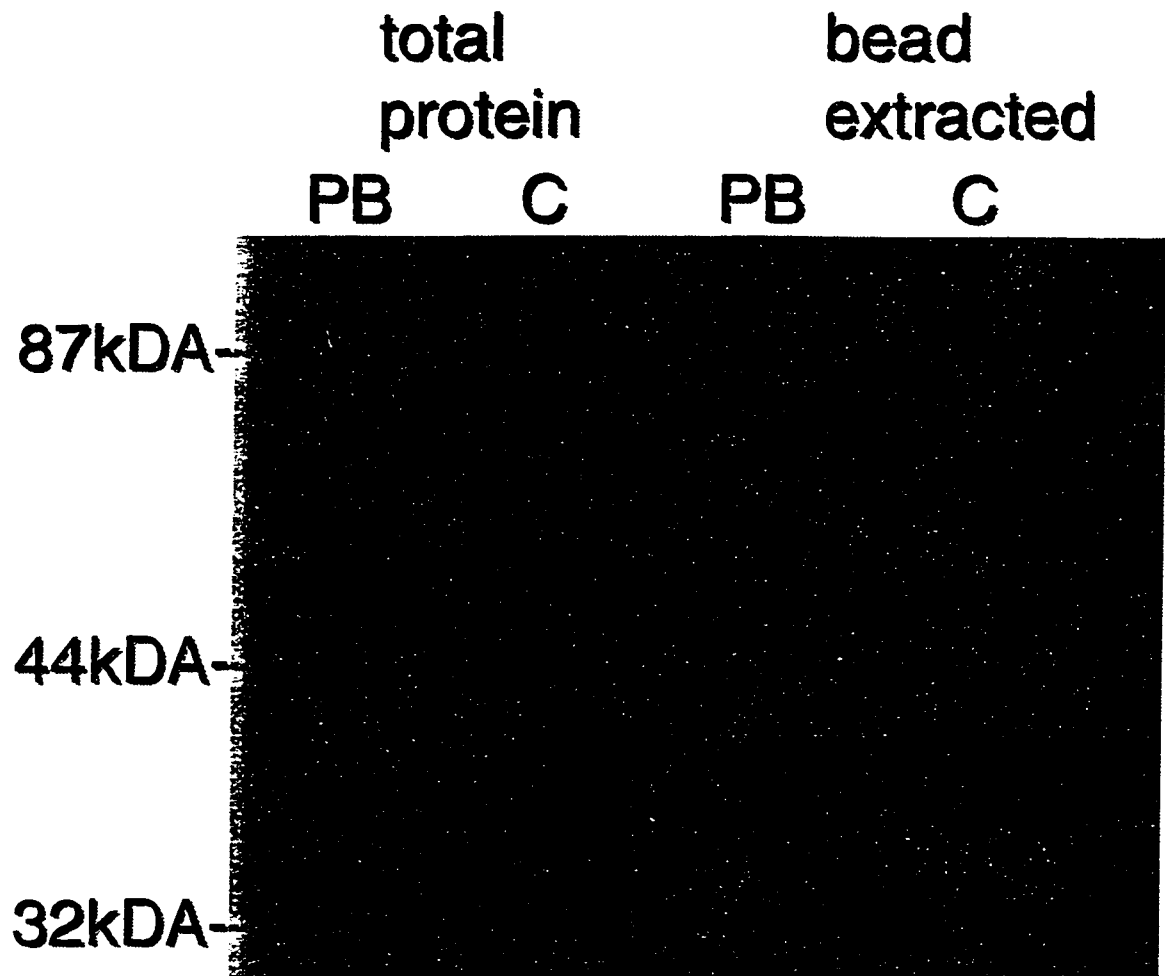


Figure 15. ETS Protein Expression.

This figure shows the expression of ETS proteins in total protein and affinity purified (bead extracted) nuclear protein samples from Control and PB treated rat livers. Molecular weight markers, in kilodaltons, are shown to the left of the immunoblot.

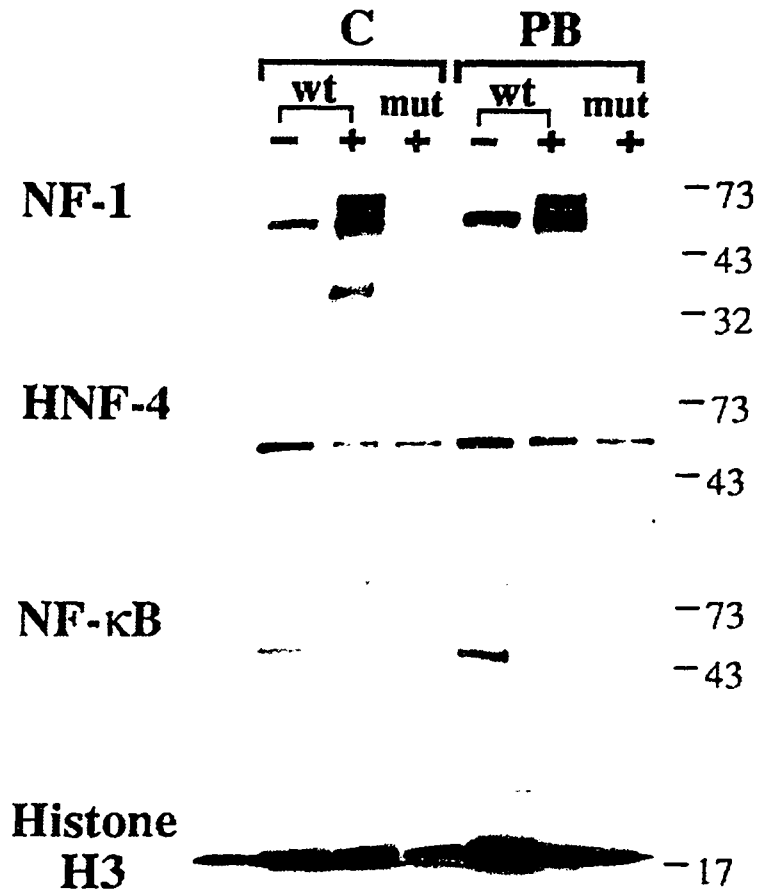


Figure 16. Immunoblot Analysis of Proteins Binding the PBRU.

Rat nuclear proteins from C or PB treated livers were affinity purified (+) against the wild type (wt) CYP2B2 -2271/-2186 DNA sequence or the identical fragment with specific mutations (Fig. 11) introduced to the NF-1 consensus binding site (mut). Proteins were eluted, separated by SDS-PAGE, and resulting blots were probed with specific antibodies directed against NF-1, HNF-4, NF-κB, and Histone H3. Liver nuclear proteins, C or PB, that were not subjected to affinity purification were also probed. Molecular weight markers, in kilodaltons, are shown to the right of respective immunoblots.

CHAPTER 2: PHENOBARBITAL EXPOSURES DO NOT ALTER cAMP LEVELS OR PROTEIN KINASE A ACTIVITY IN RAT PRIMARY HEPATOCYTES

Abstract

Results of previous studies have substantiated a negative modulatory role for cAMP and protein kinase A (PKA) dependent processes on the phenobarbital (PB) induction response in hepatocytes. The current study was conducted to further examine the potential role of second messenger pathways in the initial phases of induction, specifically addressing the effects of PB on the expression of intracellular cAMP levels and associated PKA activity. Using a highly differentiated primary rat hepatocyte system, cells were exposed to PB for various intervals (30 sec to 48 hrs) and levels of intracellular cAMP and subsequent PKA activity were determined. Although PB markedly induced CYP2B expression, exposure to this agent produced no detectable increases in cAMP levels and PKA activity at any of the times examined. These results demonstrate that the initial events stimulated by PB in rat hepatocytes do not include alterations of cAMP levels or associated PKA activities.

Introduction

CYP genes metabolize a broad range of endogenous and exogenous lipophilic compounds including drugs, steroids and polyaromatic hydrocarbons (Gonzalez *et al.*, 1993). Molecular mechanisms and the roles that signaling intermediates may play in determining PB induction of the CYP2B genes are not well understood, although it is well established that this process involves activation of *de novo* transcription (Hardwick, et al., 1983; Waxman and Azaroff, 1992).

The effects of PB on the liver are diverse and include cellular hypertrophy, tumor promotional effects in rodents, as well as gene induction (Peraino *et al.*, 1975; Ruch and Klaunig, 1986). In general, most liver processes are controlled by a variety of intracellular messengers and receptors that act as transducers for extracellular stimuli. Activation of protein kinases, through cyclic nucleotide stimulation, underscores many of the events regulating cellular function (Edelman *et al.*, 1987). Earlier studies (Byus, *et al.*, 1976; Canepa, *et al.*, 1985; Hutterer, *et al.*, 1975) examining the effects of PB on CYP gene induction and tumor promotion, suggested roles for the involvement of cAMP dependent pathways in these processes. However, measures of non-specific endpoints have made interpretation of these data difficult. Our laboratory has established a primary hepatocyte culture system that is highly permissive for differentiated hepatocyte function, CYP expression, and PB induction (Sidhu *et al.*, 1994; Sidhu, *et al.*, 1993; Sidhu and Omiecinski, 1995b). Using this defined system, dissection of signaling pathways is possible and a striking inhibition of PB mediated CYP gene induction by cAMP and PKA activators has been demonstrated (Sidhu and Omiecinski, 1995a). Other investigators have also begun to investigate the effects of signaling intermediates on PB induction (Dogra and May, 1996; Nirodi, *et al.*, 1996).

Our goal in the present study was to examine changes in second messenger systems that occur upon initial exposure to PB. Although recent reports (Brown *et al.*, 1995; Dogra and May, 1996; Honkakoski and Negishi, 1998a; Nirodi, *et al.*, 1996; Sidhu and Omiecinski, 1995a; Sidhu and Omiecinski, 1996) suggested a requirement for phosphorylation and dephosphorylation events in controlling PB mediated CYP expression, these studies induced cells in the presence of agents which modulate signal transduction processes. Alterations in signaling pathways immediately following cellular exposure to PB have not been investigated. In this study we examined cAMP levels and PKA activities in time course experiments subsequent to exposure of primary rat hepatocytes to PB and demonstrate that this signaling pathway is not activated in the early phases of the PB induction response.

Materials and Methods

MATERIALS

PB was obtained from the University of Washington Hospital Pharmacy Services (Seattle, WA). Dibutyryl cAMP (dBcAMP) and dexamethasone were purchased from Sigma (St. Louis, MO). Forskolin and Ro 20-1724 were purchased from the Alexis Corporation (San Diego, CA). All cell culture materials and Trizol™ were obtained from Life Technologies (Grand Island, NY). Matrigel, ITS+, and NuSerum were obtained from Collaborative Biomedical Products (Bedford, MA).

PRIMARY HEPATOCYTE ISOLATION AND CULTURE

Rat hepatocytes were isolated by a modification of the two-step collagenase perfusion *in situ* (Seglen, 1976) and cultured with modifications of the protocols as described previously (Sidhu, et al., 1993; Sidhu and Omiecinski, 1996). A dilute concentration (233 µg/ml final concentration) of ECM (Matrigel) was added as an overlay 16 hrs after initial plating of the cells. Medium changes were conducted thereafter on a daily basis and a 25 nM dexamethasone concentration was maintained in all media.

TREATMENTS

PB (1.0 M) and dBcAMP (100 mM) were dissolved in tissue culture grade water as stock solutions and stored in aliquots at -20°C. Forskolin (100 mM) and Ro 20-1724 (500 mM) were dissolved in DMSO as stock solutions and also stored at -20°C. Cells were cultured for 48 hr before the addition of any treatments. Final concentrations of each treatment were: PB, 500 µM; dBcAMP, 25 µM; Forskolin, 100 µM; and Ro 20-1724, 200 µM; unless otherwise stated. For all analyses, representative data are shown from multiple studies performed independently with different hepatocyte preparations.

CAMP ANALYSIS

Intracellular cAMP was measured using the Biotrak™ cAMP enzyme immunoassay system from Amersham (Arlington Heights, IL). Cells were stimulated for the indicated times and cell extracts were prepared. Briefly, cells were washed twice with cold PBS and then lysed and scraped in 800 µl of ice cold 70% ethanol. Cell debris was pelleted at 2000 x g and the resulting supernatant was subsequently lyophilized and stored at -20°C. For analysis, resulting pellets were resuspended in 500 µl of the required assay buffer. Intracellular cAMP analysis was performed in duplicate for each sample evaluated. Protein concentrations were determined using bovine serum albumin as a standard with the BCA protein assay reagent (Pierce, Rockford, IL).

MEASUREMENT OF PKA ACTIVITY

Protein Kinase A activity was assessed by measuring the transfer of ³²P-labeled phosphate to a biotinylated Kemptide (LRRASLG) substrate using Promega's Signa-TECT™ PKA assay system (Madison, WI). At specified timepoints after treatments, cells were washed twice and then scraped off the plates in cold PBS. Cells were pelleted at 500 x g at 4°C and resuspended in 100 µl of ice cold buffer (25 mM Tris pH 7.5, 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM sodium orthovanadate, and 10 mM MgCl₂). The resulting cell suspension was lysed by brief (7 sec.) sonication on ice, and the cell debris was pelleted at 15,000 x g at 4°C. Five µl of supernatant was assayed for 5 min at 30°C with: 5 µl PKA assay buffer (Promega), 0.1 mM ATP, 5 µl γ-[³²P]ATP (6,000 Ci/mmol), and 0.1 mM PKA biotinylated Kemptide substrate, with or without activation by 5 µM cAMP. Mixtures without exogenous cAMP measured endogenously activated PKA, while mixtures with added cAMP measured the total available PKA in the cells. Reaction termination, spotting onto membranes, and washing were carried out as directed by the manufacturers' protocol. PKA activity, in the absence of exogenously added cAMP, was determined in duplicate for each sample evaluated. Protein concentrations were determined using bovine serum albumin as a standard with the Bio-Rad Protein assay (Hercules, CA).

RNA ANALYSIS

Total RNA was isolated (Chomczynski and Sacchi, 1987) using Trizol as described by the supplier (Life Technologies). For slot blot evaluation, 5 μg of total RNA was applied directly to a Genescreen Plus nylon membrane (DuPont) under vacuum and denaturing conditions using a Minifold II apparatus (Schleicher and Schuell, Keene, NH). The membranes were hybridized with specific ^{32}P -radiolabeled oligonucleotides for CYP2B1, CYP2B2, and 18s rRNA, as described previously (Omiecinski *et al.*, 1990; Sidhu and Omiecinski, 1996).

Results

In the experiments conducted here, we examined the effects of PB on intracellular cAMP levels and subsequent PKA activity in primary rat hepatocytes. In Figure 17, data are presented demonstrating that treatment of primary rat hepatocytes with either forskolin or dBcAMP results in up to 50 fold stimulation of intracellular cAMP levels relative to control (untreated) cells. For dBcAMP this increase is sustained over a 24 hr period, and maintains the elevated level even at 48 hrs. The increase in cAMP produced by forskolin is more transient and begins to approach basal levels within 24 hrs. These results appear consistent with the relative resistance to phosphodiesterase degradation offered by the dibutyryl moiety of dBcAMP. Unlike the cAMP analog or the known adenylase cyclase activator, forskolin, PB exposures (500 μM) did not result in any detectable transient or sustained increase in intracellular cAMP levels. As shown in figure 18, the inductive effects of PB upon CYP2B1 expression are easily detected within an 8 hr period, yet PB treated cells harvested as early as 30 seconds and as late as 48 hrs did not show any corresponding increases, or decreases, in intracellular cAMP levels as compared to control cells.

We used a synthetic Kemptide substrate to assess increases in PKA activity following treatment with either dBcAMP, forskolin, or PB. As shown in figure 19, dBcAMP and forskolin treatments resulted in marked increases in PKA activity that are clearly detectable

within 30 seconds of treatment. The increases produced by forskolin were more transitory than those elicited by the cAMP analog, returning to near basal levels within 30 minutes. In contrast, treatment with PB resulted in no detectable changes in PKA activity levels. The data provided in Table 3 further demonstrate the lack of PB effect upon the total cellular PKA activity or the fraction of PKA activated by endogenous levels of cAMP.

Discussion

In this report we have investigated the effects of PB on intracellular cAMP levels and PKA activity in a highly defined primary hepatocyte system. The results presented here clearly demonstrate that the addition of PB to primary hepatocytes does not result in any detectable alterations in either cAMP level or subsequent PKA activity.

Results from early *in vivo* experiments and studies with hepatocytes in suspension, previously indicated that PB may activate a cAMP dependent protein kinase that in turn might increase the levels of CYP gene expression (Byus, et al., 1976; Canepa, et al., 1985; Hutterer, et al., 1975). However, these studies are now dated by their use of imprecise endpoints and the utilization of super-physiologic doses of cAMP. Of interest from the tumor promotion standpoint, PB was reported to inhibit the phorbol ester induced translocation of protein kinase C (PKC) to the plasma membrane in hepatocytes from rats that had ingested PB in their water (Brockenbrough, et al., 1991). In addition, it was suggested that PB inhibits PKC by occupying the diacylglycerol binding site (Chauhan and Brockerhoff, 1987).

Work from our laboratory has yielded a different spectrum of conclusions. In primary rat hepatocyte cultures, cAMP analogs and adenylase cyclase activators, such as forskolin, dramatically inhibited the PB induction response of CYP2B genes (Sidhu and Omiecinski, 1995a). Evidence was provided that the second messenger pathway was PKA, rather than PKG or PKC (Sidhu and Omiecinski, 1995a). Additionally, it was determined that okadaic acid markedly potentiated the repressive effects of cAMP on the PB induction process (Sidhu and Omiecinski, 1997). Recently, similar repression also was reported in mouse hepatocytes by other investigators examining PB-inducible expression of the CYP2B10 gene (Honkakoski and Negishi, 1998a).

Thus, current data are strongly supportive of the involvement of cAMP and PKA in modulating PB responsiveness. Investigators have attempted to examine the roles that specific signaling pathways may be playing in CYP gene regulation by either blocking or enhancing specific signal transduction pathways (Brown, et al., 1997; Dogra and May, 1996; Nirodi, et al., 1996; Sidhu and Omiecinski, 1995a; Sidhu and Omiecinski, 1997). The current study is unique in that it addresses the potential role of signaling intermediates, specifically cAMP and PKA, upon exposure of hepatocytes to PB. Despite results of previous studies showing that activators of cAMP pathways, or inhibitors of protein phosphatases, modulate the PB induction response (Brown, et al., 1997; Nirodi, et al., 1996; Sidhu and Omiecinski, 1995a; Sidhu and Omiecinski, 1997), we were surprised to find that PB itself did not alter cAMP levels and subsequent PKA activity. Thus, the issues of how PB is mediating its effects and what intracellular signaling pathways are involved remains unclear. However, despite their involvement in modulating the induction process, the results shown here demonstrate that the initial events stimulated by PB in rat hepatocytes do not include alterations of cAMP levels or associated PKA activities.

Table 3. Activity of cAMP Dependent Protein Kinase in Treated Rat Hepatocytes

Time	Treatment	<u>-cAMP^a</u> (pmol ATP/min)		<u>+cAMP^a</u> (pmol ATP/min)		% of Total Activated ^b
30 seconds	Control	0.708 ± 0.078		9.710 ± 1.018		7.294 ± 0.448
	PB	0.660 ± 0.169		10.765 ± 1.795		6.446 ± 2.600
	Forskolin	1.843 ± 0.163		7.129 ± 2.054		27.129 ± 6.144
	dBcAMP	3.461 ± 0.298		11.245 ± 3.007		31.979 ± 6.004
5 minutes	Control	0.901 ± 0.227		12.592 ± 1.632		7.084 ± 0.993
	PB	0.721 ± 0.155		12.574 ± 0.212		5.718 ± 1.142
	Forskolin	2.482 ± 0.376		8.508 ± 2.121		29.761 ± 3.275
	dBcAMP	5.189 ± 1.669		12.672 ± 3.477		40.528 ± 2.255
15 minutes	Control	0.851 ± 0.054		14.477 ± 1.556		5.906 ± 0.366
	PB	0.636 ± 0.159		15.775 ± 2.582		3.988 ± 0.363
	Forskolin	2.126 ± 0.494		7.090 ± 1.256		29.778 ± 2.063
	dBcAMP	4.564 ± 1.762		12.770 ± 5.349		36.190 ± 1.756
1 hour	Control	0.349 ± 0.078		13.486 ± 2.084		2.700 ± 0.990
	PB	0.603 ± 0.100		17.179 ± 6.398		4.079 ± 2.065
	Forskolin	0.973 ± 0.085		7.843 ± 2.291		13.423 ± 4.766
	dBcAMP	4.126 ± 1.792		11.066 ± 2.958		36.041 ± 6.784
8 hours	Control	0.452 ± 0.042		13.610 ± 2.145		3.390 ± 0.674
	PB	0.523 ± 0.089		15.852 ± 3.440		3.512 ± 1.305
	Forskolin	0.439 ± 0.198		6.573 ± 1.604		7.513 ± 4.846
	dBcAMP	2.798 ± 1.497		7.431 ± 1.503		35.71 ± 13.00
24 hours	Control	0.412 ± 0.049		14.510 ± 1.334		2.847 ± 0.355
	PB	0.456 ± 0.113		15.328 ± 0.828		3.012 ± 0.888
	Forskolin	0.235 ± 0.173		9.338 ± 0.226		2.487 ± 1.795
	dBcAMP	3.116 ± 0.836		8.221 ± 0.233		38.14 ± 11.24

^a Values are mean ± standard deviation from at least 2 individual hepatocyte preparations. Kinase activities are expressed as enzyme-specific activity, in pmol ATP incorporated into substrate/min.

^b The % of total activated was determined by dividing the endogenous cAMP levels (-cAMP) by exogenously activated samples (+cAMP).

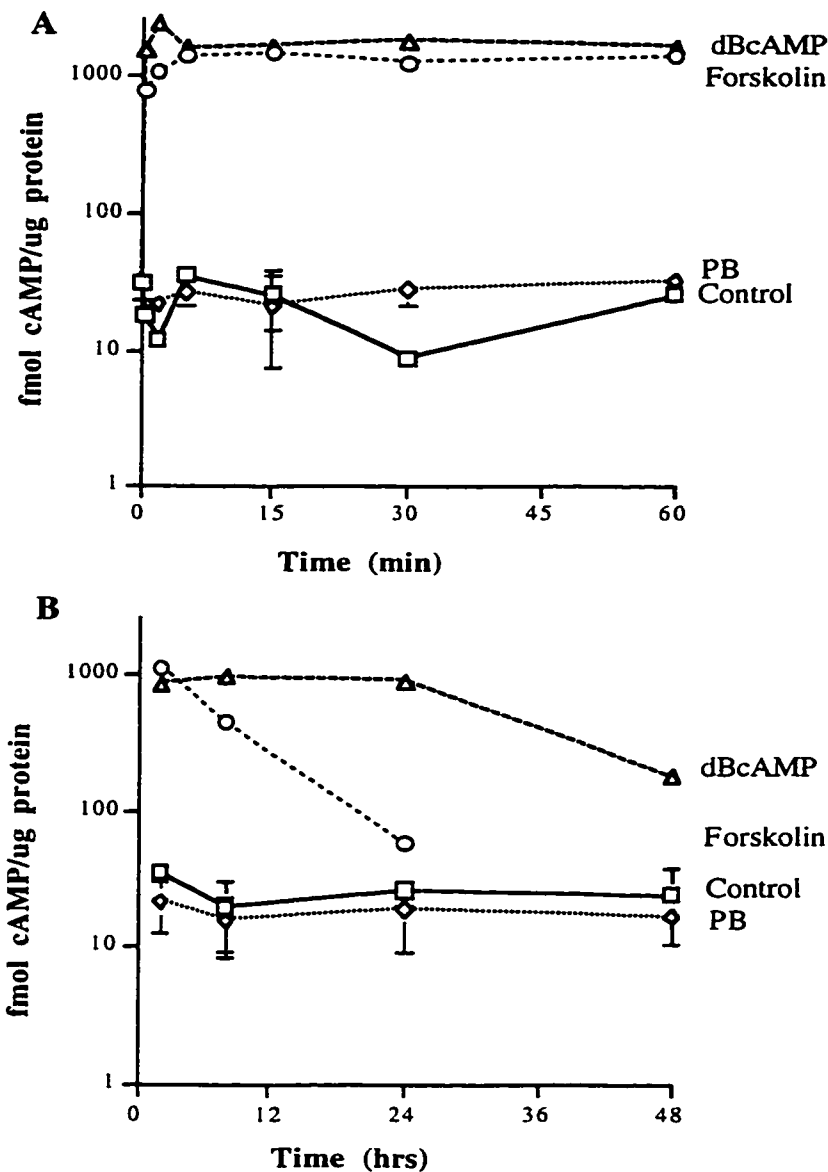


Figure 17. The Effects of dBcAMP, Forskolin, and PB on Intracellular cAMP Levels.

Panel A represents the effects on intracellular cAMP after brief treatment intervals, 30 sec. - 1 hr. *Panel B* represents the effects of longer treatment periods, 2-48 hrs. As the dBcAMP and forskolin treatments functioned as positive controls in these experiments, the phosphodiesterase inhibitor Ro-20-1724 (200 μ M) was added to these treatments. Values represent the average \pm standard deviation from at least 3 independent hepatocyte preparations.

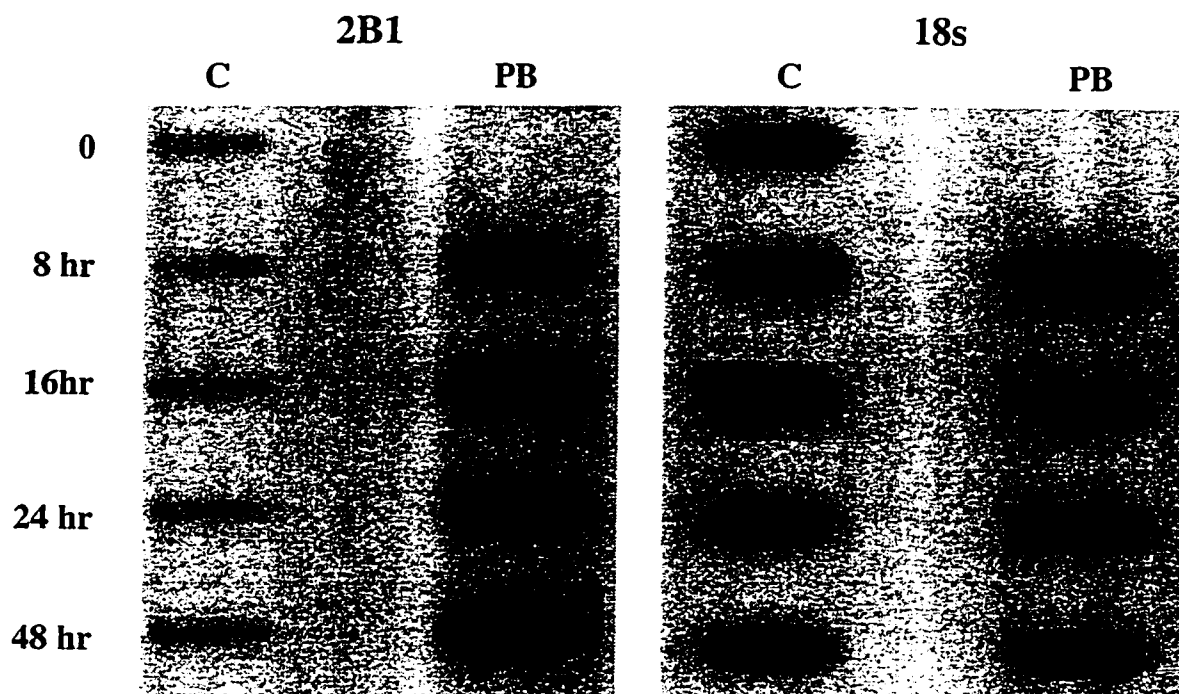


Figure 18. The Effects of PB upon the Induction of CYP2B1 in Primary Rat Hepatocytes.

Cultures were seeded for 48 hrs before the addition of PB (500 μ M). Total RNA was isolated and evaluated as stated under "methods and materials." Ribosomal 18s RNA hybridization levels were used to demonstrate equal loading between samples.

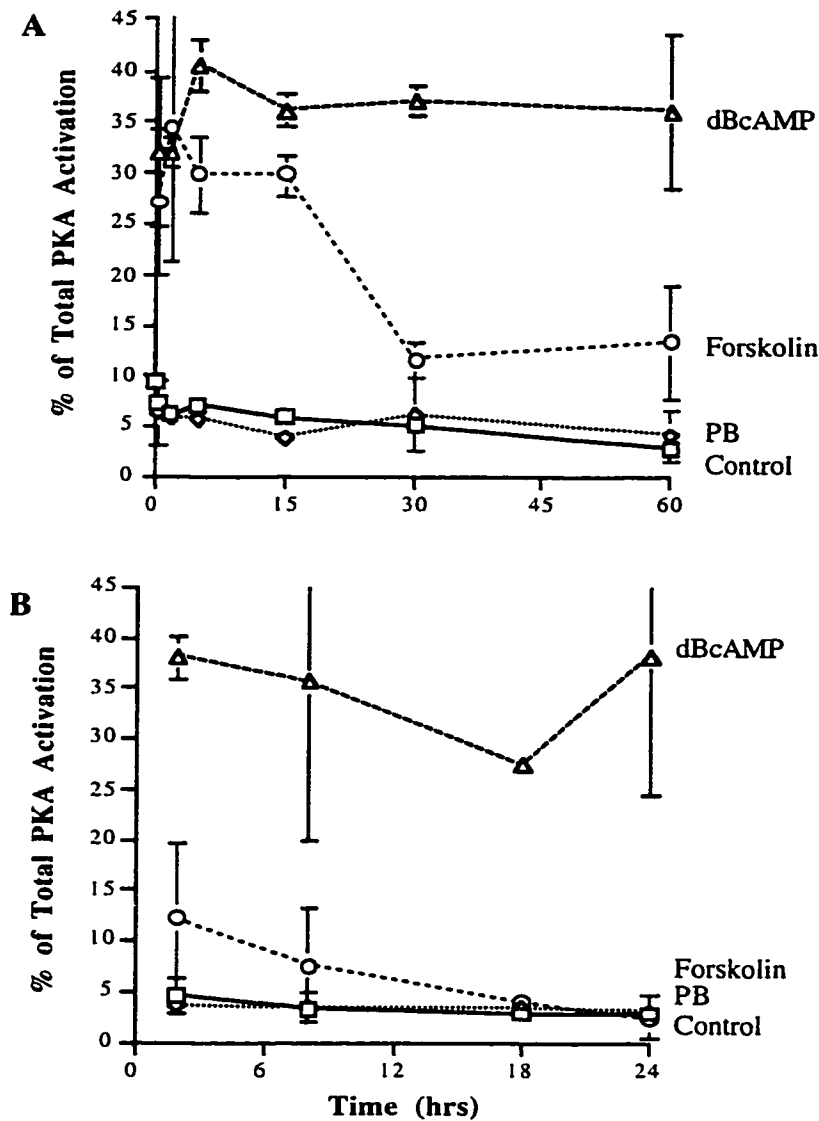


Figure 19. The Effects of dBcAMP, Forskolin, and PB on PKA Activity Levels.

Panel A represents the effects on PKA activity over short term treatments, 30 sec. - 1 hr. *Panel B* represents the effects of longer treatment intervals, 2-48 hrs. The phosphodiesterase inhibitor Ro 20-1724 (200 μ M) also was added to the dBcAMP and forskolin treatments. % of total PKA activation is the amount of endogenous PKA activity / the amount of total available PKA as determined by the addition of exogenous cAMP (5 μ M) to the sample. Values represent the average \pm standard deviation from at least 3 independent hepatocyte preparations.

CHAPTER 3: THE EFFECTS OF BACULOVIRUS MEDIATED GENE DELIVERY ON RAT PRIMARY HEPATOCYTES: AN EXAMINATION OF CYP INDUCTION AND CYTOKINE EXPRESSION

Abstract

Baculovirus transfection strategies have proven successful at transferring foreign DNA into hepatoma cells and primary hepatocytes. In the process of testing the applicability of these methodologies for the study of the phenobarbital mediated gene activation response, we discovered that the presence of the baculovirus disrupts the PB induction process. Upon further investigation it became apparent that PB responsiveness of primary hepatocytes was not compromised when heat-inactivated baculovirus was substituted for native virus, although transfection efficiencies were severely compromised by this manipulation. In concert with previous reports from our laboratory showing that increases in cAMP levels could repress the induction process, we measured cAMP concentrations and PKA activities in primary cells after exposure to baculovirus. Neither parameter was affected by the presence of the virus. To evaluate whether immune response modulation was triggered by baculovirus exposure, we used RNase protection assays and discovered that the baculovirus infections led to activation of IL-1 α , IL-1 β , and TNF- α expression in the primary hepatocyte cultures. The production of cytokines is likely due to the presence of small numbers of Kupffer cells present in the cultures. Previous reports have indicated that these cytokines are capable of repressing the PB induction response, and thus are likely to be responsible for the repressive effects of baculovirus noted here. Interestingly, heat-inactivated virus produced the identical pattern of cytokine expression in the culture system, with the exception that IL-10 expression was also observed. These results indicate that baculovirus vectors enhance the expression of inflammatory cytokines in primary hepatocyte cultures and therefore raise concerns as to whether the baculovirus vectors will

be useful tools for further studies of CYP gene regulation *in vitro*, as well as for liver-directed gene therapy in humans.

Introduction

As the liver is the predominant organ responsible for the metabolism of most endogenous and exogenous compounds, understanding the factors governing liver-specific gene regulation is crucial. Recent advances in the ability to culture primary hepatocytes under serum-free conditions have allowed for the preservation of differentiated hepatocyte function (Schuetz, et al., 1988; Sidhu, et al., 1993; Sinclair, et al., 1990). The optimization of hepatocyte culture conditions now allows researchers to pursue various *in vitro* assays to further study the mechanisms governing many liver processes. Despite this progress, the inability to efficiently transfect hepatocyte cultures has strongly hindered the understanding of the liver and liver specific processes. To fully understand how genes are regulated, it is necessary to examine controls at the transcriptional level and transfection studies provide a mechanism by which to overexpress, manipulate, or track the expression of a particular gene.

In our laboratory we are specifically interested in understanding the mechanisms which control the PB-mediated induction of the CYP2B gene family. As primary hepatoma cell lines have largely lost the capacity to respond to PB, the use of primary hepatocyte cultures to probe gene regulation processes is necessary. To increase transfection efficiencies in our hepatocyte cultures, we explored the use of baculovirus (*Autographa californica* nuclear polyhedrosis virus) mediated transfections in our rat primary cultures. Previous studies have reported that baculovirus vectors can be used to shuttle foreign genes into mammalian cells (Boyce and Bucher, 1996; Hofmann, et al., 1995; Shoji, et al., 1997). Interestingly, the uptake of the baculovirus was reported to be specific for hepatic cell types and the transfection efficiency in primary rat hepatocytes was shown to be over 70% (Boyce and Bucher, 1996).

In this report, we describe our results with the baculovirus vectors and discuss the effects of the baculovirus on primary hepatocyte cultures. Although highly efficient at delivering foreign genes into rat primary hepatocytes, the presence of the baculovirus blocked the PB mediated induction of the CYP2B genes. We conducted further studies to explore the mechanisms by which the baculovirus disrupts CYP induction, and discovered that the presence of the baculovirus leads to the expression of multiple cytokines in primary hepatocyte cultures. Baculovirus vectors did not affect cAMP expression or PKA activity levels in primary cells. The results described in this report raise concerns as to whether the baculovirus vectors will be useful tools for further studies of CYP gene regulation *in vitro*, as well as for liver-directed gene therapy in humans.

Materials and Methods

MATERIALS

PB was obtained from the University of Washington Hospital Pharmacy Services (Seattle, WA). Dexamethasone was purchased from Sigma (St. Louis, MO). Forskolin and Ro 20-1724 were purchased from the Alexis Corporation (San Diego, CA). Recombinant human TNF- α was purchased from Genzyme Diagnostics (Cambridge, MA). Recombinant human IL-10 was purchased from R&D Systems (Minneapolis, MN). KU-1 antibody was a generous gift from Dr. Jonathan Reichner (Rhode Island Hospital and Brown University, Providence, RI). All insect and hepatocyte cell culture material and TrizolTM were obtained from Life Technologies (Grand Island, NY). Matrigel, ITS+, and NuSerum were obtained from Collaborative Biomedical Products (Bedford, MA).

VIRUS CONSTRUCTION

A TK-luciferase (TK-luc) construct was constructed by inserting the TK promoter of the pBRAMSCAT2 vector into the PGL2 basic vector (Promega, Madison, WI) at the *Hind*III site. Viral constructs were made using the pFastBac1 plasmid and the Bac-To-BacTM

Baculovirus Expression System (Life Technologies). To make the luciferase-FastBac (Lfast) construct, the PGL2 basic cloning vector was digested with *Mlu*I and *Sal*I to isolate the luciferase cassette. Fragments were separated by electrophoresis in agarose then excised, treated with Agarase (Boehringer Mannheim, Indianapolis, IN), and then purified with QIAquick PCR columns (Qiagen, Valencia, CA). The purified fragment was ligated to the pFastBac vector, which was previously digested with *Bss*HIII and *Sal*I. For TK-Luciferase-FastBac (TKLfast) construction, TK-luc was digested with *Mlu*I and *Sal*I to isolate the TK promoter and the luciferase cassette. The isolated fragment was purified and ligated into the pFastBac vector as described for the Lfast construct. All final constructs were confirmed with restriction digestion and PCR with at least 3 different primer sets. Once the recombinant FastBac constructs were made they were individually transformed with competent DH10Bac *E.coli* cells (Life Technologies) to make the recombinant Bacmid. Correct orientation and sequences of the recombinant Bacmid DNA constructs were confirmed with PCR and cycle sequencing. Sf9 insect cells (ATCC, Rockville, MD) were transfected with the recombinant Bacmid DNA using Lipofectin (Life Technologies) and virus particles were isolated (King and Possee, 1992). Viral DNA was extracted with 1% SDS and two phenol-chloroform extractions. Viral DNA sequences were again confirmed using multiple PCR primer sets.

INSECT CELL CULTURE, VIRUS AMPLIFICATION, AND VIRUS PURIFICATION

Sf9 cells were cultured at 28°C in ambient air. Cells were grown and maintained in Graces media supplemented with 9% fetal bovine serum, 90 µg/ml NaCl, 0.1 mg/ml streptomycin sulfate, 0.16 mg/ml penicillin G, and 0.56 µg/ml Amphotericin B. Virus was titered in Sf9 cells using the plaque assay technique as described by King and Possee (1992) with minor variation. Cells were maintained in supplemented Graces media and after applying the agarose overlay the cells were incubated for six days before staining plaques. To amplify the virus, a multiplicity of infection (MOI) of 0.1 was used. Viral stocks were always titered and stored at 4°C in the dark. To purify virus, cell debris was removed by a brief centrifugation (10 min.) at 2000 rpm. Cleared virus was layered over 27% sucrose and centrifuged at 24000 rpm for 75 min in SW28 tubes. The pellet was resuspended in TE, pH 8, sonicated on ice for 20 sec., and then layered over a 20- 60% sucrose gradient in

SW 27.1 tubes. After centrifugation at 27,000 rpm for 155 min. the purified viral band was isolated using a syringe filter with a 20 gauge needle. To concentrate the virus and remove any residual sucrose, the virus was mixed with TE and pelleted in SW 27.1 tubes by centrifugation at 27,000 rpm for 150 min. The final pellet was resuspended in TE, pH 8, briefly sonicated on ice, filtered through a 0.45 μm syringe filter and stored at 4°C in the dark. Purified virus was titered using the plaque assay technique. Heat-inactivated virus samples were heated at 56°C for 20 min.

PRIMARY HEPATOCYTE ISOLATION AND CULTURE

Rat hepatocytes were isolated by a modification of the two-step collagenase perfusion *in situ* (Seglen, 1976) and cultured with modifications (Sidhu, et al., 1993; Sidhu and Omiecinski, 1996) of the protocols as described previously. A dilute concentration (233 $\mu\text{g/ml}$ final concentration) of ECM (Matrigel) was added as an overlay 16 hrs after initial plating of the cells or as stated. Medium changes were conducted on a daily basis and a 25 nM dexamethasone concentration was maintained in all media.

CELL LINES

Huh-7 cells were cultured at 37°C in 95% air/5% CO₂. Cells were maintained in DMEM/F-12 media supplemented with 2.2 mg/ml tissue culture grade sodium bicarbonate, 5% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin.

TREATMENTS

PB (1.0 M) was dissolved in tissue culture grade water as a stock solution and stored in aliquots at -20°C. Forskolin (100 mM), dexamethasone (25 μM), β -naphthoflavone (BNF) (12 $\mu\text{g}/\text{ul}$) and Ro 20-1724 (500 mM) were dissolved in DMSO as stock solutions and also stored at -20°C. TNF- α was diluted in complete Williams E media (Sidhu, et al., 1993) and stored at -80°C in 10 ng/ μl aliquots. IL-10 was reconstituted in complete Williams E

media and used immediately. Cells were cultured for 24-48 hrs before the addition of any treatments. Final concentrations of each treatment were: PB, 500 μ M; Forskolin, 100 μ M; BNF, 22 μ M; and Ro, 20-1724 200 μ M; unless otherwise stated. TNF- α and IL-10 treatments were variable and were as stated in the following sections. For all analyses, representative data are shown from multiple studies performed independently with different hepatocyte preparations.

TRANSFECTIONS

Lipid mediated transfections were carried out using Lipofectin (Life Technologies). Liver cells (primary hepatocytes or HUH-7's), were washed and put in serum free media for transfections. For primary hepatocytes, transfections were performed in 60 mm dishes 16 hr after plating and before the addition of Matrigel. 10 μ l of plasmid DNA plus 2.5 μ l of a CMV-secreted-alkaline phosphatase plasmid or a CMV- β -galactosidase plasmid were cotransfected into cells in 3 mls of media (with PB treatments as noted) as suggested by Life Technologies. After 6 hrs, 3 mls of serum containing media and Matrigel were added to each dish to stop the transfection. After overnight incubation, the media was replaced with fresh media. For cell lines, serum containing media was added instead of Matrigel to stop the transfections. Cells were harvested 48 hrs after transfection for either RNA or protein. For β -galactosidase or luciferase staining, the cells were fixed with 3% paraformaldehyde. For viral transfections, cells were grown in 35 mm dishes at a confluency of 1.5×10^6 cells/dish and transfections were performed 16 hr after plating, before the addition of Matrigel. Virus, at a MOI of 10-200 was added to the cells in 1.5 mls of complete media and was left on the cells and incubated at 37°C for 2 hrs. After this period, 1.5 mls of complete media was added and the cells were incubated overnight. The next morning, the virus and media were removed as fresh media, with or without stated treatments, was added in addition to Matrigel. 48 hrs after the addition of the virus the cells were harvested for RNA and protein, or fixed for luciferase, β -galactosidase, and KU-1 staining.

LUCIFERASE ACTIVITY

Cells were scraped in PBS, centrifuged at 500 rpm and resuspended in 100 μ l of reporter lysis buffer (Promega). After 1 freeze-thaw cycle, cellular debris was pelleted and 25 μ l of supernatant was measured for luciferase activity using an LB9507 luminometer (EG&G Berthold). Promega luciferase assay substrate, 100 μ l, was used. Luciferase levels, relative light units (RLU) were normalized to protein levels which were measured using the BCA assay (Pierce, Rockford, IL).

β -GALACTOSIDASE MEASUREMENTS AND ANTIBODY STAINING

To measure transfection efficiency with β -galactosidase, fixed cells were stained with a potassium ferricyanide/ferrocyanide solution (Ausubel, et al., 1994) and the stained cells were counted using phase-contrast light microscopy. To examine luciferase expression, fixed cells were permeabilized with 0.1% Triton-X and incubated at room temperature with a 1:200 dilution of anti-luciferase antibody (Promega) in a 1% BSA solution. After 2 hrs cells were washed with PBS and a goat-anti rabbit IgG-FITC secondary antibody (Sigma) was added. For detection, nuclei were stained with propidium iodide and cells were examined using scanning laser cytometry on the ACAS 570 laser cytometer (Meridian Instruments, Okemos, MI). For kupffer cell detection, fixed cells were permeabilized with 0.1% saponin and incubated with a 1:2 dilution of KU-1 antibody in 1% goat serum for 2 hrs. A goat anti-mouse-FITC conjugated secondary antibody was then added. For nuclei detection, a Hoechst 33342 solution (2 μ g/ml in PBS) was added immediately before examining the cells with scanning laser cytometry.

CAMP ANALYSIS

Intracellular cAMP was measured using the Biotrak™ cAMP enzyme immunoassay system from Amersham (Arlington Heights, IL). Cells were stimulated for the indicated times and cell extracts were prepared. Briefly, cells were washed twice with cold PBS and then lysed and scraped in 800 μ l of ice cold 70% ethanol. Cell debris was pelleted at 2000 x g and the

resulting supernatant was subsequently lyophilized and stored at -20°C . For analysis, resulting pellets were resuspended in 500 μl of the required assay buffer. Protein concentrations were determined using bovine serum albumin as a standard with the BCA protein assay reagent (Pierce).

MEASUREMENT OF PKA ACTIVITY

Protein Kinase A activity was assessed by measuring the transfer of ^{32}P -labeled phosphate to a biotinylated Kemptide (LRRASLG) substrate using Promega's Signa-TECT™ PKA assay system (Madison, WI). At specified timepoints after treatments, cells were washed twice and then scraped with cold PBS. Cells were pelleted at 500 x g at 4°C and resuspended in 100 μl of ice cold buffer (25 mM Tris pH 7.5, 5 mM β -glycerophosphate, 2 mM DTT, 0.1 mM sodium orthovanadate, and 10 mM MgCl_2). The resulting cell suspension was lysed by brief (7 sec.) sonication on ice, and the cell debris was pelleted at 15,000 x g at 4°C . Five μl of supernatant was assayed for 5 min at 30°C with: 5 μl PKA assay buffer (Promega), 0.1 mM ATP, 5 μl γ - ^{32}P ATP (6,000 Ci/mmol), and 0.1 mM PKA biotinylated Kemptide substrate, with or without activation by 5 μM cAMP. Mixtures without exogenous cAMP measured endogenously activated PKA, while mixtures with added cAMP measured the total available PKA in the cells. Reaction termination, spotting onto membranes, and washing were carried out as directed by the manufacturers' protocol. Protein concentrations were determined using bovine serum albumin as a standard with the Bio-Rad Protein assay (Hercules, CA).

RNA ANALYSIS

Total RNA was isolated (Chomczynski and Sacchi, 1987) using Trizol™ as described by the supplier. For slot blot evaluation, 5 μg of total RNA was applied directly to a Genescreen Plus nylon membrane (DuPont) under vacuum and denaturing conditions using a Minifold II apparatus (Schleicher and Schuell, Keene, NH). The membranes were hybridized with specific ^{32}P -radiolabeled oligonucleotides or cDNA probes for CYP2B1,

CYP2B2, CYP3A1, CYP1A1, albumin, and 18s rRNA. as described previously (Omiecinski, et al., 1990; Sidhu and Omiecinski, 1996). To examine cytokine expression in primary hepatocytes multi-probe RNase protection assays were performed using the rCK-1 template with the Riboquant™ multi-probe RNase protection assay system (Pharmingen, San Diego, CA). 15 µg of target RNA were used for each sample and assays were performed according to the manufacturers specifications.

Results

TRANSFECTION EFFICIENCY STUDIES

Transformed cell lines, such as COS-1 and Huh-7, are only marginally PB inducible, if inducible at all. This makes them an inappropriate model for examination of CYP gene induction by PB. To characterize the PB activation sequences in the 5' upstream region of the rat CYP2B genes it is necessary that transfection studies be performed in primary hepatocyte cells. Previous experimentation in our laboratory (which will not be discussed here) has involved studies with diverse transfection techniques and cationic lipids including: electroporation, calcium phosphate, Dosper, Lipofectamine, Lipofectin, and DOTAP. Our best results were obtained using lipofectin as our DNA delivery lipid. Using β-galactosidase staining of our primary hepatocytes we quantitated transfection efficiency and found that it was approximately 0.04% (data not shown). A similar low level of transfection efficiency has been seen by other investigators (Ourlin, et al., 1997). The levels in hepatoma cell lines are also low, ranging from 3% to 8% transfection efficiency (Ourlin, et al., 1997).

We tested a baculovirus mediated transfection technique in an attempt to improve transfection efficiencies. As seen in Figure 20, when virus carrying TK-Luciferase DNA is used to transfect Huh-7 cells, a very high level of luciferase expression was detected by laser cytometry using an Anti-luciferase antibody. As the titer (MOI) of virus added to the cells was increased a clear increase in luciferase expression resulted. Similarly, the fluorescence intensity levels (pixels/cell), as well as the luciferase activity of harvested cells, increased as the MOI of the baculovirus was increased (Table 4). This trend was

consistent between multiple preparations of virus and was also demonstrated in transfection studies with multiple hepatocyte preparations.

CYP INDUCTION IN BACULOVIRUS TRANSFECTED PRIMARY HEPATOCYTES

Figure 21 shows the PB mediated induction of CYP2B1, dexamethasone mediated induction of CYP3A1 and BNF mediated CYP1A1 induction. Increasing titers of baculovirus added to the cultures, resulted in a repression of CYP2B1 (panel A) and CYP3A1 mRNA induction (panel B). CYP1A1 induction was not affected by the presence of baculovirus (panel C). As seen in all panels, although the 18s RNA levels remain constant, as the MOI of baculovirus was increased, there was a corresponding decrease in albumin mRNA levels in the primary cells.

As our intent was to study the regulation of the CYP2B genes, we conducted further studies to try to rescue the PB induction response. Interestingly, addition of heat-inactivated baculovirus (56° for 20 min) did not compromise the CYP2B1 induction or albumin expression of the hepatocytes (Figure 22). There was some variability in the extent to which the heat-inactivation rescued the induction response; the data shown are representative of the average response that occurred. Unfortunately, heat-inactivation of the baculovirus severely compromised its ability to transfect hepatocytes with high efficiency.

EFFECTS OF BACULOVIRUS cAMP AND PKA PATHWAYS

Previous results reported by our laboratory demonstrated that cAMP analogs and activators significantly repress the PB mediated CYP2B induction event (Sidhu and Omiecinski, 1995a). To further examine the response of the baculovirus on primary hepatocytes, we investigated the effects of the native and heat-inactivated baculovirus on cAMP levels and subsequent PKA activity. Figure 23 shows treatment of primary rat hepatocytes with TE pH 8 (our control), baculovirus (MOI 100), heat-inactivated baculovirus (MOI 100), or forskolin (positive control). Within 5 minutes there was a 90 fold increase in intracellular cAMP levels upon forskolin treatment. Despite this capacity for response, the baculovirus and inactivated baculovirus exerted no observable effects on cAMP levels. Similar results were obtained when treatment periods were extended to 24

hrs. Higher MOI's of baculovirus also were without effect on cAMP levels (data not shown).

We used a synthetic Kemptide substrate to assess increases in PKA activity following treatments with either TE pH 8, baculovirus (MOI 100), heat-inactivated baculovirus (MOI 100), or forskolin. As shown in Figure 24, only the forskolin treatment produced an increase in PKA activation in the primary hepatocyte system. Baculovirus and inactivated baculovirus were without effect on PKA activity. The results presented are representative of results obtained from 3 separate hepatocyte preparations.

EFFECTS OF BACULOVIRUS ON CYTOKINE EXPRESSION

To further understand the effects of the baculovirus and heat-inactivated baculovirus on primary hepatocyte cultures, we examined cytokine expression levels. The use of multiprobe RNase protection analysis enabled screening for multiple cytokine messages with the same RNA sample. Figure 25 shows a representative assay result. In Control or PB treated cells there was no detectable cytokine expression (lanes 1 and 6). In the presence of virus detectable levels of TNF- α and low levels of IL-1 β were detected. With the heat-inactivated baculovirus TNF- α , IL-1 α , and IL-1 β were detected along with a marked increase in IL-10 expression. In some hepatocyte preparations, virus treatment produced detectable levels of IL-1 α and IL-1 β , although this expression was variable between hepatocyte preparations (data not shown). We were unable to detect TNF- β , IL-3, IL-4, IL-5, IL-6, IL-2, or INF- γ in any of the samples. In all lanes there is detectable L32 and GADPH, confirming that an equal level of mRNA was present within each lane.

EFFECTS OF TNF- α AND IL-10 ON CYP2B EXPRESSION

To further investigate the effects of cytokines on CYP2B expression, we examined TNF- α and IL-10 in more detail. As expected, when purified TNF- α was added to the hepatocyte cultures a decrease in CYP2B expression resulted (Figure 26), although not as pronounced as the repression demonstrated upon addition of baculovirus (Figure 21).

When purified IL-10 was added to hepatocyte cultures that had been treated with either TNF- α or baculovirus, we were unable to rescue the CYP2B1 responsiveness of the cells (Figure 26).

KUPFFER CELL DETECTION

To investigate the potential origins of cytokine production, we examined hepatocyte cultures using an antibody directed against the Kupffer cell, KU-1. As seen in Figure 27, although their abundance was low, Kupffer cells were present in the cell population. The level of kupffer cells varied from 0 to 8 cells and in fields of approximately 100 hepatocytes.

Discussion

The low transfection efficiencies obtained with primary cell transfection have been a major hurdle for many researchers. Even with the development of lipofection mediated gene delivery techniques (Felgner *et al.*, 1987; Felgner and Ringold, 1989), primary hepatocytes have remained difficult to transfect (Ourlin, et al., 1997). When conducting studies to examine gene regulation, it is very important that the transfected cells are representative of the total cell population of interest. Unfortunately, when transfection efficiencies of primary hepatocytes are below 1.0%, this becomes very difficult. It has been suggested (Ourlin, et al., 1997) that cell cycle division is a driving factor for lipofection mediated gene delivery; since hepatocytes are in a quiescent G0 state, this could explain their low transfectability.

The application of viral gene delivery strategies, including retrovirus and adenovirus vectors, has had a marked impact in the gene therapy arena. As retrovirus vectors require cellular proliferation to achieve high transfection efficiencies (Ferry *et al.*, 1991), their use for hepatic gene transfer is limited. Adenovirus does not require cellular division for infection, but these vectors do not specifically target the liver and cell toxicity has been documented (Yang *et al.*, 1996; Yang and Wilson, 1995). Recent improvements

in adenoviral vectors have limited the toxicity (Yeh *et al.*, 1996) and the advent of liver specific promoters should help target these vectors to the liver.

Baculovirus has historically been used as a vector for protein overexpression in insect cells (King and Possee, 1992; Luckow and Summers, 1988). The risk of infection to humans is very minimal as the baculovirus can only replicate in invertebrates and baculovirus expression in mammals and plants has never been reported (Luckow and Summers, 1988). Highly successful gene delivery into hepatocytes and liver cell lines, presumably by an endosomal uptake pathway, has made baculovirus mediated transfection very appealing for the study of CYP gene regulation (Boyce and Bucher, 1996; Hofmann, *et al.*, 1995). Indeed, in our hands a very high level of transfection efficiency resulted from the use of this vector (figure 20, table 4) compared to lipofection or calcium phosphate mediated transfection methods. Since luciferase reporter expression was not localized, it was difficult to quantify the transfection efficiency on a per cell basis, but clearly over 50% of the cells were transfected when the viral titer was increased. Although no overt toxicity was noted in the presence of the baculovirus, there appeared to be a slight, yet detectable, dose-dependent decrease in albumin expression levels. At low infectivity levels, MOI 10, where no decreases in albumin expression occurred, the baculovirus repressed PB inducible CYP2B1 gene expression. Similar repression of the dexamethasone inducible CYP3A1 expression was produced whereas BNF inducible CYP1A1 expression was not affected by the presence of baculovirus. Thus the response we observed appeared specific to the PB/dexamethasone induction pathways.

To determine if the active baculovirus itself was responsible for repression of CYP induction, we used heat-inactivated baculovirus and then re-examined CYP expression. Heat inactivation of baculovirus was shown previously to result in a five-log reduction in virus titer, or infectivity (Hartig *et al.*, 1992), and ablated the toxicity of the baculovirus itself as opposed to toxicity caused by viral gene expression (Hartig, *et al.*, 1992). As presented previously, the heat-inactivated virus no longer repressed either CYP induction or albumin expression. The measured level of CYP expression with the inactivated virus was variable in that some batches of inactivated virus were fully permissive with respect to PB responsiveness while other inactivated preparations of baculovirus still partially repressed the induction response. This variation may be due more to variability in the hepatocyte preparation (e.g. altered numbers of Kupffer cells) than the virus itself. The

data shown are representative of the average rescue response seen in multiple hepatocyte preparations.

To further understand why the virus was selectively repressing CYP2B1 and CYP3A1 expression, we examined pathways that were known effectors of these genes. Research from our laboratory has demonstrated that cAMP analogs and activators of cAMP inhibit PB mediated CYP2B and CYP3A1 gene expression (Sidhu and Omiecinski, 1995a). As the baculovirus vectors themselves did not affect cAMP expression levels or associated PKA activity levels, it does not appear that the baculovirus mediated repression of the CYP gene induction is occurring through the cAMP signaling pathway.

In vivo attempts for gene transfer using baculovirus vectors have been unsuccessful in mice and rats either with systemic, intraportal, or direct injection into the liver parenchyma (Sandig *et al.*, 1996). It was suggested that the baculovirus was being inactivated by a classic complement cascade, similar to the pathway which inactivates retrovirus gene transfer and long-term expression. Further research has confirmed that it is the complement system which is responsible for baculovirus inactivation in human serum (Hofmann and Strauss, 1998). Using antibodies directed against specific complement components, baculovirus survival in the presence of human serum was demonstrated. Although this result allows for possible solutions to overcome a key issue determining the success of baculovirus mediated human gene transfer, there are many other issues that may arise. The effects of baculovirus vectors on cytokine expression in hepatocytes have not been examined previously. It is well established that certain cytokines act directly on human and rat hepatocytes and *in vivo* in rat liver to depress inducible CYP gene expression (Abdel *et al.*, 1995; Abdel *et al.*, 1993; Morgan *et al.*, 1994). CYP2B expression and activity is repressed by several cytokines including IL-6, IL-1 α , IL-1 β , TNF- α and INF- γ (Abdel, *et al.*, 1995; Clark *et al.*, 1995; Clark *et al.*, 1996; Monshouwer *et al.*, 1996). As baculovirus disrupts CYP expression in our hepatocytes, we examined the specific effects of baculovirus, as well as heat-inactivated baculovirus, on cytokine expression. Not surprisingly, we were unable to detect any of the examined cytokines in our control or PB treated hepatocytes.

Expression of TNF- α , IL-1 α , and IL-1 β was detected upon exposure to the baculovirus and IL-10 was additionally detected in the presence of inactivated baculovirus. This response was quite interesting as IL-10 is classified as an inhibitory cytokine based on its ability to suppress the action of many cytokines, including IL-1 β , TNF- α , IL-6, IL-12

and $\text{INF-}\gamma$ (Fiorentino *et al.*, 1991; Knolle *et al.*, 1997). In several experimental models, treatment with IL-10 protects against acute shock that is induced in the presence of endotoxin (Berg *et al.*, 1995; Howard *et al.*, 1993). Endotoxin contamination in our baculovirus preparations was ruled out as limulus amoebocyte lysate (LAL) testing determined that the endotoxin levels in the baculovirus samples were extremely low (19 ng/ml). Furthermore, when we tested the effects of a 100 fold higher concentration of purified lipopolysaccharide (LPS) on primary hepatocytes, a repression of CYP induction was not detected (data not shown). The presence of $\text{TNF-}\alpha$ and IL-1 in our baculovirus treated hepatocytes may explain the observed decreases in albumin expression as both these cytokines have been shown to depress albumin expression (Brenner *et al.*, 1990; Mackiewicz *et al.*, 1991; Muntan'e-Relat *et al.*, 1995).

At this point, it is difficult to speculate as to exactly why heat inactivation of the baculovirus activates expression of IL-10. IL-10 gene expression is regulated by a negative autoregulatory feedback loop and receptor expression was shown to be constitutive and functional in human and murine Kupffer cells (Knolle *et al.*, 1998). It was suggested that this negative autoregulation, which keeps IL-10 production low, helps to keep the liver microenvironment responsive to new inflammatory stimuli. Possibly, the heat-inactivated baculovirus may have undergone a conformational change that interferes with or allows the baculovirus to interfere with IL-10 regulation and its receptor, thus disrupting the feedback loop and allowing for higher IL-10 expression.

IL-10 has been shown to down regulate $\text{TNF-}\alpha$ expression by increasing the $\text{TNF-}\alpha$ mRNA instability (Grewe *et al.*, 1994). In the studies presented here, there was variability in the $\text{TNF-}\alpha$ expression, relative to IL-10 levels. In some hepatocyte preparations, the inactivated baculovirus led to a marked increase in IL-10 levels and a noticeable decrease in $\text{TNF-}\alpha$ levels; but, in other preparations a decrease in $\text{TNF-}\alpha$ levels was not always detected. It is quite possible that our RNase protection assay was not sensitive enough to detect very subtle changes in $\text{TNF-}\alpha$ expression levels. As hepatocytes treated with heat-inactivated baculovirus did not exhibit decrements in albumin expression, it is possible that the IL-10 inhibits the effects of the $\text{TNF-}\alpha$, despite our inability to detect a change in mRNA levels. There was also some variability in the expression of IL-1 α and IL-1 β levels in our baculovirus and inactivated baculovirus treated hepatocytes, but there did not appear to be a correlation with IL-10 expression levels; again, this may be due to the sensitivity of the techniques employed or differences in hepatocyte preparations.

To test the ability of IL-10 to protect against TNF- α induced CYP2B repression, we used purified TNF- α and IL-10 in our primary hepatocyte system. Although the expected CYP repression was seen with TNF- α , IL-10 was unable to relieve this repression in a manner similar to that of the heat-inactivated baculovirus. It is possible that results relates to the time course of the experiment. In our studies, hepatocytes were exposed to IL-10 for 12 hrs before the addition of TNF- α . In certain systems, it was found that the protective effects of IL-10 were apparent only when the IL-10 treatment was added within 30 minutes of LPS stimulation (G'erard *et al.*, 1993; Howard, et al., 1993), whereas other studies have conducted longer pretreatments to see a blocking effect (Knolle *et al.*, 1995). IL-1 α and IL-1 β may also be playing a modulatory role that effects PB inducible CYP expression, but we have not yet tested the effects of these cytokines in the primary hepatocyte cultures.

In a study of two different hepatocyte cell lines and human hepatocytes, all expressed numerous cytokines after stimulation with chemokines; IL-1 α and IL-10 were not among the detected cytokines (Rowell *et al.*, 1997). In fact, there are very little data demonstrating the ability of hepatocytes to express cytokines as most studies have focused on the Kupffer cell. Representing the largest population of tissue macrophages, the Kupffer cells of the liver take on many important functions including the presentation of antigen, the clearance of endotoxin and the production and release of many cytokines and eicosanoids. A rat liver contains approximately 12 Kupffer cells for every 100 hepatocytes (Knook and Sleyster, 1976). Although our hepatocyte isolation procedure is optimized through the use of differential centrifugation and subsequent culturing conditions to yield cultures which contain over 95% hepatocytes, the potential for Kupffer cell contamination does exist. Using a monoclonal antibody, KU-1, we did in fact determine that Kupffer cells were present at very low levels in the primary hepatocyte cultures, from 0 to 8 cells per 100 hepatocytes. As the Kupffer cell is strongly responsive to an inflammatory challenge, it is possible that the cytokine expression we detected upon exposure to baculovirus is emanating from these cells. Differences in the levels of Kupffer cells present in individual hepatocyte preparations may explain the variable levels of TNF- α , IL-1 α , and IL-1 β noted between experiments. Studies which used gadolinium chloride to deplete Kupffer cell populations, have demonstrated a change in the balance of inflammatory cytokine expression, including a decrease in IL-10 induction and a sustained overexpression of TNF- α (Rai *et al.*, 1997; Rai *et al.*, 1996). Future studies using

gadolinium chloride to ablate the Kupffer cell population will be able to address the question of exactly which cells are responsible for the baculovirus mediated inflammatory response and subsequent changes in CYP gene expression.

The implications of this work create a large obstacle that must be overcome before baculovirus mediated gene transfer can become a tool of the future. The antagonization of CYP induction and expression will certainly modulate the detoxification capabilities of the liver and the expression of cytokines will have multiple effects. For instance, IL-10 expression has been shown to inhibit ICAM-1 expression which will subsequently effect leukocyte migration and adhesion (Shrikant *et al.*, 1995). TNF- α has been shown to increase the levels of c-fos and c-jun proteins and to also increase their binding to an AP-1 element in the CYP19 aromatase gene (Zhao *et al.*, 1996). Additionally, it has been suggested that the effects of TNF- α on CYP2B expression are mediated through nitric oxide (Khatsenko *et al.*, 1997; Muller *et al.*, 1996); the induction of nitric oxide in the liver will certainly have multiple consequences. Future studies addressing the effects of baculovirus on cytokine expression in the liver will be needed if baculovirus vectors are to be considered as a tool for human gene therapy.

Table 4. Luciferase Expression and Activity in Transfected Cells

Baculovirus MOI	Fluorescence Pixels/Cell	Luciferase Activity RLU
0	4.8	25
100	12	596
200	64	1204

Luciferase Expression and Activity in Transfected cells.

Fluorescence (pixels/cell) was measured using the ACAS laser cytometer. The FITC (luciferase) expression was a measure of total expression within the field. This value was divided by the number of cells within the field as measured by Propidium Iodide staining. Values shown are the averages of 3 scans from 2 individual plates for each treatment. Luciferase activity, RLU, (Relative Light Units) was measured using a luminometer. Values shown are representative of values seen from 3 independent experiments. All assays were conducted in duplicate.

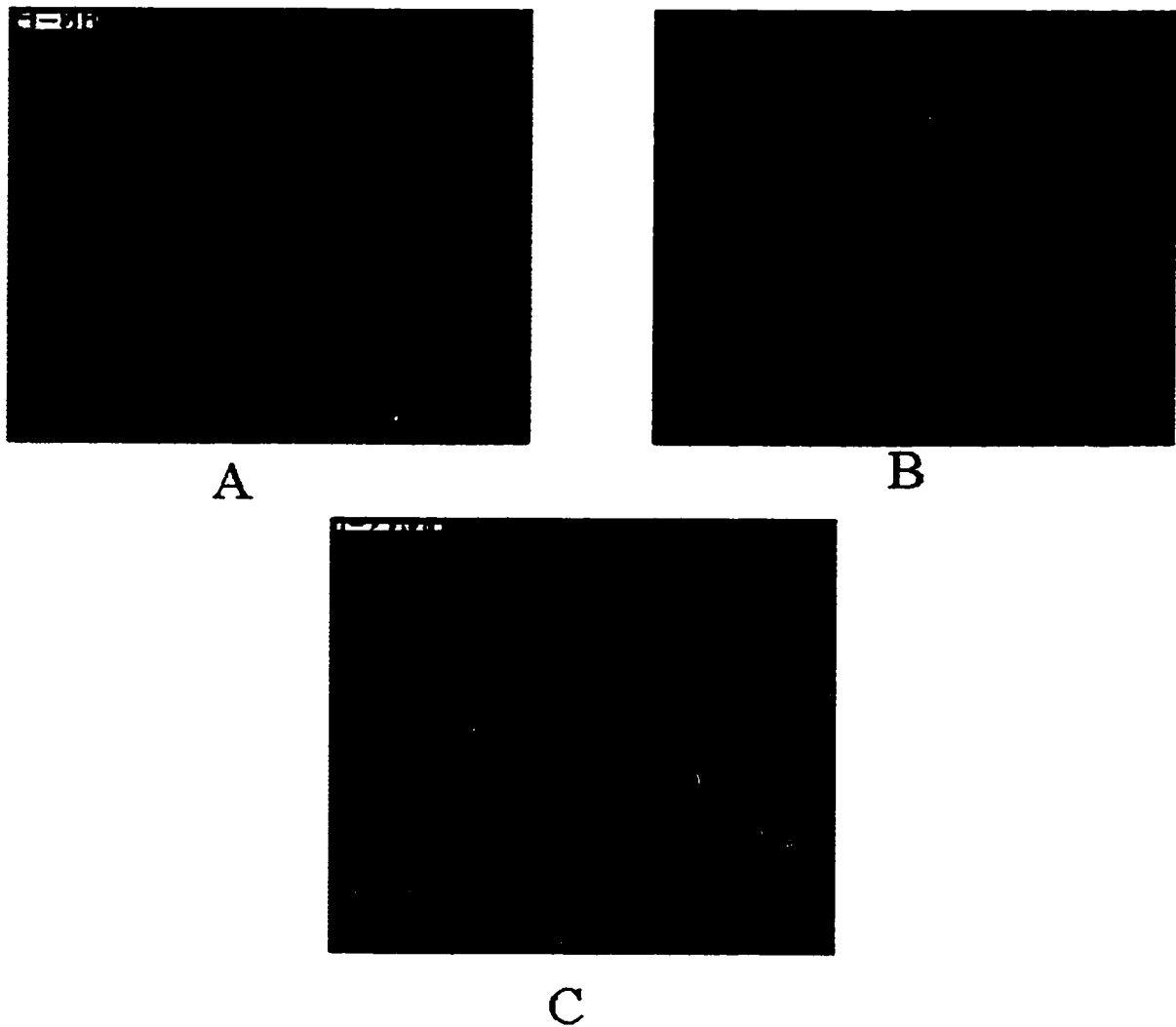


Figure 20. Baculovirus-Mediated Transfections in Huh-7 Cells.

Huh-7 cells were exposed to TK-Luc virus and examined 24 hrs later for luciferase expression using laser cytometry. Propidium iodide was used for nuclei detection (red signal). FITC conjugated luciferase antibody allows detection of luciferase expression (green/yellow signal). Panel A, no virus; Panel B, TK-Luc virus MOI 100; Panel C, TK-Luc virus MOI 200.

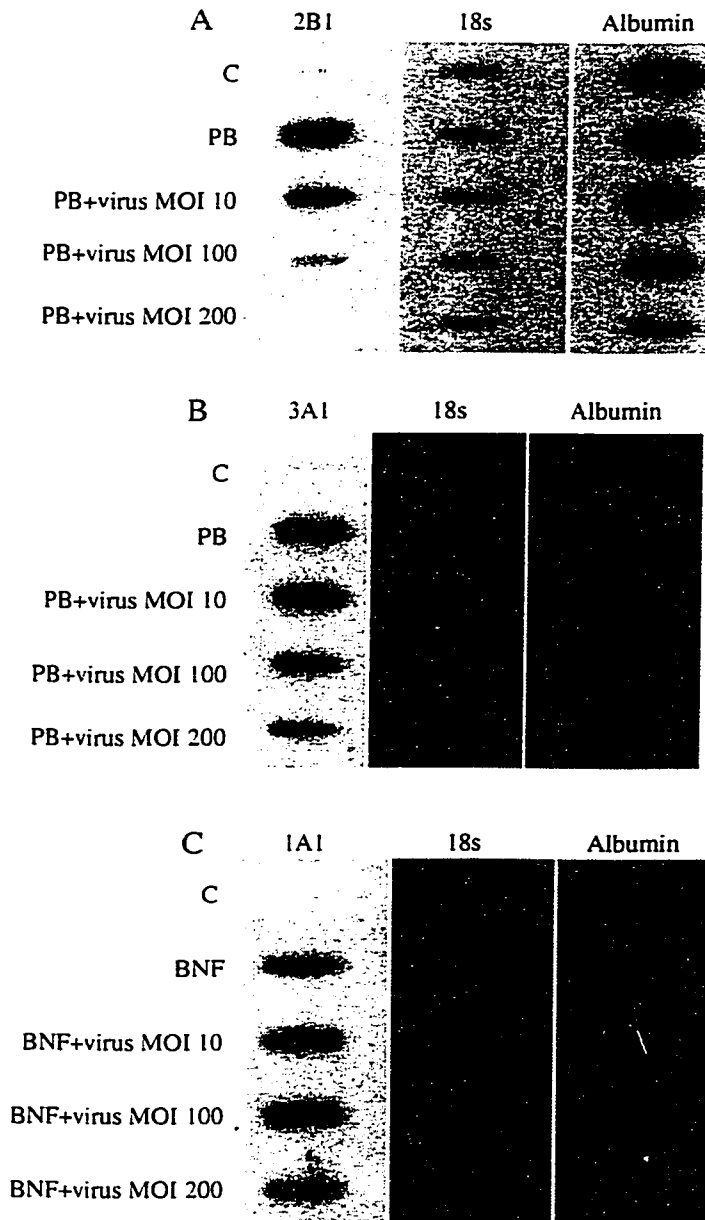


Figure 21. CYP mRNA Expression in Baculovirus Transfected Primary Hepatocytes.

Primary hepatocytes were either untreated (C) or exposed to PB (500 μ M) or BNF (μ M) alone or in the presence of TK-Luc virus, MOI 10-200 as stated. Panel A shows CYP2B1 mRNA expression; Panel B shows CYP3A1 mRNA expression; Panel C shows CYP1A1 mRNA expression. Corresponding 18s ribosomal RNA levels and Albumin levels are shown for each sample. Blots shown are representative of data obtained from at least 3 independent hepatocyte preparations and each treatment was performed in duplicate.

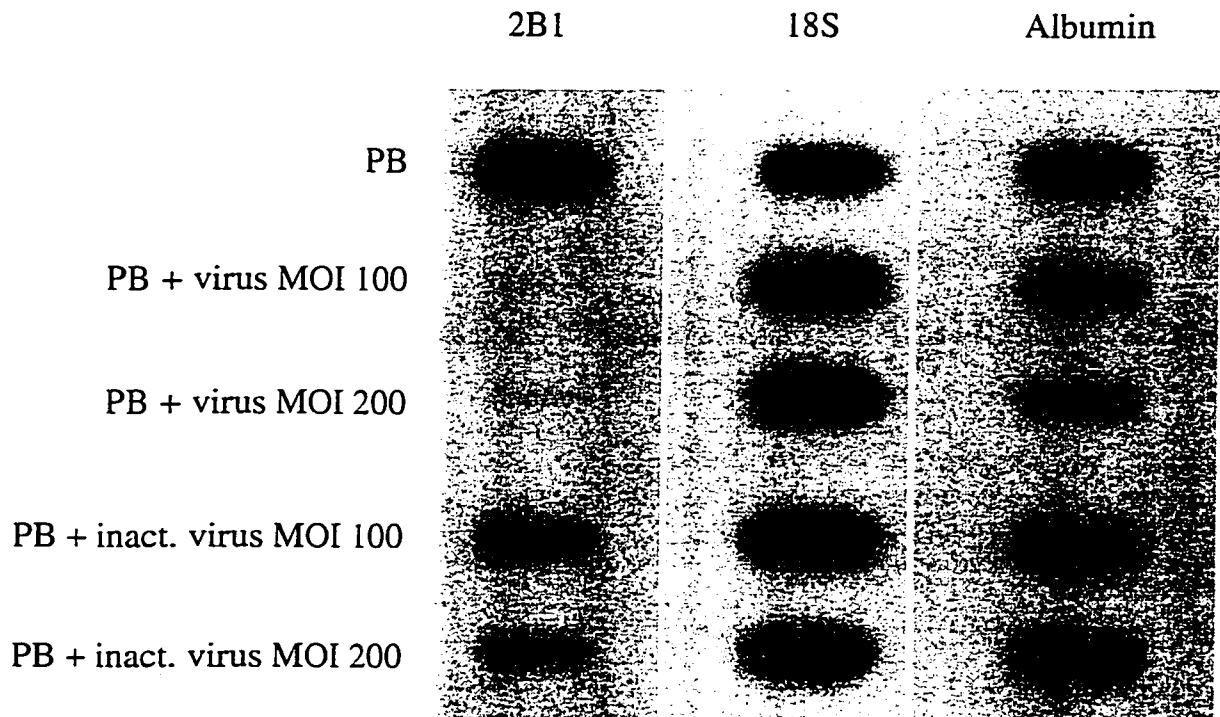


Figure 22. Heat-inactivation of Baculovirus Rescues CYP2B1 mRNA Expression.

Primary hepatocytes were treated with PB (500 μ M) and transfected with TK-Luc baculovirus or heat-inactivated (56°, 20 min) baculovirus. mRNA was isolated after 24 hrs. CYP2B1 expression and corresponding 18s and Albumin levels are shown. The blot shown is representative of data obtained from at least 6 independent hepatocyte and virus preparations.

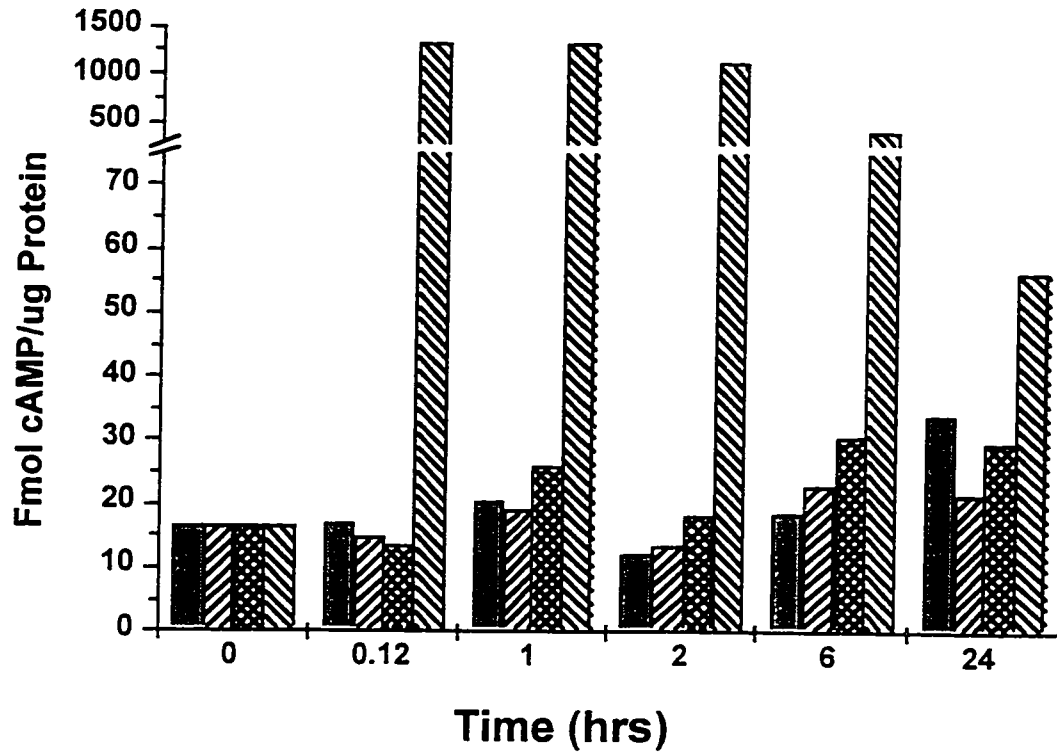


Figure 23. Effects of Baculovirus on Intracellular cAMP Levels in Primary Hepatocytes.

Primary rat hepatocytes were plated 24 hrs before treatments were initiated. The effects of TE pH 8 (Control, grey bars) baculovirus MOI 100 (right hatches), heat-inactivated baculovirus MOI 100 (cross-hatches), and Forskolin (100 μ M) (left hatches) on intracellular cAMP levels are shown. Forskolin treatment acted as a positive control. Values represent data obtained from 3 individual virus preparations and hepatocyte isolations. Samples were assayed in duplicate for cAMP levels.

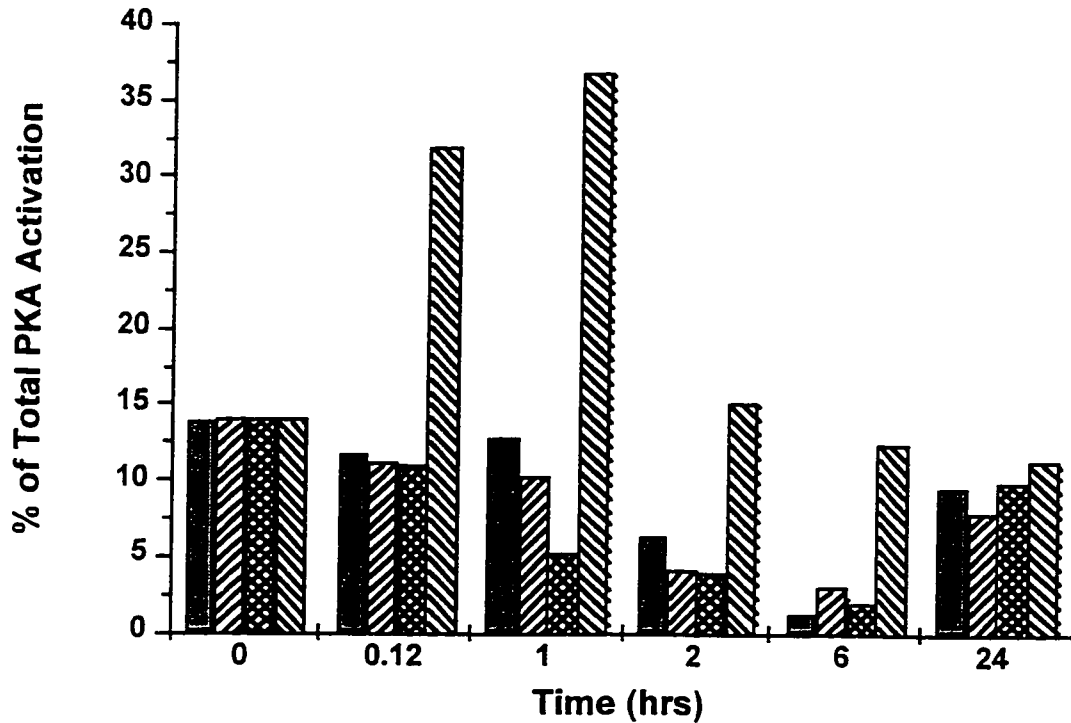


Figure 24. Effects of Baculovirus on PKA Levels in Primary Hepatocytes.

Primary rat hepatocytes were plated 24 hrs before treatments were initiated. The effects of TE pH 8 (Control, grey bars) baculovirus MOI 100 (right hatches), heat-inactivated baculovirus MOI 100 (cross-hatches), and Forskolin (100µM) (left hatches) on PKA activity levels are shown. Forskolin treatment acted as a positive control. Values represent data obtained from 3 individual virus preparations and hepatocyte isolations. % of total PKA activation is the amount of endogenous PKA activity / the amount of total available PKA as determined by the addition of exogenous cAMP (5µM) to the sample. PKA activity assays, in the absence of exogenously added cAMP, were performed in duplicate for each sample evaluated.

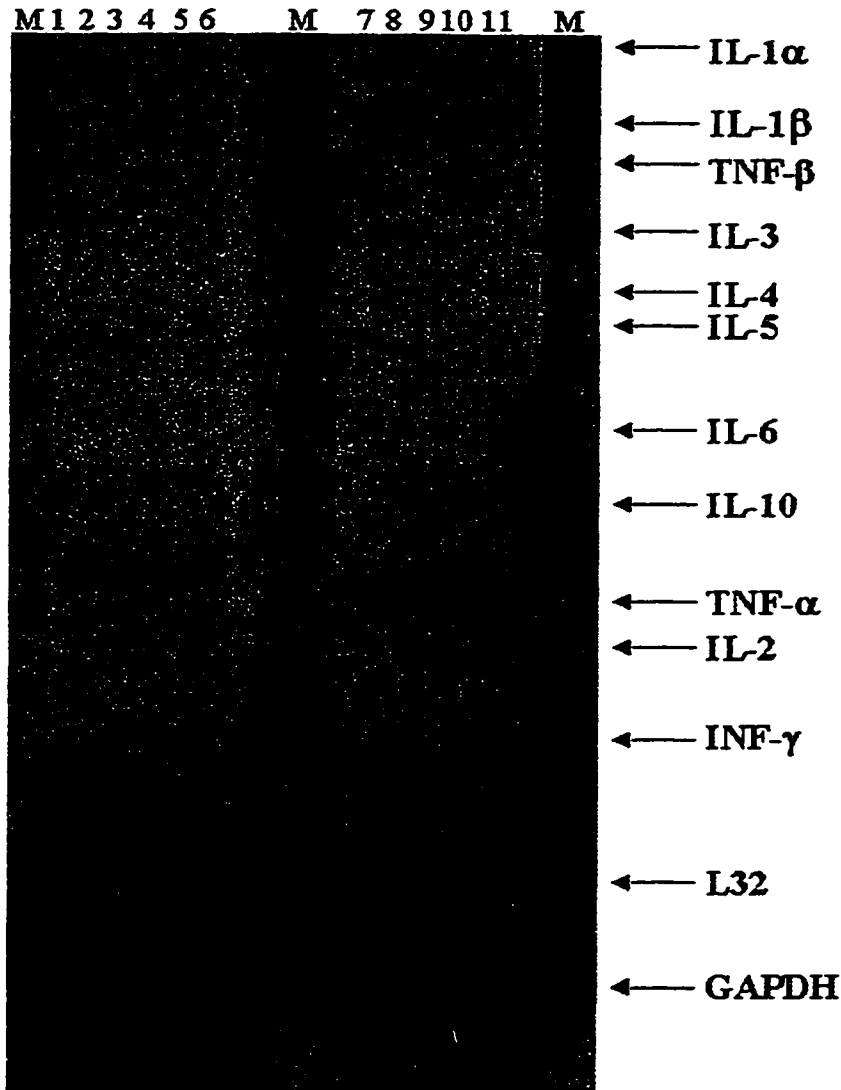


Figure 25. Effects of Baculovirus on Cytokine Expression.

Multi-probe RNase protection assays examined the effects of baculovirus (BV) and heat-inactivated BV on cytokine expression in primary hepatocytes. Cells were transfected with TK-Luc BV or heat-inactivated TK-Luc BV. RNA was isolated 24 hrs after transfections. Lane 1, TE pH8 (C); lanes 2 & 4, C + virus MOI 100; lanes 3 & 5, PB + virus MOI 100; lane 6, PB; lane 7, PB + virus MOI 100; lane 8, PB + heat-inact. virus MOI 100; lane 9, PB + virus MOI 200; lane 10, PB + heat-inact. virus MOI 200; lane 11, PB + virus MOI 200. Virus used in lanes 2-10 were from the same preparation. The arrows depict sizes of the expected protected probes for individual cytokines. M represents the ladder of unprotected probe fragments, each has a known length and can serve as a size marker to compare the protected probe sizes. Earlier exposures of the films were conducted to determine that RNA levels were equivalent by examining L32 and GAPDH levels.

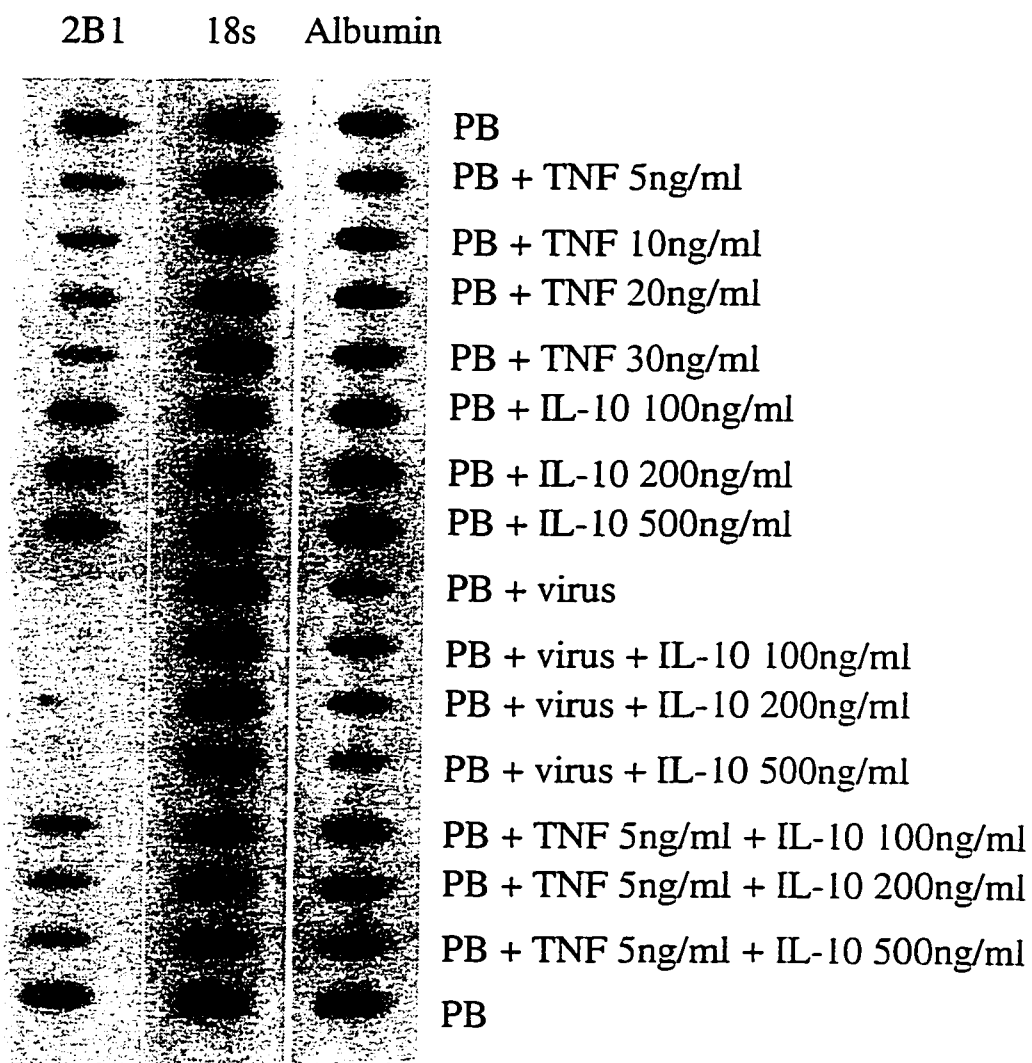


Figure 26. Effects of TNF- α and IL-10 on CYP2B1 Expression.

CYP2B1 and corresponding 18s ribosomal RNA and Albumin levels were measured in RNA extracted from primary hepatocytes treated with PB (500 μ M) and differing levels of TNF- α and/ or IL-10 as stated. Cells that received virus and IL-10 were pretreated with IL-10 for 12 hrs before the addition of baculovirus (TK-Luc. MOI 100). Similar pretreatments were done for cells exposed to both IL-10 and TNF- α . In all cases, the IL-10 treatment levels were maintained in the cells until they were harvested. Cells were harvested for RNA 24 hrs after treatment.

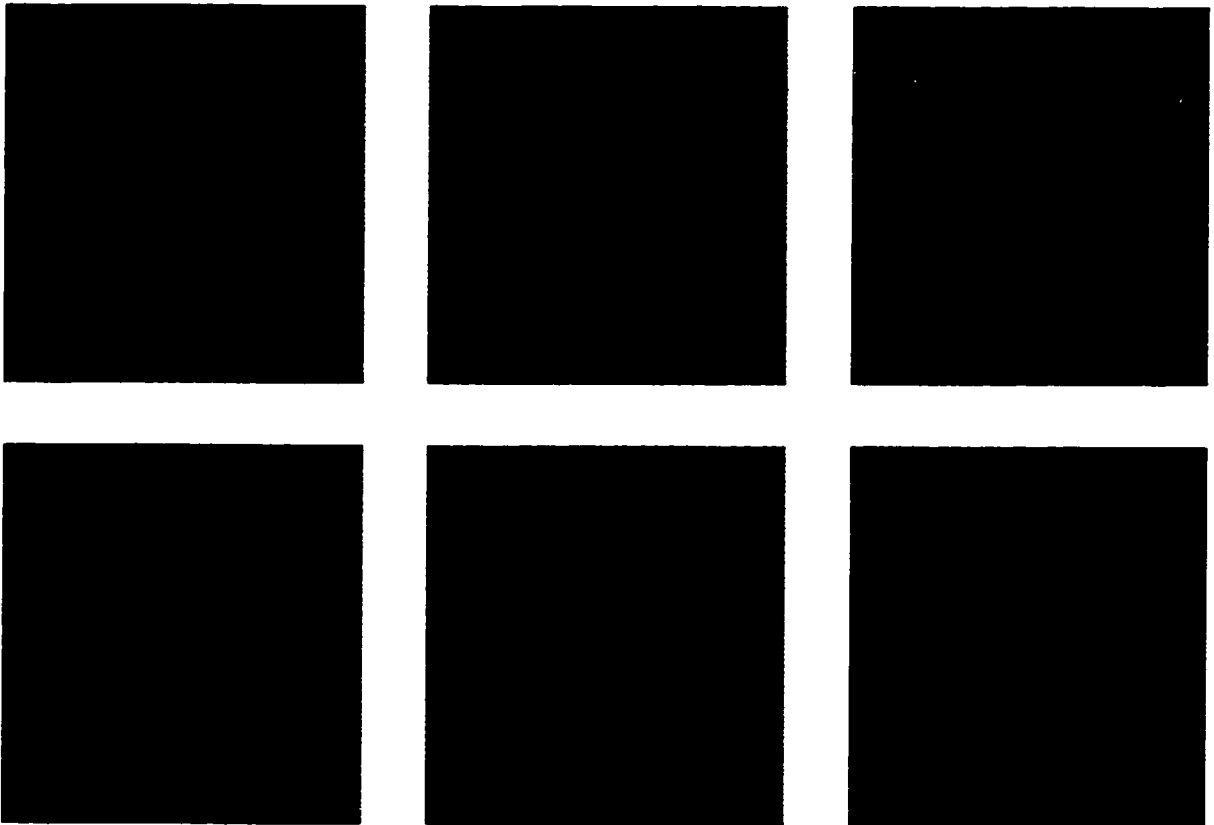


Figure 27. Kupffer Cell Detection in Primary Hepatocytes.

After 72 hrs in culture, primary hepatocytes were fixed and incubated with KU-1 antibody. Expression levels were detected using a FITC conjugated secondary antibody. Using laser cytometry, the Kupffer cells are recognized by their green staining and small size. Red pseudocolor represents hepatocyte nuclei as detected using Hoechst dye.

CHAPTER 4: THE EFFECT OF HISTONE DEACETYLATION ON THE PHENOBARBITAL RESPONSIVE CYTOCHROME P450 2B1 GENE

Abstract

Phenobarbital (PB) and 'PB-like' inducers modulate the expression of many biotransformation enzyme genes, including the CYP2B1 and CYP2B2 genes. This modulation is known to occur at the level of transcription. A PB responsive unit (PBRU) has been identified in the 5' upstream region of the CYP2B genes and characterizations of the proteins binding in this region, such as NF-1 and CAR-RXR, are beginning to shed light on how this region is modulating PB induction. Histone acetylation has been implicated in controlling the transcription of many genes, including those coding for other liver enzymes such as CYP1A1. To understand and address potential avenues of PB mediated gene regulation, we have initiated studies to examine histone acetylation as a potential modulator of transcription. In the present investigation, we examined the effects of the highly specific global histone deacetylase inhibitor, Trichostatin A (TSA), on the expression of the CYP2B1 gene in rat primary hepatocyte cultures. This deacetylase inhibitor was ineffective as a modulator of the PB induction response, although TSA did markedly increase expression of both 18s and albumin mRNA.

Introduction

In eucaryotes, all DNA is packaged into chromatin by a group of basic proteins known as histones. DNA is complexed with a histone octamer to form nucleosomes, the basic unit of chromatin. 146 bp of DNA are tightly wound around this core which consists of the H2A, H2B, H3 and H4 histone proteins. H1 and H5, the linker histones, further constrain the DNA between the nucleosomes (Van Holde and Zlatanova, 1996). The linker histones bind externally to the nucleosome core; it is believed that they shield the charge of the DNA which links the nucleosomes and also act to stabilize the DNA strands as they wind around the octamer. H1 is hypothesized to further compact the chromatin into a higher ordered structure.

The nucleosome limits the access of *trans* acting transcription factors to the transcriptional machinery, thus regulating gene activity. A large body of literature points to histones and histone modifications as key modulators of gene transcription. The N-terminal domains of histones are subject to several post-translational modifications including: methylation, phosphorylation, ubiquitination, polyADP-ribosylation, and acetylation. Acetylation levels are likely determined by the dynamic equilibrium that exists between competing acetylase and deacetylase activities. It is believed that these activities decide the longevity of the unfolded, or transcriptionally active, nucleosomal form (Walia *et al.*, 1998). The current theory is that these highly charged N-terminal extensions interact strongly with the negatively charged DNA phosphodiesterase backbone and upon acetylation the positive charge on the histones is neutralized thus weakening interactions between the DNA and nucleosomal core, therefore allowing access to transcription factor binding. Upon acetylation, it has been demonstrated that the shape of the nucleosome is altered so as to decrease the number of times the DNA winds around the nucleosome (Bauer *et al.*, 1994). There are 26 N-terminal lysine sites per nucleosome that can be acetylated, hence giving rise to significant heterogeneity with respect to patterns of modifications (Loidl, 1994).

In 1988, using specific antibodies to acetylated histone H4, a direct link between core histone acetylation and transcriptionally active chromatin was shown (Hebbes *et al.*, 1988). Further studies have demonstrated that acetylation of H4 plays a key role in stimulating transcription factor binding to the nucleosomal DNA (Vettese *et al.*, 1996). In 1990, it was demonstrated that CpG islands, which represent a model of active

transcription, are depleted in histone H1, represent nucleosome free regions, show hyperacetylation of histones H3 and H4, and have a less compact structures (Tazi and Bird, 1990). Immunolabeling studies established that the inactive X chromosome virtually lacks all histone H4 acetylation (Jeppesen and Turner, 1993). DNase I sensitivity analysis of the chicken β -globin locus was found to map to areas that precisely matched hyperacetylated areas (Hebbes *et al.*, 1994). Examinations of the 5S RNA gene have determined that acetylation of core histones allows TFIIIA to bind the histone-DNA complex without any impediments, thus allowing gene transcription (Lee and Masson, 1993).

The recent discovery of Trichostatin A (TSA), a very specific histone deacetylase inhibitor (Yoshida *et al.*, 1995; Yoshida *et al.*, 1990), has enabled researchers to study the specific effects of histone acetylation. TSA treatment produces increased activation of many genes including: the tissue-type plasminogen activator (Arts *et al.*, 1995), the mouse mammary tumor virus (Bartsch *et al.*, 1996), the HIV-1 promoter (Van Lint *et al.*, 1996a) and the *MDR1* gene (Jin and Scotto, 1998). Recently, genes silenced by retroviral and adeno-associated viral sequences were shown to be reactivated in tissue culture models after treatment with TSA (Chen *et al.*, 1997). In rat liver primary hepatocytes, TSA treatment increased the inductive effects of TCDD on CYP1A1 gene expression over 200% (Xu *et al.*, 1997). Furthermore, in hepatoma cell lines that were deficient in CYP1A1 induction, TSA treatment restored AH receptor expression as well as CYP1A1 inducibility (Zhang *et al.*, 1996). Potentiation of thyroid hormone and retinoic acid mediated growth hormone expression have also been demonstrated upon treatment with TSA (Garcia *et al.*, 1997).

In the CYP2B genes, a 51 bp region determined to contain the phenobarbital responsive unit (PBRU) has been identified (Trottier, *et al.*, 1995). In the center of this region, there is a consensus site for the NF-1 transcription factor. *In vivo* footprinting studies have shown that the NF-1 footprint is extended in nuclear extracts from PB treated rats as compared to controls (Kim and Kemper, 1997). This result has led to further speculation regarding the involvement chromatin modification may be playing in PB induced gene expression. *In vitro*, NF-1 exhibits a strong binding interaction to this region (Ramsden *et al.*, unpublished results). Protein affinity purification studies also have demonstrated that histone H3 binds in this region (Ramsden *et al.*, submitted). Previous studies have also determined that the CTF/NF-1 family interacts with histone H3 and this interaction results in an alteration of the nucleosomal core (Alevizopoulos *et al.*, 1995). It

was suggested that CTF/NF-1 is a modulator of histone H1 binding as the predicted molecular structure suggests that CTF/NF-1 may function as a histone acetylase (Oikarinen, 1991; Oikarinen and Mannermaa, 1990). Other researchers have demonstrated that NF-1 can prevent repression of DNA replication by changing the local distribution of nucleosomes and thus increase accessibility to transcriptional initiation factors (Cheng and Kelly, 1989).

The recent finding that p300/CBP has histone acetyltransferase activity (Ogryzko *et al.*, 1996) is quite intriguing as cAMP additions or phosphatase inhibitors dramatically inhibit the phenobarbital mediated induction of the CYP2B genes (Sidhu and Omiecinski, 1995a). The N-terminal region of p300/CBP can interact with the ligand binding domain of RXR, an orphan receptor recognized as heterodimerizing with the CAR protein (Chakravarti *et al.*, 1996; Kamei *et al.*, 1996). CAR proteins have recently been identified as binding partners in the PBRU of the CYP2B genes in mouse hepatocytes (Honkakoski, *et al.*, 1998b).

To understand and address potential avenues of PB mediated gene regulation, we have initiated studies to examine histone acetylation as a potential modulator of transcription. The cAMP modulation of the CYP2B genes and the strong binding of NF-1 to the PBRU, both suggest a role for histone acetylation in transcriptional activation. The studies presented in this report examined the effects of TSA on the PB mediated induction of the CYP2B1 genes. Although histone acetylation modulates the transcriptional activity of other liver enzymes, TSA did not appear to have any effect on the PB mediated CYP2B1 activation. Although we can not rule out a potential role for the involvement of histone acetylation in the gene induction process, acetylation alone is not sufficient to activate or potentiate transcription of the CYP2B1 gene.

Methods and Materials

MATERIALS

PB was obtained from the University of Washington Hospital Pharmacy Services (Seattle, WA). Dexamethasone, Trichostatin A (TSA), 7-benzyloxyresorufin, ethoxyresorufin, and dicumarol were purchased from Sigma (St. Louis, MO). All hepatocyte cell culture material and Trizol™ were obtained from Life Technologies (Grand Island, NY). Matrigel, ITS+, and NuSerum were obtained from Collaborative Biomedical Products (Bedford, MA).

PRIMARY HEPATOCYTE ISOLATION AND CULTURE

Rat hepatocytes were isolated by a modification of the two-step collagenase perfusion *in situ* (Seglen, 1976) and cultured with modifications (Sidhu, et al., 1993; Sidhu and Omiecinski, 1996) of the protocols as described previously. A dilute concentration (233 µg/ml final concentration) of ECM (Matrigel) was added as an overlay 16 hrs after initial plating of the cells or as stated. Medium changes were conducted on a daily basis and a 25 nM dexamethasone concentration was maintained in all media.

TREATMENTS

PB (1.0 M) was dissolved in tissue culture grade water as a stock solution and stored in aliquots at -20°C. Dexamethasone (25 µM), β-naphthoflavone (BNF) (12 µg/ul), BROD (5 mM), EROD (5 mM), and TSA (10 µM) were dissolved in DMSO as stock solutions and also stored at -20°C. Dicumarol was prepared as a 10 mM solution in 0.2 N NaOH, and back titrated to pH 7.4. For activity assays, EROD and BROD were used at 10 µM and the dicumarol concentration was 25 µM. Cells were cultured for 24-48 hrs before the addition of any treatments. Final concentrations of each treatment were: PB, 500 µM, and BNF, 22 µM, unless otherwise stated. TSA was used at stated concentrations for each treatment. For all analyses, representative data are shown from multiple studies performed

independently with different hepatocyte preparations. Treatments at each time point and concentration were always performed in duplicate or triplicate.

ACTIVITY ASSAYS

For enzymatic activity assays, cells were washed twice with EBSS buffer that was supplemented with 5 mM MgCl₂, 2.5 mM CaCl₂, 15 mM HEPES, pH 7.4, and then gassed for 15 min with 95% O₂/ 5% CO₂. Cells were then incubated at 37°C for 30 min. in the above EBSS buffer plus 25 μM dicumarol. Ethoxyresorufin or benzyloxyresorufin was then added at a 10 μM final concentration. After 60 min at 37°C, the buffer was then collected and stored at -20°C until further analysis. O-dealkylase activity in the collected supernatants was measured using 535 nm excitation and 585 nm emission on a LS 50 luminescence spectrometer (Perkin Elmer, Foster City, CA). Resorufin (Sigma) was used to create a standard curve for activity levels.

RNA ANALYSIS

Total RNA was isolated (Chomczynski and Sacchi, 1987) using Trizol™ as described by the supplier. For slot blot evaluation, 5 μg of total RNA was applied directly to a Genescreen Plus nylon membrane (DuPont) under vacuum and denaturing conditions using a Minifold II apparatus (Schleicher and Schuell, Keene, NH). The membranes were hybridized with specific ³²P-radiolabeled oligonucleotides or cDNA probes for CYP2B1, CYP2B2, CYP1A1, Albumin and 18s rRNA, as described previously (Omiecinski, et al., 1990; Sidhu and Omiecinski, 1996). Resulting films from autoradiography were scanned densitometrically with a Howtek flatbed scanner and analyzed with BioImage™ Whole Band Analysis software (Millipore, Bedford, MA).

STATISTICAL ANALYSIS

Statistical analysis was performed using Prism analysis software (GraphPad, San Diego, CA). Repeated measures ANOVA followed by Dunnett's post comparison testing was used.

Results

EFFECTS OF TSA ON O-DEALKYLASE ACTIVITY

Preliminary studies demonstrated that TSA alone was unable to turn on CYP2B1 expression (data not shown). We therefore used variable amounts of TSA (10 nM to 10 μ M) in the presence of PB, either 100 μ M or 500 μ M, to look for a potentiation of BROD activity. Examination of activity levels was conducted after 24 hrs and 48 hrs of treatment in the presence of TSA. Figure 28 depicts the increases in BROD activity that were seen using PB treatment alone as our 100% baseline. The 10 μ M TSA treatment was able to significantly increase BROD activity levels after 48 hrs with 100 μ M PB (15%) and after 24 and 48 hr incubations with 500 μ M PB. The maximum increase in activity that we detected was 20%. As previous work has shown that TSA could potentiate the TCDD mediated induction of CYP1A1 (Xu, et al., 1997), examinations of EROD activity, after treatment with BNF and TSA were also conducted. As seen in Figure 29, increasing TSA concentrations did lead to a potentiation of EROD activity. This potentiation was maximal after 24 hrs of treatment with TSA and was approximately 150% when 10 μ M TSA was used. Our potentiation level was not quite as high as previously determined (Xu, et al., 1997), but differences in hepatocyte culture techniques and subsequent induction levels may account for this discrepancy.

EFFECTS OF TSA ON MRNA EXPRESSION LEVELS

Figure 30 shows the effects of TSA treatment on PB mediated CYP2B1 induction after 24 and 48 hrs. Interestingly, after 48 hrs treatment with 500 μ M PB and 10 μ M TSA, the TSA appeared to decrease the levels of CYP2B1 expression. As seen in Figure 31,

scanning densitometry revealed that this decrease was significant. Changes in CYP2B1 expression were not seen with any other TSA co-treatments. To normalize for equal loading of RNA, we also examined 18s ribosomal RNA and albumin expression. In conjunction with PB treatment, the 10 μ M TSA treatment appeared to visibly increase 18s and albumin mRNA levels. Upon treatment of 500 μ M PB and 1 μ M TSA, there is a 25% increase in 18s gene expression after 24 hrs. Similarly, the 10 μ M TSA treatment resulted in a 50% increase in 18s gene expression at 48 hrs (Figure 32). Albumin expression was increased more than 100% with the lower 10 nM TSA treatment and showed a 350% increase when hepatocytes were incubated with 500 μ M PB and 10 μ M TSA for 48 hrs (Figure 33).

The effects of TSA on BNF mediated CYP1A1 induction were not very striking, although a trend towards increasing expression upon incubation with increasing TSA concentrations was observed (Figures 34 and 35). In the presence of BNF, TSA also led to an increase in 18s and albumin mRNA expression levels (Figures 36 and 37). These increases were quite striking and obvious.

Discussion

Histones not only provide the necessary infrastructure for the transcriptional machinery, but their modulation is hypothesized to strongly impact the binding and accessibility of the *trans*-acting factors responsible for activating on gene expression. The studies presented in this paper demonstrated that the global histone deacetylase inhibitor, TSA, is unable to markedly increase CYP2B1 gene expression when primary hepatocytes are treated with TSA alone or in the presence of PB. Although statistically significant increases in BROD activity were seen with the very highest dose of TSA, 10 μ M, the increase was less than 20% and corresponding increases in messenger RNA levels were not observed. In fact, at the highest TSA dose with 100 μ M PB, the mRNA levels at 24 hrs actually decreased.

Therefore, it is likely that this result may be due to secondary effects that TSA can have on differentiation and cell cycling (Yoshida, et al., 1990).

Using differential display techniques, Van Lint et al. observed that less than 5% of the genes they examined (340 total) were changed after treatment with TSA (Van Lint *et al.*, 1996b). Interestingly, this number corresponds with the fraction of histones whose acetyl groups turnover rapidly (Wolffe, 1992). As histones are also modified by phosphorylation, ubiquitination, methylation and polyADP-ribosylation, it is quite possible that for the CYP2B genes acetylation alone is not enough to induce activation. Methylation has been demonstrated to be a cause of gene repression and it is possible that the balance of methylation and acetylation may finely tune the regulation of specific genes, with acetylation relieving methylation associated repression (Razin, 1998). Examination of the relationship between histone H3 phosphorylation and acetylation have shown that mitogen regulated kinases and butyrate sensitive deacetylases can act on a common population of nucleosomes, but the actions of the kinases and deacetylases are not coordinately regulated (Barratt *et al.*, 1994). Histone acetylation may be a requirement for gene activation, but the involvement of other post-translational modifications, as well as the recruitment of other critical coactivators may also be involved.

A surprising outcome of these studies was the dramatic increases in 18s and albumin mRNA expression that were seen upon co-treatment of hepatocytes with either BNF or PB with TSA. The large increases that were seen in albumin mRNA levels are likely due to the effects that TSA has on the induction of differentiation and the blocking of the cell cycle at both G1 and G2 (Yoshida, et al., 1990). Further studies are needed to understand how the acetylation process is affecting the regulation of 18s ribosomal RNA. It will also be important to examine the effects of histone acetylation on other housekeeping genes expressed by primary hepatocytes.

Although TSA, a global histone modulator, did not effect transcriptional activation by PB, we have not ruled out the possibility that highly specific changes in nucleosome and chromatin structure may occur within the proximal promoter and/or the PBRU. More definitive studies will need to be conducted to delineate specific histone acetylation patterns within these distinct regions. TSA is a global inhibitor that does not discriminate between different histones. The recent availability of multiple antibodies for specific histones, and specifically acetylated histone forms, together with enhanced discoveries of multiple

histone acetyltransferases and deacetyltransferases, will allow for more in-depth discrimination in future analyses.

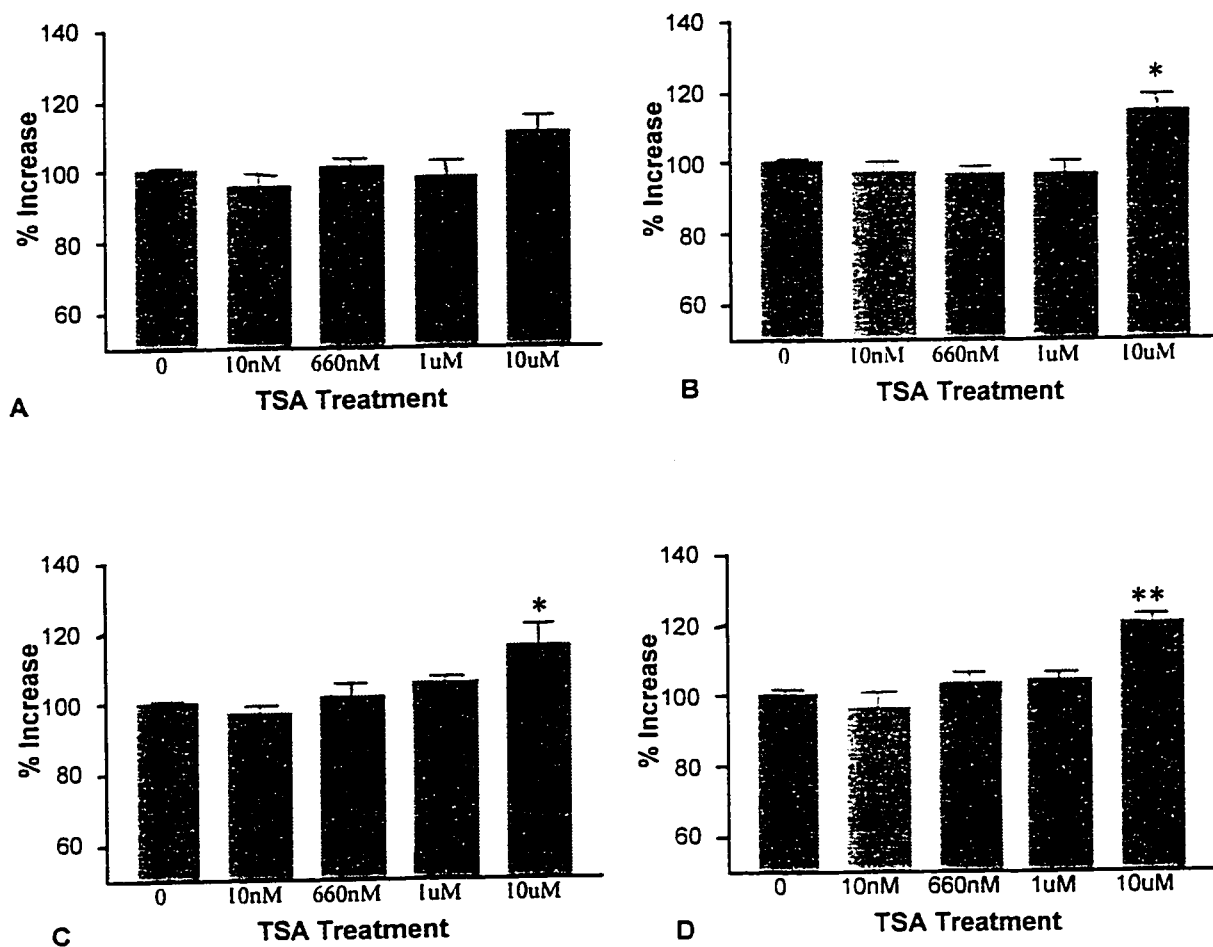


Figure 28. Effects of PB and TSA on BROD Activity.

After a period of 24 (Panels A & C) or 48 hrs (Panels B & D) in culture with PB and increasing TSA concentrations, BROD activity levels were measured in rat primary hepatocytes. Normalized values (% increase) are expressed relative to the PB induction response in the absence of TSA treatment. PB concentrations were either 100 μM (Panels A & B) or 500 μM (Panels C & D). Data represent the mean and SEM obtained from at least 2 independent rat hepatocyte preparations.

* p value < 0.05

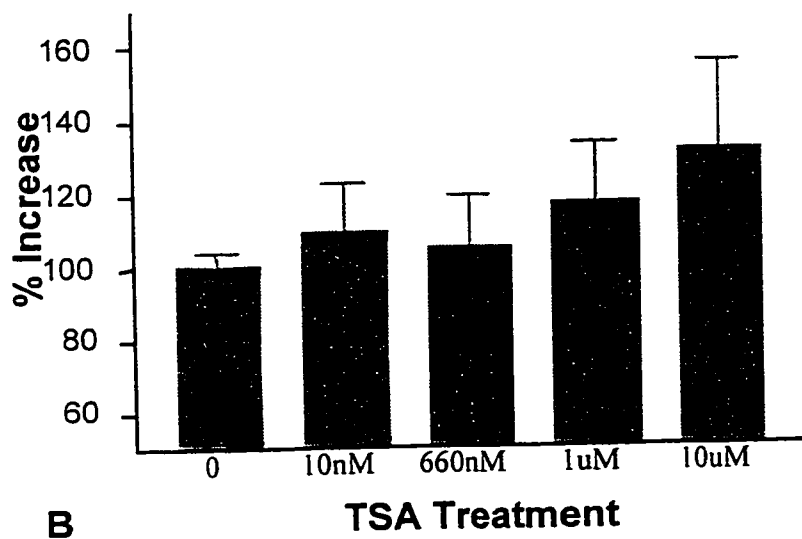
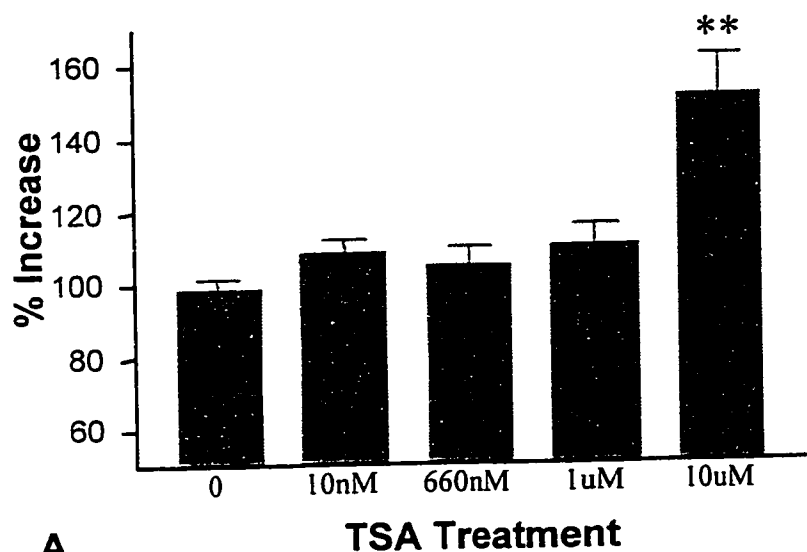


Figure 29. Effects of BNF and TSA on EROD Activity.

After a period of 24 (Panel A) or 48 hrs (Panel B) in culture with BNF and increasing TSA concentrations, EROD activity levels were measured in rat primary hepatocytes. Normalized values (% increase) are expressed relative to the BNF induction response in the absence of TSA treatment. BNF was used at 22 μ M with all treatments. Data represent the mean and SEM obtained from at least 2 independent rat hepatocyte preparations.

* p value < 0.05; ** p value < 0.01

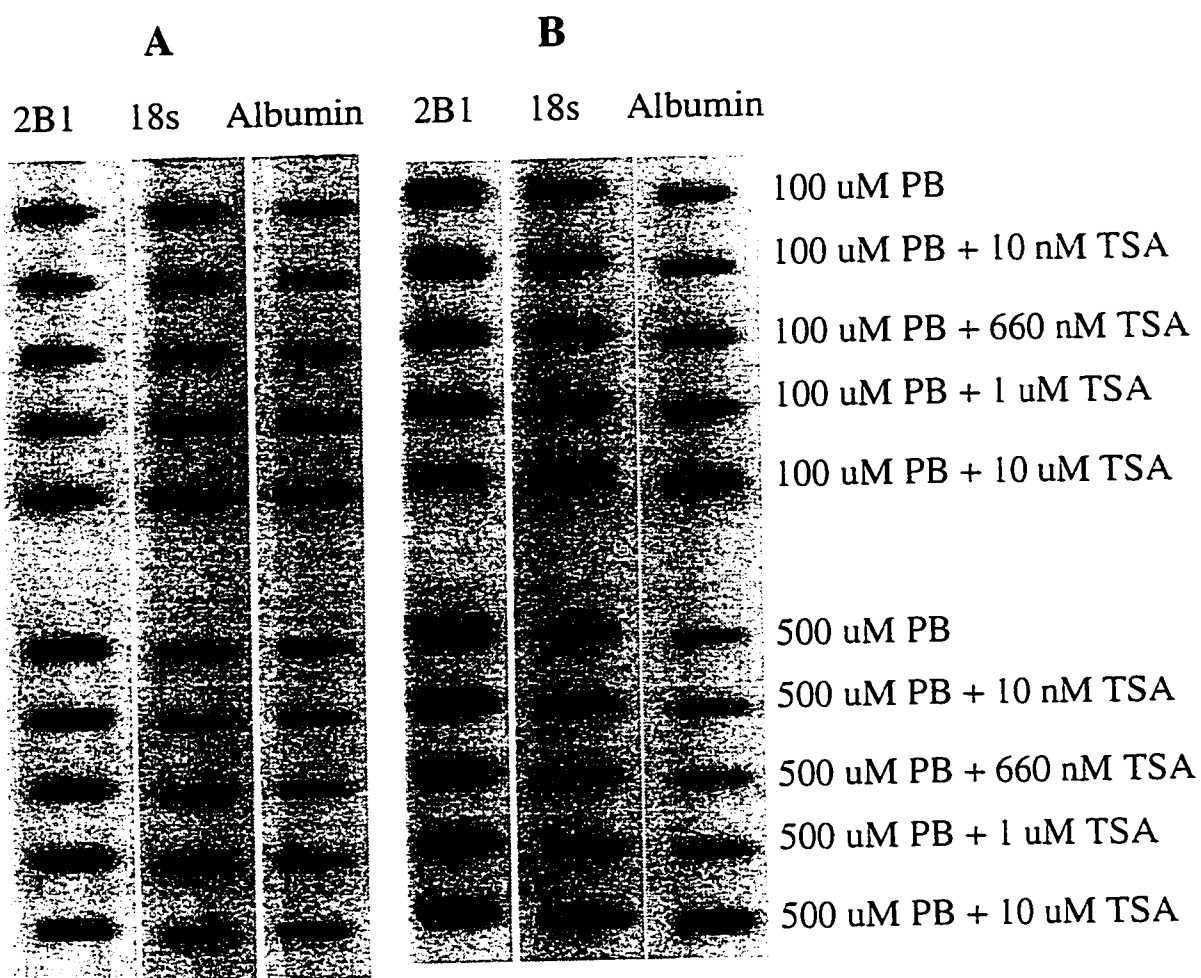


Figure 30. The Effects of PB and TSA on CYP2B1, 18s, and Albumin mRNA Expression.

After a period of 48 hrs in culture, primary rat hepatocytes were treated with PB and increasing concentrations of TSA for either 24 hrs (Panel A) or 48 hrs (Panel B). Total RNA was then isolated and evaluated by slot-blot hybridization analysis. Blots shown are representative of the response that was demonstrated in multiple hepatocyte preparations.

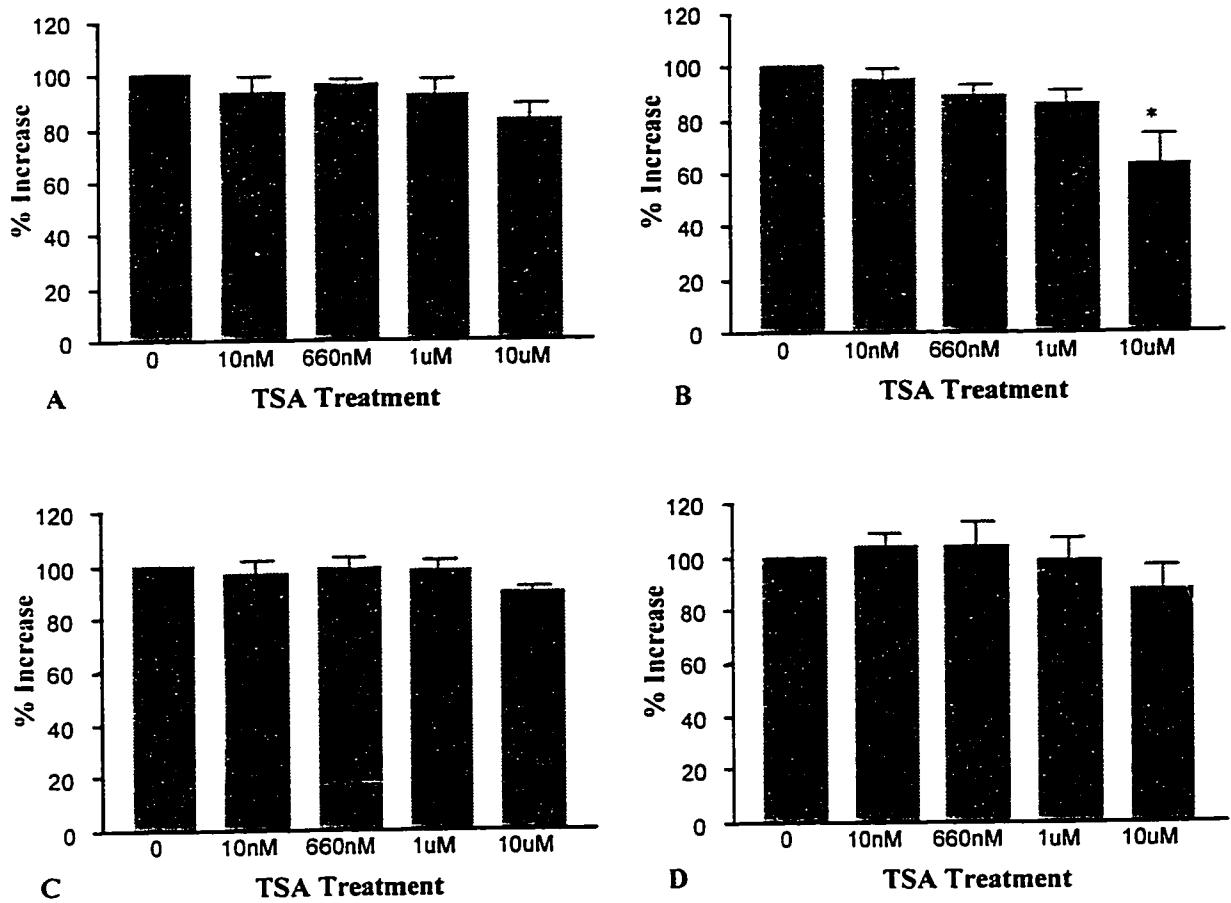


Figure 31. Densitometric Evaluation of Slot Blot Data for the TSA Mediated Modulation of PB Inducible CYP2B1 Gene Expression.

Autoradiographic data were quantified by whole band analysis as described in Materials and Methods. Values were then expressed as a percent increase relative to the PB treatment alone. Panel A, 100 μM PB and 24 hr TSA co-treatments; Panel B, 100 μM PB and 48 hr TSA co-treatments; Panel C, 500 μM PB and 24 hr TSA co-treatments; Panel D, 500 μM PB and 48 hr TSA co-treatments. Data represent the mean and SEM obtained from at least 2 independent rat hepatocyte preparations.

* p value < 0.05

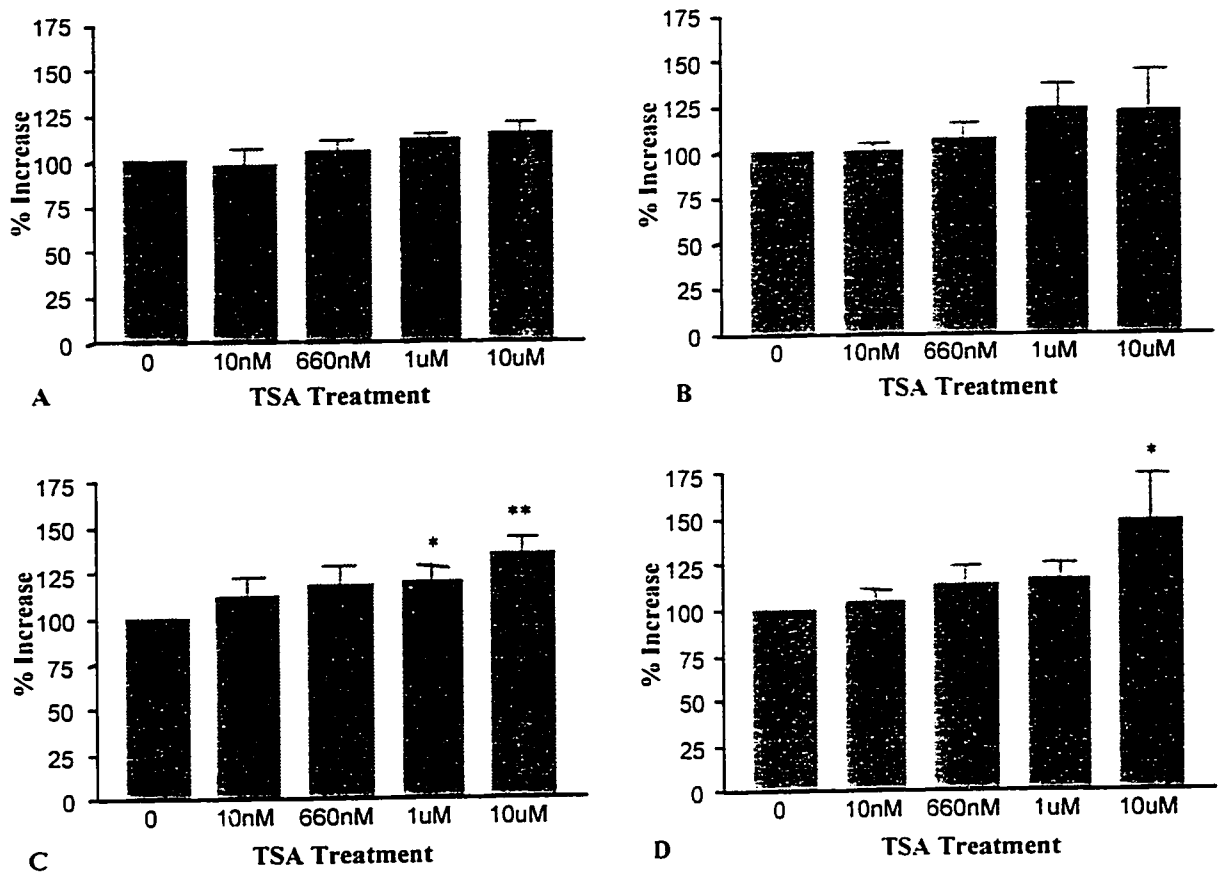


Figure 32. Densitometric Evaluation of Slot Blot Data for the PB and TSA Mediated Modulation of 18s Gene Expression.

Autoradiographic data were quantified by whole band analysis as described in Materials and Methods. Values were then expressed as a percent increase relative to the PB treatment alone. Panel A, 100 μM PB and 24 hr TSA co-treatments; Panel B, 100 μM PB and 48 hr TSA co-treatments; Panel C, 500 μM PB and 24 hr TSA co-treatments; Panel D, 500 μM PB and 48 hr TSA co-treatments. Data represent the mean and SEM obtained from at least 2 independent rat hepatocyte preparations.

* p value < 0.05

** p value < 0.01

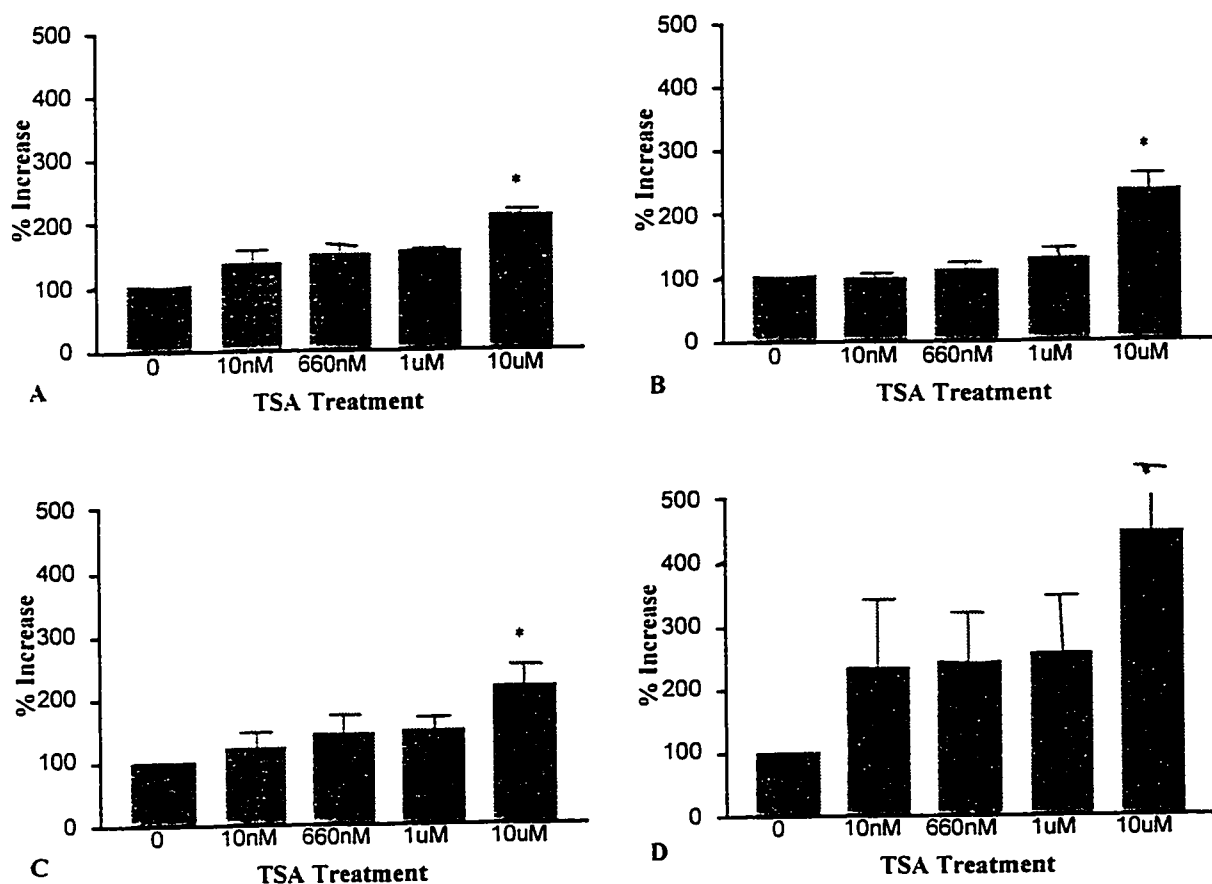


Figure 33. Densitometric Evaluation of Slot Blot Data for the PB and TSA Mediated Modulation of Albumin Gene Expression.

Autoradiographic data were quantified by whole band analysis as described in Materials and Methods. Values were then expressed as a percent increase relative to the PB treatment alone. Panel A, 100 μ M PB and 24 hr TSA co-treatments; Panel B, 100 μ M PB and 48 hr TSA co-treatments; Panel C, 500 μ M PB and 24 hr TSA co-treatments; Panel D, 500 μ M PB and 48 hr TSA co-treatments. Data represent the mean and SEM obtained from at least 2 independent rat hepatocyte preparations.

* p value < 0.05

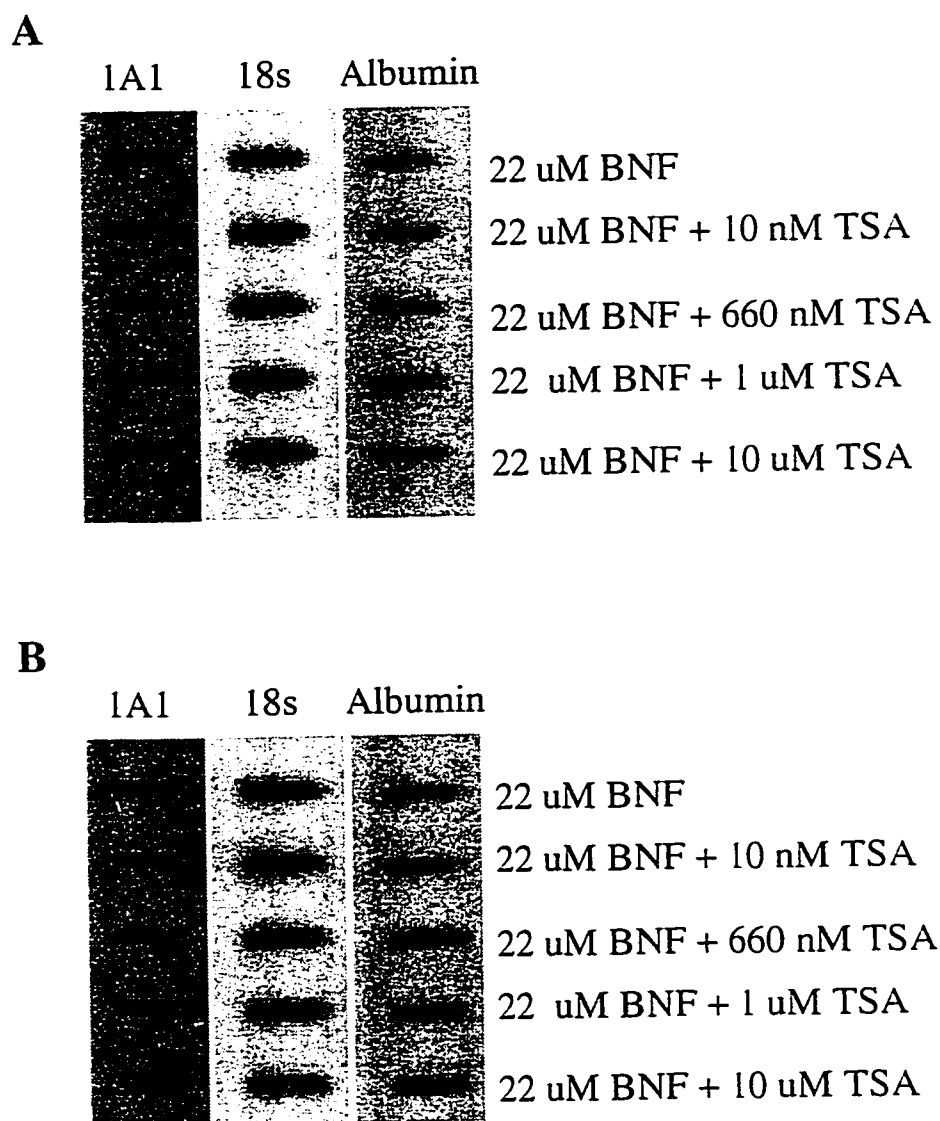


Figure 34. The Effects of BNF and TSA on CYP1A1, 18s, and Albumin mRNA Expression.

After a period of 48 hrs in culture, primary rat hepatocytes were treated with BNF and increasing concentrations of TSA for either 24 hrs (Panel A) or 48 hrs (Panel B). Total RNA was then isolated and evaluated by slot-blot hybridization analysis. Blots shown are representative of the response that was demonstrated in multiple hepatocyte preparations.

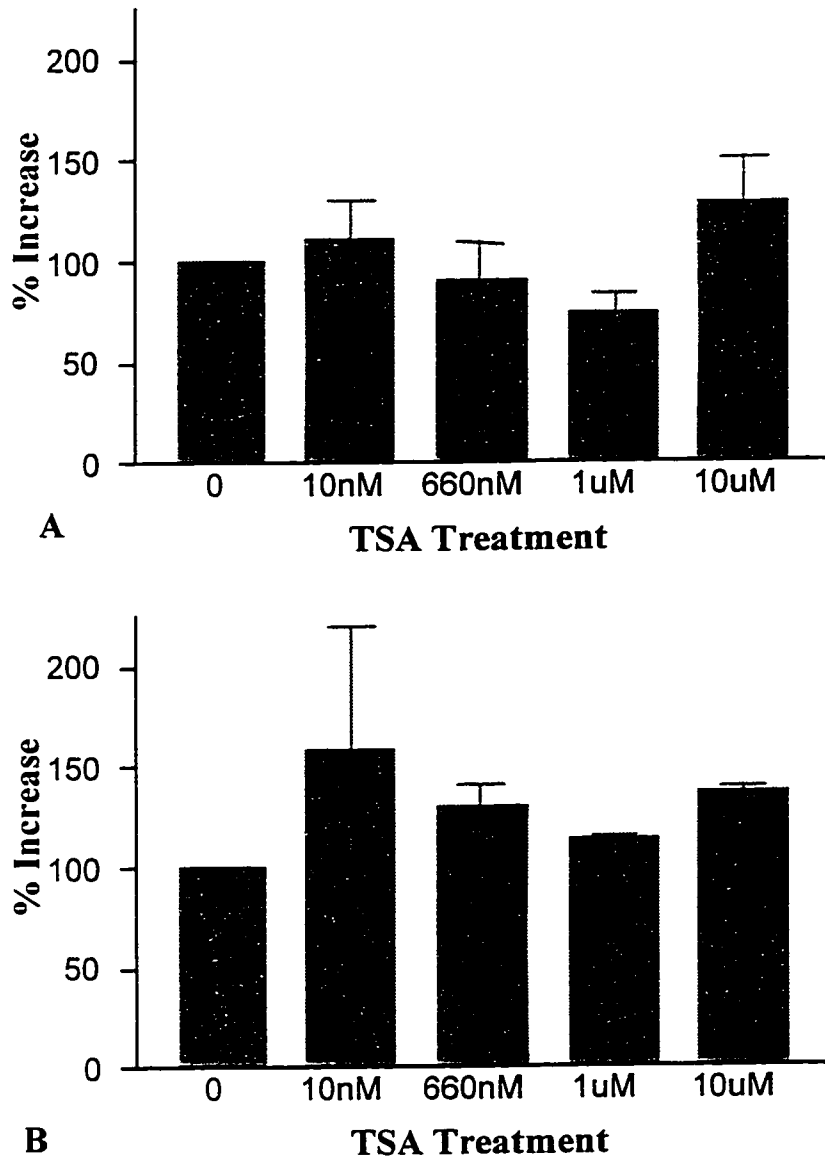
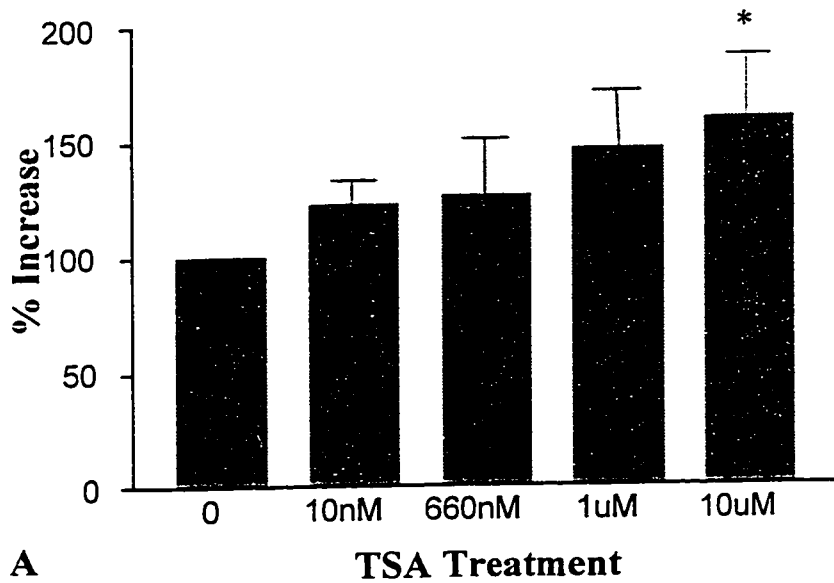
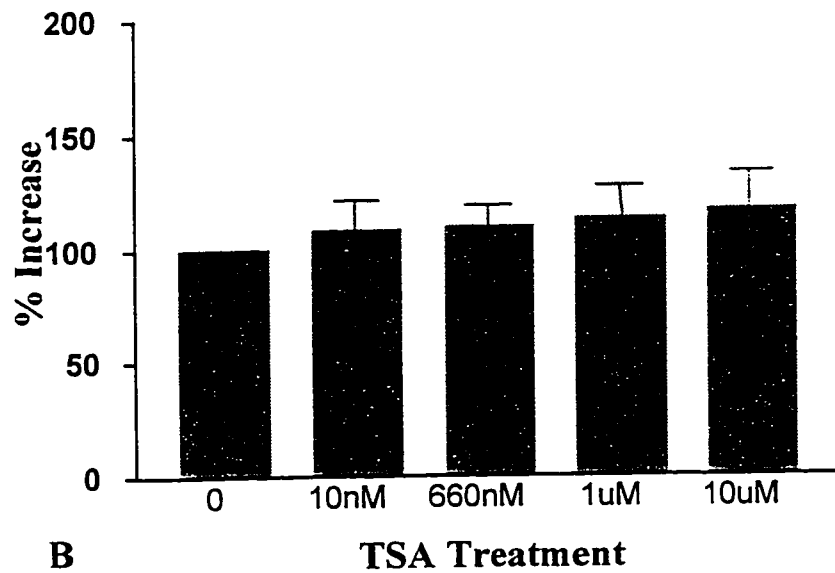


Figure 35. Densitometric Evaluation of Slot Blot Data for the TSA Mediated Modulation of BNF Inducible CYP1A1 Gene Expression.

Autoradiographic data were quantified by whole band analysis as described in Materials and Methods. Values were then expressed as a percent increase relative to the PB treatment alone. Panel A, 22 μ M BNF and 24 hr TSA co-treatments; Panel B, 22 μ M BNF and 48 hr TSA co-treatments. Data represent the mean and SEM obtained from at least 2 independent rat hepatocyte preparations.



A



B

Figure 36. Densitometric Evaluation of Slot Blot Data for the BNF and TSA Mediated Modulation of 18s Gene Expression.

Autoradiographic data were quantified by whole band analysis as described in Materials and Methods. Values were then expressed as a percent increase relative to the PB treatment alone. Panel A, 22 μ M BNF and 24 hr TSA co-treatments; Panel B, 22 μ M BNF and 48 hr TSA co-treatments. Data represent the mean and SEM obtained from at least 2 independent rat hepatocyte preparations. * p value < 0.05

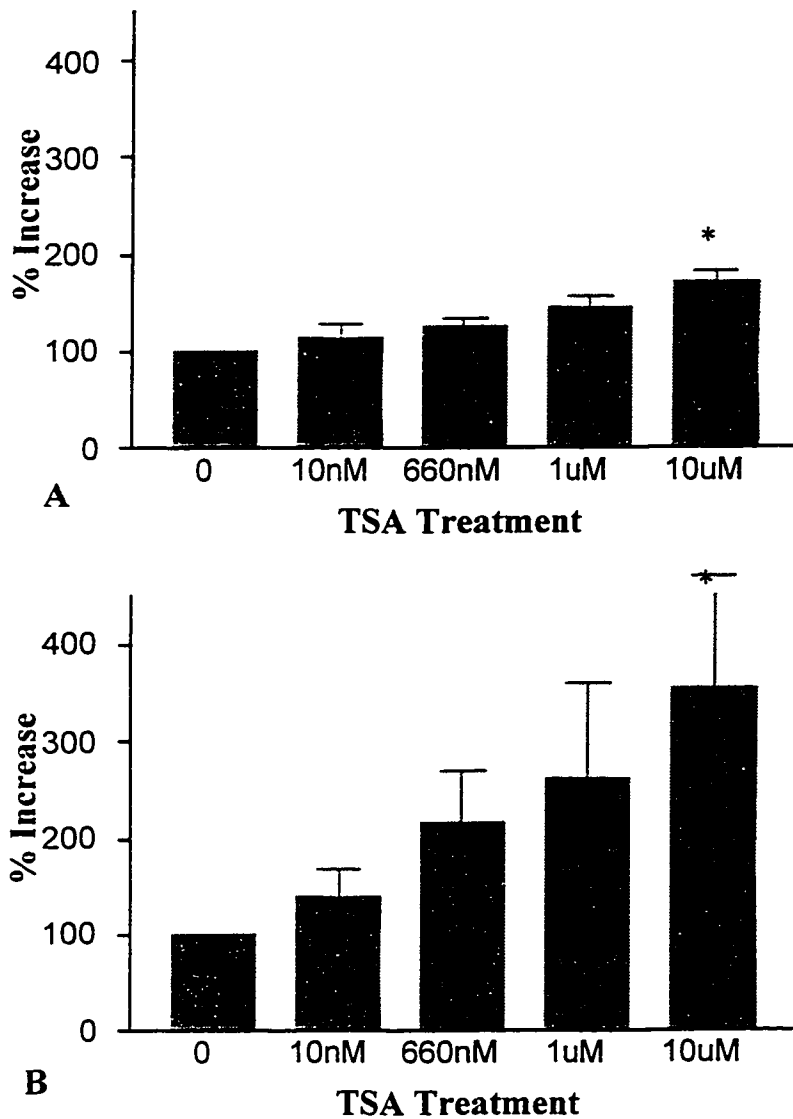


Figure 37. Densitometric Evaluation of Slot Blot Data for the BNF and TSA Mediated Modulation of Albumin Gene Expression.

Autoradiographic data were quantified by whole band analysis as described in Materials and Methods. Values were then expressed as a percent increase relative to the PB treatment alone. Panel A, 22 μ M BNF and 24 hr TSA co-treatments; Panel B, 22 μ M BNF and 48 hr TSA co-treatments. Data represent the mean and SEM obtained from at least 2 independent rat hepatocyte preparations. * p value < 0.05

CHAPTER 5: SUMMARY AND CONCLUSIONS

Understanding the mechanisms by which PB regulates CYP2B gene expression has not been a straightforward and simple task. When I first began my research, very little was understood about possible mechanisms of control. The induction response was considered to be a 'black box' brimming with unknowns. To address these multiple unknowns, my dissertation work approached the problem from three distinct angles.

1) FUNCTIONAL CHARACTERIZATION WORK

The first angle took a functional approach. Encompassing Specific Aim 1, I sought to identify the 5'-upstream regions that were critical for the regulation of the PB induction response. As primary hepatoma cell lines do not respond to PB, it was necessary to use primary hepatocyte cultures to probe gene regulation processes. Significant effort was expended in trying to successfully transfect primary cells. My achievements matched those stated in the published literature, but the transfection efficiencies obtained were too low to conduct the types of studies I had envisioned. Baculovirus mediated transfection protocols seemed very promising, and indeed I did have great success in using baculovirus vectors as a tool for gene delivery. Unfortunately, the baculovirus compromised the PB induction response and again probing the gene regulation processes was not possible. Fortunately, significant progress was made within our laboratory, as well as others, that helped to identify critical regions for induction.

The baculovirus system, although not useful for functional characterization, did yield useful data. This was in the area of cytokine expression and CYP2B activation. Although there is a large body of literature examining cytokine expression and CYP induction, relatively little has been published on the CYP2B gene family and the role cytokines may play in regulating gene induction. My work has made a significant contribution in this area by documenting how a battery of cytokines can repress the PB mediated CYP2B induction response. Additionally, as baculovirus mediated gene delivery is being considered as a method for liver directed gene therapy in humans, the

characterization of baculovirus effects on hepatocytes has yielded some very important information, specifically that the presence of the baculovirus induces an inflammatory response which includes the activation of IL-1 α , IL-1 β , and TNF- α in primary hepatocyte cultures. Further research need to be conducted before human gene therapy studies can be deemed safe and effective.

2) PROTEIN-DNA BINDING STUDIES

My second approach, encompassing Specific Aim 2, involved examination of protein-protein and protein-DNA interactions. This work was quite fruitful as affinity purification was used to examine proteins binding to the PBRU. An ideal result would have allowed us to identify a particular protein that was binding only in the PB or control state, but in all the protein-DNA binding experiments conducted the binding levels were equal. However, these studies did allow us to rule out the importance of certain transcription factors, i.e. NF- κ B, in regulating PB responsiveness. As recent literature had been implying the importance of the NF- κ B factor, these studies in conjunction with transgenic mice analyses, will help to direct the research attention to other areas of the PBRU. Further work is now underway in the laboratory, under the direction of other laboratory members, to examine protein-protein and protein-DNA interactions among factors other than NF- κ B.

3) EXAMINATION OF POST-TRANSLATIONAL MODIFICATIONS

Investigation of the roles that post-translational modifications may play in modulating PB responsiveness encompassed Specific Aims 3 through 5. Although a PBRU was characterized, distinct proteins critical for mediating CYP2B induction have not yet been absolutely identified. This fact has precluded the examination of the phosphorylation status of specific PB-modulatory proteins.

Instead, I examined the changes in second messenger systems that occur upon the initial exposure of hepatocytes to PB. Multiple recent reports have suggested a requirement for phosphorylation and dephosphorylation events in controlling PB mediated CYP2B expression, but they are not all in agreement as to the specific mechanisms involved. Although cAMP itself has clearly been shown to modulate the PB induction process, the studies presented here demonstrated that PB itself does not activate this pathway. PB

markedly induced CYP2B expression without producing any detectable increases in cAMP levels or PKA activity.

Further examination of post-translational modifications included the examination of the effects of TSA, a specific histone deacetylase inhibitor, on the PB mediated CYP2B induction process. Histone acetylation has been shown to increase gene expression of many diverse gene products, including other CYP genes. Additionally, cofactors involved in controlling cAMP mediated gene activation, such as p300/CBP, have been shown to act as histone acetyltransferases. Since cAMP is an effective modulator of CYP2B induction, I examined the effects of changes in histone acetylation on the PB induction process. Although TSA did not appear to have any effect on PB mediated CYP2B activation in the studies presented here, we can not rule out a potential role for the involvement of histone modifications in the gene induction process as highly specific changes in nucleosome and chromatin structure may occur within the proximal promoter and/or the PBRU. The recent availability of multiple histone acetyltransferases and deacetyltransferases, as well as multiple antibodies for specific histones and specifically acetylated histone forms will allow for more in-depth analysis of these factors in the future.

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APPENDIX A: PHENOBARBITAL RESPONSIVENESS CONFERRED BY THE 5'-FLANKING REGION OF THE RAT CYP2B2 GENE IN TRANSGENIC MICE

The following manuscript has been accepted to *Gene* pending revisions which are in progress. The authors are: Richard Ramsden, Nancy B. Beck, Karen M. Sommer, and Curtis J. Omiecinski.

Abstract

Phenobarbital (PB) is a prototype for a class of agents that produce marked transcriptional activation of a number of genes, including certain cytochrome P450s. We used transgenic mouse approaches and multiple gene reporters to assess the functional consequences of specific deletions and site-specific mutations within the 2.5kb 5'-flanking region of the rat *CYP2B2* gene. Protein-DNA interactions at the PBRU domain also were characterized. Using the transgenic models, we demonstrate that sequences between -2500 and -1700 bp of the *CYP2B2* gene are critical for PB induction; mice with 1700 or 800 bp of 5'-flanking *CYP2B2* sequence are not PB responsive. DNA affinity enrichment techniques and immunoblotting and electromobility shift assays were used to determine that nuclear factor 1 (NF-1) interacts strongly with a site centered at -2200 bp in the PB responsive unit (PBRU) of *CYP2B2*. To test the functional contribution of NF-1 in PB activation, we introduced specific mutations within the PBRU NF-1 element and demonstrated that these mutations completely ablate the binding interaction. However, transgenic mice incorporating the mutant NF-1 sequence within an otherwise wild-type -2500/*CYP2B2* transgene maintained full PB responsiveness. These results indicate that NF-1 interaction

within the PBRU is not a critical factor directing PB transcriptional activation. Other factors were detected that interact with this region, including HNF-4, and likely play key regulatory roles in the PB induction process.

Introduction

Phenobarbital (PB) is a prototype for a class of agents that produce marked transcriptional activation of a number of genes, including certain cytochrome P450s (CYP), aldehyde dehydrogenase, NADPH-P450 oxidoreductase, and individual phase II enzyme systems (Waxman and Azaroff, 1992; Frueh et al., 1997). The PB induction response occurs in many species, including humans (Morel et al., 1990; Hansen et al., 1989; Wen and Fulco, 1987; Honkakoski and Lang, 1989). The rat is a well-characterized animal model of gene induction. In particular, the rat *CYP2B1* and *CYP2B2* genes exhibit basal expression levels that are extremely low, but are induced markedly in the liver within a few hours after PB treatment (Omiecinski et al., 1985). The PB induction response is transcriptionally mediated (Hardwick et al., 1983), however, the molecular mechanisms underlying the activation process are not clearly defined.

It is noteworthy that hepatocytes are heterogeneous in their PB induction character, with perivenous cells being much more responsive than cells in the periportal region of the liver lobule (Baron et al., 1981; Hassett et al., 1989). The heterogeneity of expression is maintained in primary hepatocyte cultures, suggesting that the factors that facilitate the induction response are imprinted zonally within the liver lobule (Bars et al., 1992).

A 17 bp "barbie box" sequence was identified within the promoter regions of several PB-inducible genes and implicated in barbiturate regulation of the cytochrome P450_{BM-1} gene in *Bacillus megatarium* (Shaw and Fulco, 1993; He and Fulco, 1991). However, the role of this P450_{BM-1} element and the corresponding Bm1P1 protein regulator in the induction process has recently been questioned (Shaw et al., 1998). An accumulating body of evidence suggests that the barbie box is not a critical regulator of PB induction in

mammalian liver cells (Stoltz et al., 1998; Honkakoski and Negishi, 1997a; Park et al., 1996a). Rather, experimental results from rat and mouse studies have implicated a PB responsive unit (PBRU) far upstream of the barbie box element as modulating PB activation. In primary rat hepatocyte gene transfection studies, Anderson and co-workers (Trottier et al., 1995) initially delineated a PBRU as residing between -2317 bp to -2155 bp upstream of the transcription start site of the *CYP2B2* gene. Similar findings were obtained in transfection assays performed *in situ* in rat liver (Park et al., 1996a). In transfected mouse hepatocytes, an analogous region from -2258 to -2170 of the *Cyp2b10* gene was identified with transfected gene constructs as conferring PB responsiveness (Honkakoski and Negishi, 1997a). DNA transfection efficiencies achieved in these studies were not reported.

In the mouse hepatocyte studies, the PBRU was characterized as containing two critical binding motifs, a nuclear receptor site and another for nuclear factor 1 (NF-1) (Honkakoski and Negishi, 1997a). This region exhibits 91% sequence identity with the corresponding area of the rat *CYP2B2* gene PBRU, where an NF-1 site also has been implicated as an element regulating PB induction (Stoltz et al., 1998). Since transfection efficiencies in primary hepatocytes are likely to be extremely low (< 1%) (Ourlin et al., 1997), and levels of PB induction for transfected DNA constructs in hepatocytes do not approach that of the endogenous liver genes (Trottier et al., 1995; Honkakoski and Negishi, 1997a), uncertainties remain with respect to the extrapolation of these transfection results to the *in vivo* situation

In this study, we used transgenic mouse approaches to assess the functional consequences of specific deletions and site-specific mutations within the 2.5 kb upstream region of the rat *CYP2B2* gene. Protein-DNA interactions at the PBRU domain also were characterized. Using the transgenic models, we demonstrate that sequences between -2500 and -1700 bp of the *CYP2B2* gene are critical for PB induction. Based on several lines of evidence implicating the NF-1 element of the PBRU as a PB response modulator, we constructed lines of transgenic mice with mutated and non-functional NF-1 binding sites within the *CYP2B2* PBRU. The transgenes in these mice remained highly PB inducible. We conclude that the NF-1 regulatory component of the PBRU is not a critical contributor to the PB induction response.

Materials and Methods

IN VITRO DNASE I FOOTPRINTING

A pBluescript clone containing CYP2B2 5' flanking gene sequence encompassing -2271/-2186 was used for *in vitro* DNase I footprinting. *Hind* III (sense strand) or *Xba* I (anti-sense strand) digestions were followed by a DNA polymerase I (Klenow fragment) fill-in reaction in the presence of α -³²P-dCTP. Plasmids were then digested with *Xba* I (sense strand) or *Hind* III (anti-sense strand) followed by separation of the *CYP2B2* probe from vector by non-denaturing acrylamide gel electrophoresis. Footprinting assays were performed as described (Anonymous 1994) with slight modifications of the binding buffer composition. Crude nuclear protein extracts from liver of control or PB-treated rats (1, 2, or 5 μ g) were incubated with 50,000 cpm of the labeled probe in 50 mM NaCl, 5 mM MgCl₂, 1 mM CaCl₂, 4 μ g·ml⁻¹ poly dIdC, 0.1 mM EDTA, 20 mM HEPES (pH 7.6), 10% glycerol, 0.5 mM DTT and 50 mg·ml⁻¹ bovine serum albumin (BSA). DNase I digestion was performed using a concentration of 0.02 units per 200 μ l reaction.

GENE CONSTRUCTS AND TRANSGENIC MICE

A *Bst* XI/*Xho* I fragment comprising +25 to -2499 bp of the *CYP2B2* gene 5'-flanking region was derived from an isolate (clone 26-1) of a Sprague-Dawley rat genomic library described previously (Ramsden et al., 1993). This fragment was blunt-ended with the Klenow fragment of DNA polymerase I and ligated to the 2158 bp human growth hormone gene obtained from plasmid Tf-hGH (a gift from Dr. GS McKnight, University of Washington). The construct was then ligated into vector pBS', a slightly modified pBluescript plasmid (Ramsden et al., 1993). The structure of the resulting clone, designated -2500/hGH, was verified by restriction digestion and DNA sequencing. The

junction of the two fragments was as follows: 5' (*CYP2B2*) ACCGT GGTTA CAACCA GATCC CAAGG CCCAA (hGH) 3'.

A genomic fragment encompassing 2500 bp of the *CYP2B2* 5' flanking sequence, plus all *CYP2B2* exons and introns, and approximately 5000 bp of 3' flanking sequence, was constructed by ligating three fragments: the *Bam* HI/*Ksp* I fragment from -2500/hGH (-802/-2499 plus linker), the *Bam* HI fragment (-802/exon 4) and the *Bam* HI/*Ksp* I fragment (exon 4/3' plus vector pBS'), each from clone pBS'-19E (Ramsden et al., 1993). The structure of the resulting 21,500 bp clone, designated -2500/2B2, was verified by restriction digestion and partial DNA sequencing. Another construct also was prepared, designated NF1 mut-2500/2B2, and was identical to the -2500/2B2 clone except for two nucleotide substitutions within the putative NF-1 binding site (deVries E. et al., 1987): 5' (-2216)....T[G→t]GCACAGTGC[C→a]A.... (-2204) -3' (the NF-1 binding site is bolded; site-directed mutations are indicated in lower case). The NF-1 mut-2500/2B2 clone was constructed as described, except that the *Bam* HI/*Ksp* I fragment was mutated by the QuikChange™ method (Stratagene) using the mutagenic primers: NF1mutFP, 5'-TTCCT GACCT TtGCA CAGTG CaACC ATCAA CTTG-3', and NF1mutRP, its corresponding complement. The structure of the NF-1 mut-2500/2B2 21, 500 bp construct was verified as described.

All constructs were restricted with the appropriate enzymes and the gene fragments purified as described (Ramsden et al., 1993). To produce truncated 5'-flanking region derivatives, the -2500/hGH plasmid was cut with *Xho* I to produce the -2500/hGH transgene, *Xho* I/*Eco* RV to produce the -1700/hGH construct, and with *Xho* I/*Bam* HI for the resulting -800/hGH derivative. The -2500/2B2 plasmid and its NF-1 mutated counterpart were restricted with *Not* I. Microinjection of the gel-purified linear DNA molecules and oocyte implantation was performed by the University of Washington Department of Comparative Medicine. Transgenic founder mice were crossed to C57BL/6J mice and F1 animals were screened by PCR using oligonucleotide primers specific for the transgenes (Ramsden et al., 1993). Copy number was determined by phosphorimaging of a Southern blot probed with a ³²P-labeled transgene fragment using a Bio-Rad GS-525 Molecular Imager.

RNA ANALYSIS

Mice of at least six weeks of age were treated intraperitoneally (i.p) with PB at 80 mg/kg body weight or with saline for control, 17 h prior to sacrifice. Partial hepatectomy was performed on several animals, removing ~50 mg liver tissue for control analysis followed immediately by PB administration according to standard methods. RNA was isolated, then Northern blot or slot blot hybridizations were performed as described (Ramsden et al., 1993). Blots were probed with ³²P-labeled oligonucleotides specific for CYP2B2, CYP2B1, *Cyp2b10* and 18S ribosomal RNA (Ramsden et al., 1993). The hGH samples were probed with a ³²P-labeled hGH cDNA. Phosphorimage analyses was performed to generate relative signal intensity data after normalization to the 18S RNA standard.

ISOLATION OF NUCLEAR PROTEIN

Crude nuclear protein extracts from whole rat livers were obtained from Sprague-Dawley rats 4h after i.p. injection with 80 mg/kg body weight PB (in saline) or with saline alone (control). Nuclear proteins were obtained as described previously (Gorski et al., 1986), with slight modifications (Luckow and Schutz, 1987). Whole livers were initially homogenized in a buffer consisting of 50 mM Tris-HCl (pH 7.5), 20 mM KCl, 5 mM MgCl₂, 0.5 M sucrose, 0.15 mM spermine, 0.5 mM spermidine, 10 mM sodium fluoride, 1 mM sodium orthovanadate, and a protease inhibitor (Sigma) cocktail which contained antipain, chymostatin, and pepstatin (2 µg/ml each), aprotinin and leupeptin (5 µg/ml each), trypsin inhibitor (10 µg/ml), and phenylmethanesulfonyl fluoride (0.1 mM). Homogenized cells were layered over 50 mM Tris-HCl (pH 7.5), 20 mM KCl, 5 mM MgCl₂, 0.88 M sucrose, 0.15 mM spermine, and 0.5 mM spermidine, 10 mM sodium fluoride, 1 mM sodium orthovanadate, and protease inhibitor cocktail and centrifuged at 4000 x g in an SW 28 rotor (Beckman) for 10 min at 4°C. Pellets were resuspended in 10 mM HEPES (pH 7.6), 25 mM KCl, 1 mM EDTA, 2 M sucrose, 1% glycerol, 0.15 mM spermine, and 0.5 mM spermidine, 10 mM sodium fluoride, 1 mM sodium orthovanadate, and the protease inhibitor cocktail and the remaining extraction performed as described (Gorski et al., 1986) with the addition of 10 mM sodium fluoride and 1 mM sodium orthovanadate in the nuclear lysis and dialysis buffers. In addition, RNA was extracted

from a small portion of all liver tissue and analyzed in hybridization studies in order to confirm the PB induction response.

AFFINITY PURIFICATION OF DNA-BINDING PROTEINS

Enrichment of nuclear extracts for proteins with specificity for the *CYP2B2* PBRU was conducted using an affinity purification method previously described (Gabrielsen and Huet, 1993). A Klenow fill in reaction was performed on 400 μ g of *Xba* I and *Pst* I restricted plasmid *CYP2B2* -2271/-2186 pBluescript KS- in the presence of 5 nmoles biotin-16-dUTP (Boehringer Mannheim), followed by digestion with *Bst* XI and *Sac* I. Coupling of the protein binding site to magnetic beads was achieved by incubating the resulting DNA with M280-Streptavidin Dynabeads™ (Dynal) at a ratio of 10 pmoles of insert DNA·mg⁻¹ Dynabeads™ as described (Gabrielsen and Huet, 1993). A *CYP2B2* -2271/-2186 fragment containing a mutated NF-1 site was similarly labeled and complexed to magnetic beads. The mutated NF-1 site differed from the wild type at the two nucleotide positions described previously for the transgenic mice construct. Affinity purification was performed on crude nuclear extracts prepared from whole livers of control or PB-treated rats. In each experiment, 1 mg of nuclear extract was incubated at 4°C for 30 minutes with 10 mg of *CYP2B2* -2271/-2186 wild type or mutated complexed with 10 mg of Dynabeads™. Incubations were performed in TGED buffer (Gabrielsen and Huet, 1993) containing 150mM KCl, 50 mM sodium fluoride, and 0.2 mM sodium orthovanadate (150mM KCl-TGED). Dynabeads™ were washed 4 times with 150mM KCl-TGED containing poly dIdC (13 fold excess by weight). Proteins were eluted in 125 μ l of 1.5 M KCl-TGED, dialyzed 1 hour at 4°C on 0.025 μ m Millipore "V" Series Membranes against 50 mls of 40 mM KCl-TGED, and stored at -80°C.

PROTEIN ELECTROPHORESIS AND IMMUNOBLOTTING

Equal volumes of affinity purified proteins were separated via SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a Mini-Protean II 10% Tris-HCl precast gel (Bio-Rad) according to the manufacturer's protocol. Proteins were transferred onto Immobilon P

membranes (Millipore) and then probed with specific antibodies. NF- κ B antibody, directed against the p50 subunit, was generously provided by Nancy Rice at the NCI Frederick Cancer Research and Development Center. HNF-4 antibody (provided by Dr. Frances Sladek, University of California, Riverside), NF-1 antibody (Santa Cruz Biotechnology) and Histone H3 antibody (Cortex Biochem) were all used at conditions specified by the suppliers. The secondary antibody used was goat-anti-rabbit-HRP (Pierce) or rabbit-anti-sheep-HRP (Zymed) at a 1:5000 dilution. ECL (Amersham) was used for chemiluminescent visualization of specific immunoreactive proteins.

Results

Transgenic mice were developed that included 2500 bp of 5' flanking sequence of the *CYP2B2* gene fused to the *CYP2B2* structural gene itself. Four out of the five -2500/2B2 lines derived with this flanking region were responsive to PB in the liver (Figure 1); the remaining line exhibited no expression of the transgene in either untreated or PB treated animals (data not shown). When the rat *CYP2B2* gene was used as its own reporter, expression patterns of these -2500 constructs closely mimicked the expression of the endogenous gene in the rat (Omiecinski, 1986).

To test whether sequences downstream from the 5'-flanking region of the *CYP2B2* gene (e.g., intron or 3'-flanking regions) contributed to the PB induction process, we created additional mouse lines. These lines contained the identical 5' sequence (-2499/+25) but were now linked to a human growth hormone reporter gene. In addition, to further assess the contribution of the -2200 PBRU region in the PB induction process, several deletion constructs were prepared and evaluated in transgenic mice. Nine mouse lines were generated, three each containing -2500/+25, -1700/+25, or -800/+25 bp of 5' flanking *CYP2B2* gene sequence fused to hGH. Of these constructs, only the -2500/+25 hGH lines exhibited PB induction (Figure 2). It should be noted that, unlike previous results showing high constitutive expression levels when 800 bp of 5'-flanking sequence was linked to the

intact *CYP2B2* reporter gene, constitutive expression of all the 5' region transgenes was quite low when linked to the hGH reporter.

In separate studies, we also generated seven transgenic mouse lines each containing -2500/+25, -1700/+25, and -800/+25 bp of 5' flanking *CYP2B2* gene sequence linked to chloramphenicol acetyl transferase, or CAT (Luckow and Schutz, 1987), reporter gene. However, CAT mRNA expression levels from this reporter were extremely low in all the resulting transgenic animals, requiring the use of reverse transcriptase-coupled polymerase chain reaction assays for their detection. Similarly low levels of expression were observed in intron-less growth hormone transgenes, apparently due to poor nucleosomal alignment (Liu et al., 1995). In any event, only 2 of the total of 21 transgenic *Cyp2B2*/CAT lines were PB inducible; however, both of these respective lines were -2500/+25 derivatives (data not shown). These results, using the CAT reporter, were therefore consistent with our findings presented for the hGH reporter transgenes (Figure 2)

The -2271 to -2186 5'-flanking region of the rat *CYP2B1/2* genes was footprinted with *in vitro* DNase I methods. This region lies within the reported PBRU (Stoltz et al., 1998). The results are presented in Figure 3 and reveal a distinct palindromic area of protection: 5'-CC TGA CCT TGGCAC A GTGCCA CCA TCA AC-3' (inverted repeated elements are underlined). The same protected pattern was obtained with nuclear protein extracts from both untreated and PB-treated rat liver and contains a consensus NF-1 binding motif (deVries E. et al., 1987).

In vitro assays were performed to better characterize the interactions of NF-1 and other potential regulatory proteins within the -2500 to -1700 region necessary for conferring PB activation in the transgenic animals. Specifically, we focused on the *CYP2B2* -2271/-2186 region, an area that includes the putative NF-1 protein interaction and lies within the PBRU defined by others (Trottier et al., 1995; Honkakoski and Negishi, 1997a; Honkakoski and Negishi, 1997b). Affinity purification studies were conducted by coupling the -2271/-2186 region to magnetic beads. Two fragments were analyzed with these methods, a wild-type sequence and one that had been mutated at 2 bases within the core NF-1 binding DNA motif [(deVries E. et al., 1987); see Methods for details]. These DNAs were incubated with nuclear extracts from either control or PB-treated rat livers, washed extensively and

then assayed using immunoblotting techniques (Figure 4). The interaction of NF-1 with the -2271/-2186 region of CYP2B2 was confirmed using a specific antibody directed against this protein. Introduction of 2 mutant bases into the NF-1 binding motif sequence completely ablated the NF-1 interaction. HNF-4 also interacted with the -2271/-2186 fragment, however this interaction was not modified by the NF-1 mutant residues. Histone H3 was detected in both wild type and mutated DNA-affinity extracts, whereas NF- κ B did not interact with either fragment. No differences in these relative patterns of interaction were detected when using control vs. PB nuclear extracts. Electromobility shift experiments also were conducted using radiolabeled double-stranded wild type DNA fragments. NF-1 interactions that resulted were analogous to those described for the magnetic bead experiments. EMSA shifts were noted with wild type but not mutated NF-1 oligomers. Furthermore, NF-1 mutated DNA was ineffective at competing with the wild type DNA in the gel shift experiments, whereas both wild type DNA and NF-1 consensus DNA sequences (Promega) were effective competitors (data not shown). From these studies, we concluded that an NF-1 family member interacts with this region of the PBRU and that the NF-1 mutations introduced were sufficient to completely ablate the interaction. In studies on unrelated genes, others have used the same consensus site mutations we introduced to compromise the NF-1 interaction with the DNA (deVries E. et al., 1987).

To test whether the mutations introduced into the NF-1 recognition site were functionally significant to the PB induction process, we developed additional transgenic mouse lines that harbored the NF-1 mutated domain. Two lines of mice were obtained with the same -2500/2B2 transgene as previously used, except for two base mutations within the PBRU/NF-1 binding motif. Four individuals from these NF-1 mutant lines were analyzed. All of these animals were markedly PB inducible, although the constitutive levels of CYP2B2 expression were somewhat higher than in those animals with the corresponding -2500/2B2 wild-type transgene wild type-derived mice (Figure 5). A summary of the transgenic mouse lines analyzed in this study is presented in Table 1 that includes an estimate of gene copy number for each of the integrated genes.

Discussion

Earlier studies from this laboratory using transgenic mouse models of *CYP2B2* gene expression indicated that a region critical to the PB regulatory process was located upstream of -800bp in the 5'-flanking sequence of the rat gene (Ramsden et al., 1993). The induction pattern of the PB-responsive transgenes was predominantly perivenous in the liver lobule, similar to the expression character in the rat *in vivo*. Results of nitrocellulose filter-protein binding studies also performed in our laboratory indicated that the most avid *in vitro* protein/DNA interactions occurred with *CYP2B2* gene 5'-flanking sequences located between -2229 and -2186. Furthermore, this site was coincident with a region exhibiting DNase I hypersensitivity, apparent in Southern blots of genomic rat liver DNA isolated from both untreated and PB-treated animals (Ramsden and Omiecinski, unpublished results).

Reports from other laboratories have provided evidence for the importance of a PBRU as a PB transcriptional activator in mammalian systems (Trottier et al., 1995; Honkakoski and Negishi, 1997a; Stoltz et al., 1998; Honkakoski and Negishi, 1997b). In both rat and mouse *CYP2B* genes, the PBRU is positioned well upstream of the core promoter regions of these genes and contains no barbie box elements (Park et al., 1996a; Honkakoski and Negishi, 1997a). In the current study, *CYP2B2* gene deletion constructs were tested in transgenic mice. Results from these *in vivo* analyses indicate that 5' flanking sequence between -2500 and -1700 are critical for PB induction *in vivo*, a region inclusive of the PBRU.(Park et al., 1996b) These data appear to confirm results obtained using *in vitro* approaches.

The PBRU contains several putative binding motifs for nuclear regulatory proteins, including a prominent NF-1 consensus site. NF-1 proteins belong to a family of DNA-binding proteins with diverse functions in transcription promotion (Jones et al., 1987), enhancement (Auge-Gouillow et al., 1993), repression (Roy and Gu, 1994; Reifel-Miller et al., 1991), and anti-repression (Dusserre and Mermod, 1992). In a recent report, Kim and Kemper (Kim and Kemper, 1997) used *in vivo* footprinting approaches to define PB induced chromatin alterations in the -2.2 kb region of the rat *CYP2B1/2* genes, centering

around the putative NF-1 site. The authors suggested that NF-1 may bind the chromatin in the inactive state and recruit coregulators upon induction. NF-1 also has been implicated by others in the modulation of CYP2B gene induction (Honkakoski and Negishi, 1997a; Stoltz et al., 1998; Honkakoski and Negishi, 1997b).

To test the functional contribution of NF-1 in PB activation *in vivo*, we introduced specific mutations within the NF-1 binding site of the CYP2B2 gene PBRU. Using DNA affinity enrichment techniques combined with immunoblotting and electromobility shift assays, we demonstrated that the protein binding to this site cross-reacts with NF-1 antibody and that mutations introduced to the DNA consensus motif ablate the binding interaction. When this consensus mutation within the PBRU was incorporated into the otherwise wild-type – 2500/2B2 transgenes, the mutant genes remained fully PB responsive in four individual mice derived from two different lines. These results indicate that NF-1 interaction within the PBRU is not a critical factor directing PB transcriptional activation.

These results are in apparent contrast with data reported by Honkakowski and Negishi (Honkakoski and Negishi, 1997a) using transfected mouse hepatocytes. These investigators introduced a cluster of mutations simultaneously within and surrounding the NF-1 site of the Cyp2b10 PBRU that negated PB induction of a CAT reporter gene when assessed by transfection into primary hepatocytes (Honkakoski and Negishi, 1997a). However, the specific mutations responsible for these effects was not established and therefore the effects may have been contributed by sequence alterations outside of the core NF-1 domain. Recently, Stolz et al. (Stoltz et al., 1998) reported that 2 CYP2B2 gene constructs, mutated in the NF-1 PRRU region, down-regulated the PB induction response in transfected hepatocytes. The PB induction levels in our NF-1 mutant mice were not compromised, although the basal levels of transgene expression appeared elevated. Perhaps these results are reflective of inherent differences between *in vitro* and *in vivo* model systems. It remains to be determined if the apparently higher constitutive levels seen in the NF-1 mutant mice represent a significant biological response.

Other potential protein-DNA interactions, including a nuclear receptor (NR) or orphan receptor half site adjacent to the NF-1 motif in the PBRU of Cyp2b10 and CYP2B2 (Honkakoski and Negishi, 1997a; Stoltz et al., 1998), need to be further investigated for

significance to the PB induction process. In our own investigations, evidence is presented implicating additional nuclear protein interactions within the *CYP2B2* NF-1/PBRU domain, including HNF-4, histone H3 and C/EBP-family protein interactions (Figure 5 and unpublished results). Previous studies from our own laboratory demonstrated the synergistic effect of physiological concentrations of a glucocorticoid, e.g., dexamethasone, for the PB induction response (Sidhu and Omiecinski, 1995). These results are consistent with the recent report indicating that a glucocorticoid responsive element is essential for PB induction (Stoltz et al., 1998). The data available to date are consistent with a model of cooperative nuclear protein interaction in the regulation of the PB induction response. The recent isolation of the pregnane X receptor (Kliwer et al., 1998), a novel orphan nuclear protein that interacts with naturally occurring pregnanes and synthetic glucocorticoids and antiglucocorticoids, offers an interesting venue for further study with respect to potential interactions at the PBRU.

We recently established that *de novo* protein synthesis is not required for PB induction (Sidhu and Omiecinski, 1998). It is also noteworthy that results from DNase I hypersensitivity determinations, DNA affinity purification experiments, electromobility shift assays, and *in vitro* DNase I footprinting studies, all fail to reveal differences between nuclear protein extracts derived from control or PB induced liver (Honkakoski et al., 1996; Kim and Kemper, 1997; Sommer et al., 1996). Based on these considerations, a working model for PB induction is proposed that involves pre-existing regulatory factors which interact with the PBRU constitutively, but in the presence of PB, are subjected to post-transcriptional alterations (e.g., altered phosphorylation or acetylation status). These alterations in turn would enable positive interaction of the PBRU with the transcriptional initiation complex. A PB-induced dephosphorylation event within a critical nuclear regulator would be consistent with results of previous reports indicating that inhibition of protein phosphatase 1 or 2A pathways effectively repress the PB induction response (Sidhu and Omiecinski, 1997). Further analyses of these potential regulatory schemes and identification of the critical nuclear regulatory protein(s) involved are required to address these mechanistic issues.

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Table 5. Summary of Transgenic Mouse Results.

Transgene	Line	Approx. Copy No.	PB Induction in Liver
-800/hGH	4a	1	—
	4b	2	—
	4c	2	—
-1700/hGH	3a	2	—
	3b	1	—
	3c	10	—
-2500/hGH	2c	2	+
	2d	1	+
	2e	23	+
-2500/2B2	1a	3	+
	1b	2	—
	1c	3	+
	1d	1	+
	1e	1	+
NF-1 mut - 2500/2B2	5a	6	—
	5b	15	+
	5c	1	+

Multiple founders or transgenic lines were derived for each construct. Approximate number of copies of the transgenes in each line was determined by phosphorimaging of a Southern blot (see Methods). Qualitative results of the inducibility of the transgenes in the liver: "-" not responsive, "+" responsive to phenobarbital.

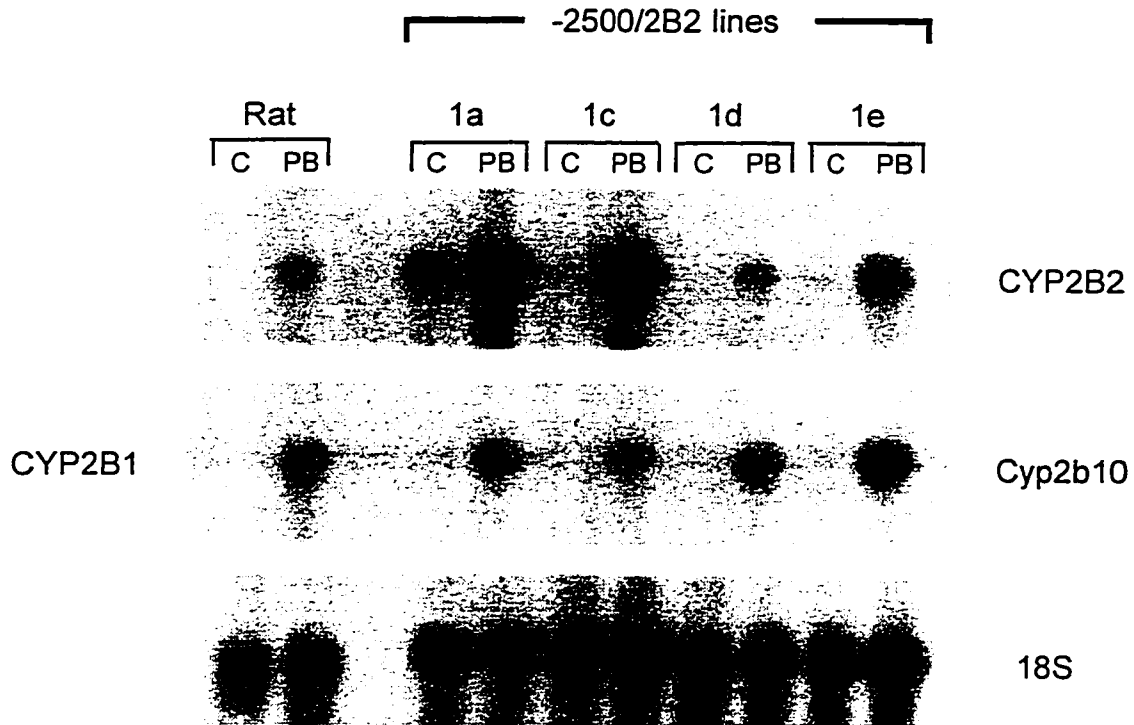


Figure 38. Northern Blot Analysis of the -2500/CYP2B2 Transgenic Mouse Lines.

Four transgenic mouse lines, each bearing the -2500/2B2 rat gene construct (see methods), were tested for CYP2B2 PB responsiveness. Fifteen μg of liver total RNA from both saline-treated control (C) and phenobarbital-treated (PB) mice and 7.5 μg of Sprague-Dawley rat RNA were electrophoresed in a 2 M formaldehyde denaturing gel, transferred to a positively-charged nylon membrane, and probed with radiolabeled oligonucleotides specific for the transgene, CYP2B2 (upper panel); for the endogenous PB-inducible mouse P450, *Cyp2b10* (middle panel); and for 18S ribosomal RNA as a loading control. The *Cyp2b10* probe also detects the rat CYP2B1 mRNA. Autoradiographs were performed using Kodak X-OMAT imaging film at -80° with one L-Plus intensifying screen for 40h, 18h, or 2h (upper to lower panels, respectively). A fifth line of mice exhibited no observable expression of the transgene in either C or PB lanes with 88 h of exposure (not shown).

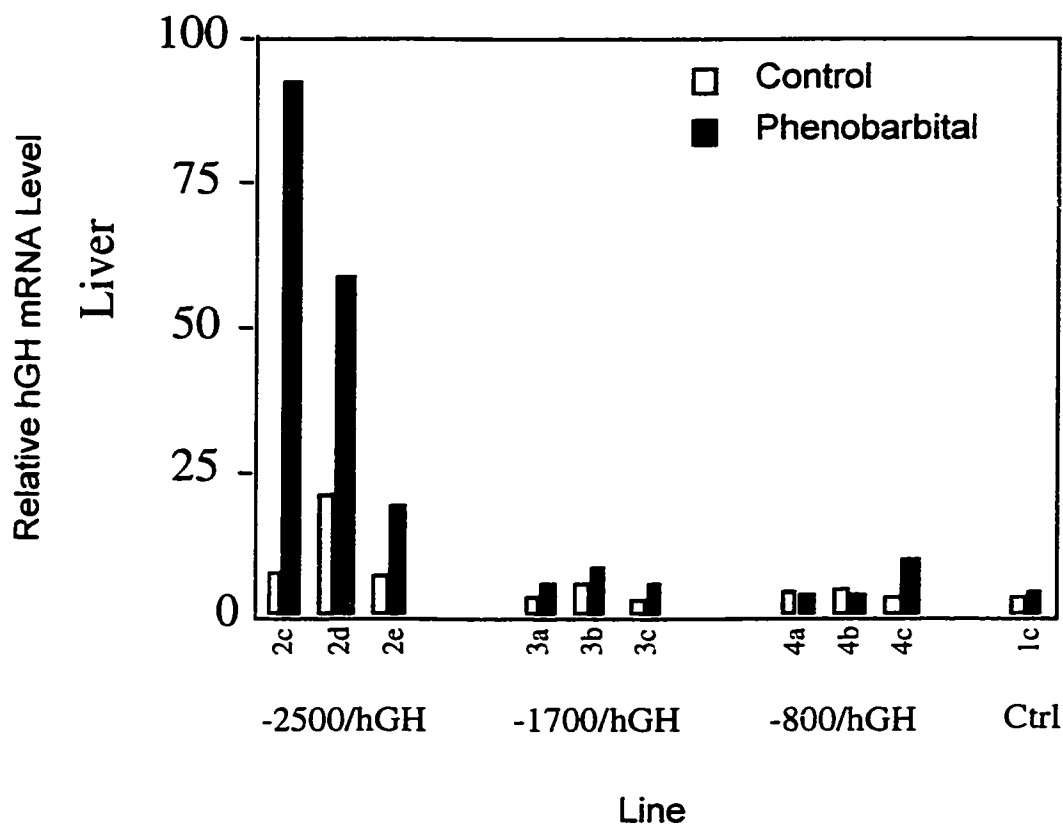


Figure 39. Determinations of Hepatic mRNA Expression Levels in Transgenic Mice Possessing the hGH Gene Reporter Linked to Varying Regions of the CYP2B2 5' Flanking Gene Sequence.

Ten μ g of liver total RNA was isolated from saline-treated controls or PB-treated mice, heated at 65° in 6M formaldehyde/10xSSC, loaded into a slotted manifold, and the denatured RNA captured under vacuum onto a nylon membrane. The membrane were probed sequentially with an hGH cDNA probe, a mouse *Cyp2b10* probe, and an 18S rRNA normalization probe. Phosphorimaging was conducted to quantify signal strength and normalized values (using 18S rRNA as standard) are presented. Three independent mouse lines were tested for each construct (-2500/hGH, -1700/hGH, and -800/hGH). The upper panel depicts normalized results for the hGH reporter transgene; the lower panel shows the expression of the endogenous PB-inducible mouse *Cyp2b10* gene in the same animals.

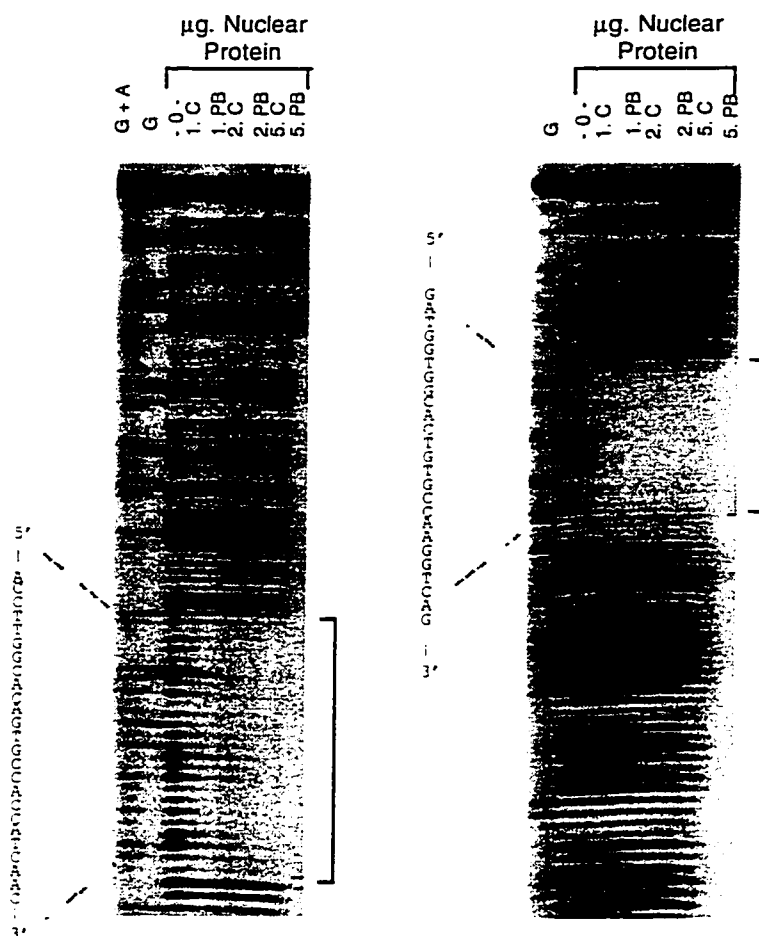


Figure 40. DNase I Footprinting of the CYP2B2 -2271 to -2186 Gene Region.

Panel A. Double stranded -2271 to -2186 CYP2B2 probes, radiolabeled on either the sense or anti-sense strand, were incubated with increasing concentrations of crude rat liver nuclear extracts as indicated at the top of each lane. 0, probe incubated with DNase I in the absence of nuclear proteins; C, control nuclear protein; PB nuclear protein from PB-treated rats. Positions of protected bases were determined by comparison to Maxam-Gilbert A+G and/or G sequencing reactions performed for each probe. The region protected from DNase I digestion by nuclear proteins is represented for each probe with brackets to the right of the respective sequences. **Panel B.** DNA sequence content of the CYP2B2 protected region within the PBRU. The bracketed region corresponds to the DNase I protected sequence. The palindromic NF-1 consensus site is indicated with bold letters. Arrows delineate inverted repeat elements that exist in the protected region. Positions of specific mutations introduced in the NF-1 sequence that were used for subsequent studies are indicated in the lower portion of the figure.

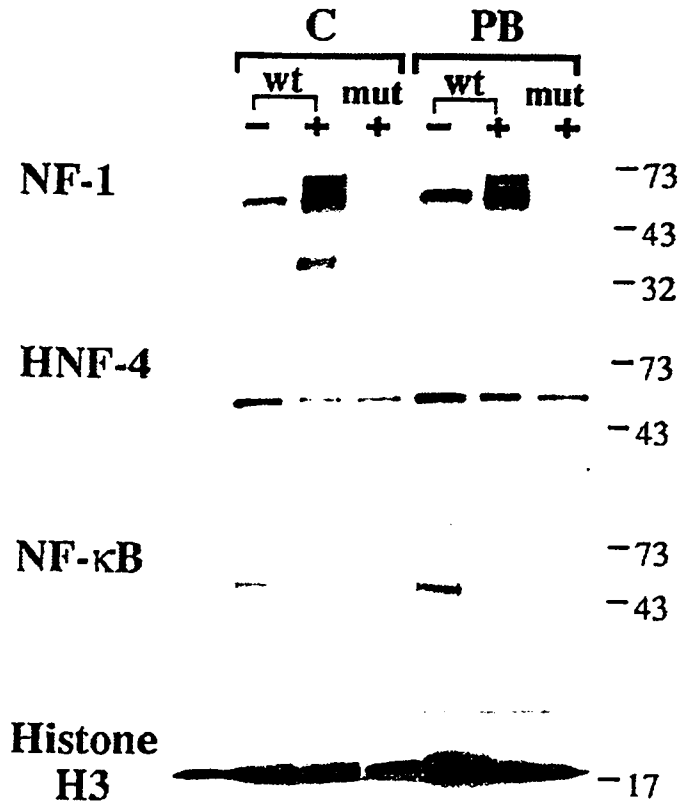


Figure 41. Immunoblot Analysis of Affinity Purified Proteins Interacting with the WT or NF-1 Mutated CYP2B2 -2271/-2186 Element.

Rat nuclear proteins from control (C) or PB-treated livers were affinity-purified (+) against the wild type (wt) *CYP2B2* -2271/-2186 DNA sequence or the identical fragment with specific mutations (see Figure 1) introduced to the NF-1 consensus binding site (mut). These proteins were eluted, separated by SDS-PAGE, and resulting blots were probed with specific antibodies directed against NF-1, HNF-4, NF-κB, and Histone H3 as described in the Methods section. Liver nuclear proteins, C or PB, that were not subjected to affinity purification (-) also were probed.

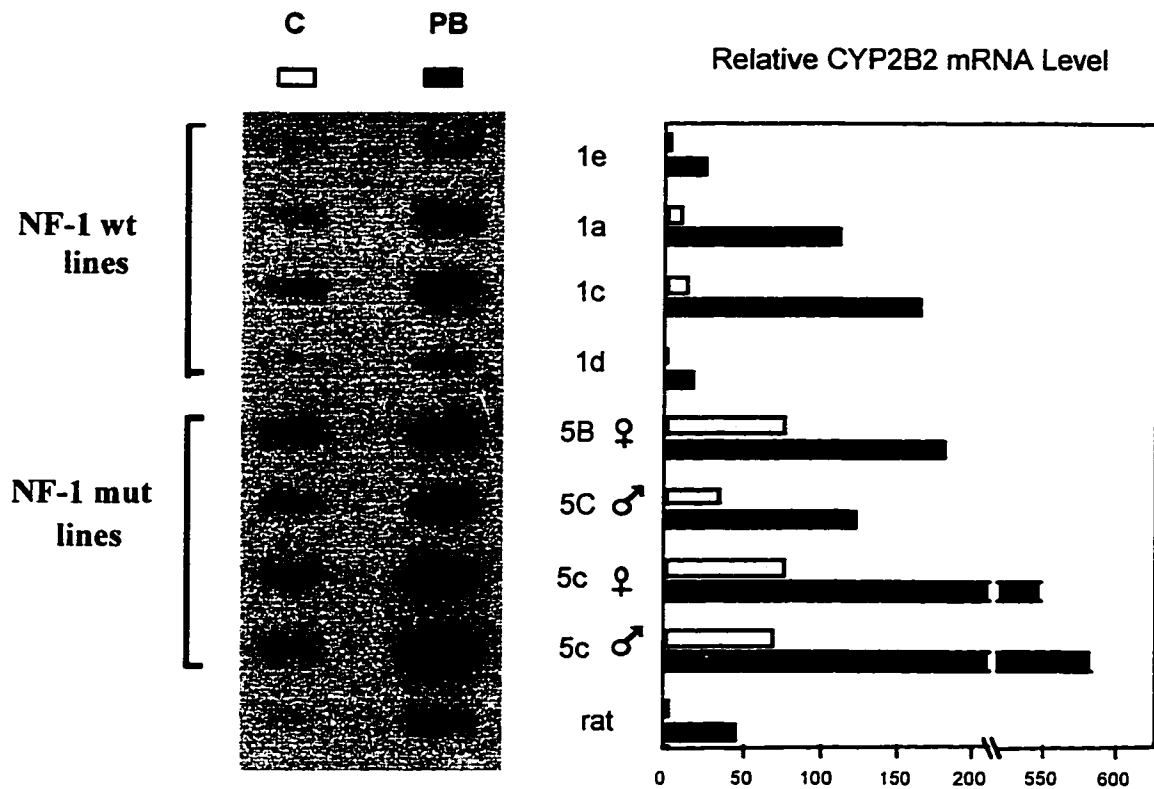


Figure 42. mRNA Slot Blot Analysis of CYP2B2 mRNA Levels in NF-1 WT and NF-1 Mutant Transgenic Mice.

Ten μ g of liver total RNA was isolated from transgenic mouse lines containing either the unaltered -2500/2B2 gene (lines 1e, 1a, 1c, 1d - all F1 males) or the same transgene with two altered bases altered (see Figure 1) in the NF-1 consensus region (founder lines 5B and 5C; 5c - F1 animals). RNA from control (C) or PB-treated mice was immobilized onto a membrane (as for Figure 3) and hybridized sequentially with probes specific for the CYP2B2 transgene and 18S rRNA. Results of the phosphorimaging for CYP2B2 transgene expression are shown on the left panel and normalized results (18S rRNA standard) are depicted on the right panel. Ten μ g of liver total RNA obtained from C or PB-treated rat liver was included as a positive control.

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- Doctoral thesis research
 - Molecular toxicology project studying the mechanisms of expression of the phenobarbital inducible Cytochrome P450 2B1 and 2B2 genes.
 - Techniques include: culturing of primary hepatocytes and cell lines, viral and lipofection mediated transfections, baculovirus expression, subcloning, PCR, RT-PCR, gel-retardation and supershift assays, cAMP/PKA analysis, histone acetylation analysis, PAGE, DNA sequencing, immunohistochemistry, RNA, DNA, and protein analysis.

TEACHING ASSISTANT**Mar.-Jun. 1993**

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- Lectured class on the disposition of chemicals in the body.
- Ran weekly review and discussion sessions.
- Tutored students on an individual basis.
- Developed exam materials and graded course work with professor for an environmental toxicology class.

RESEARCH ASSISTANT**Jun. 1991-Sept. 1992**

Dept. of Environmental Health, University of Washington, Seattle, WA

- Masters thesis research
 - Facilitated culturing of human nasal epithelial cells on microporous membranes for exposure to ozone.
 - Developed an assay to quantitate ICAM-1 levels using an ELISA technique and double staining for analysis on the ACAS 570 laser cytometer.
 - Measured IL-1 α , IL-6, IL-8, and TNF expression using two-site ELISA's.

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- Phenotyped human samples for arylesterase, paraoxonase, and chlorpyrifos-oxon activity levels.
- Collaborated in purification of rabbit paraoxonase protein.

MICROBIOLOGIST/RESEARCH SCIENTIST Aug. 1988-Sept. 1990
 Estee Lauder Companies, Melville, NY

- Initiated, coordinated, and assumed full responsibility for adaptation and in-house validation of two *in vitro* toxicity tests that have subsequently been used to replace the Draize eye irritancy test.
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COMMUNITY OUTREACH & TRAINING:

MASTER HOME ENVIRONMENTALIST Sept. 1997-present
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 Pacific Northwest Association of Toxicologists, 15th Annual Mtg
- Environmental Pathology/Toxicology Training Grant** 1993-1998
 National Institute of Environmental Health Sciences (NIEHS)
- Research Assistantship** Oct. 1990-Jan. 1993
 Department of Environmental Health,
 University of Washington, Seattle, WA
- Deans List** Aug. 1987-May 1988
 Cornell University, Ithaca, NY

LEADERSHIP ACTIVITIES:

- Graduate & Professional Student Senator** Sept. 1993-Aug. 1997
 University of Washington, Seattle, WA
- School of Public Health and Community
 Medicine Student Affairs Committee** Sept. 1994-Aug. 1996
 University of Washington, Seattle, WA
- Radiological Sciences Program Review Panel** Dec. 1994-June 1995
 University of Washington, Seattle, WA

COMPUTER SKILLS:

Highly proficient in use of Macintosh and Windows-based computers. Programs and applications include: UNIX, MS Word, Excel, Powerpoint, Persuasion, Cricket Graph, Origin, Prism, Endnote, Photoshop, Pagemaker, and Acrobat.

MEMBERSHIPS:

Society of Toxicology	1995-present
Pacific Northwest Association of Toxicologists	1992-present

PUBLICATIONS:

Beck, N.B., Tracy, J.H., Omiecinski, C.J. The Effects of Histone Deacetylase Inhibitors on the Phenobarbital Responsive Cytochrome P450 2B1 Gene. In progress.

Beck, N.B., Omiecinski, C.J. The Effects of Baculovirus Mediated Gene Delivery on Rat Primary Hepatocytes: An Examination of CYP Induction and Cytokine Expression. Submitted to *Gene Therapy*.

Beck, N.B., Omiecinski, C.J. Phenobarbital Exposures Do Not Alter cAMP Levels or Protein Kinase A Activity in Rat Primary Hepatocytes. *Biochemical Pharmacology*, In Press.

Ramsden, R., **Beck, N.B.**, Sommers, K.M., Omiecinski, C.J. Characterization of Elements Regulating the Expression of the Phenobarbital-Inducible CYP 2B Genes. Submitted to *Gene*.

Hassett, C, Robinson, K.B., **Beck, N.B.**, Omiecinski, C.J. The Human Microsomal Epoxide Hydrolase Gene (EPHX1): Complete Nucleotide Sequence and Structural Characterization. *Genomics* 23:433-442, 1994.

Beck, N.B., Koenig, J.Q., Luchtel, D.L., Altman, L.C., Orsborn, M.T., Kenney, J.S. Ozone Can Increase the Expression of Intercellular Adhesion Molecule-1 and the Synthesis of Cytokines by Human Nasal Epithelial Cells. *Inhalation Toxicology* 6:345-357, 1994.

Beck, N.B. The Effects of Ozone on Intercellular Adhesion Molecule 1 Expression in Human Nasal Epithelial cells. Masters Thesis. 1992.

PUBLISHED ABSTRACTS, PRESENTATIONS & POSTERS:

Omiecinski C.J., Ramsden, R., **Beck, N.B.**, J.S. Sidhu. Modulation of Phenobarbital Responsiveness by Protein Synthesis Inhibitors and by Site-Directed NF-1 Element Mutations. ISSX Proceedings, Vol. 13. Presented by C.J. Omiecinski at the 5th International ISSX Meeting, Cairns Australia, Oct. 1998. Poster presentation.

Beck, N.B., Omiecinski, C.J. Phenobarbital Mediated Induction of the Rat CYP2B Genes in Primary Hepatocyte Cultures. Presented at the Pacific Northwest Chapter of the Society of Toxicology, 15th Annual Meeting, Leavenworth, Washington, September 1998. Platform talk.

Beck, N.B., Ramsden, R., Sommer, K.M., Omiecinski, C.J. Transcription Factor Binding to the Phenobarbital Responsive Element (PBRE) in the P450 2B2 Gene. *Toxicological Sciences* 42(1-S) 22 abstr. 1998. Presented at Society of Toxicology Annual Meeting, Seattle, Washington, 1998. Poster presentation.

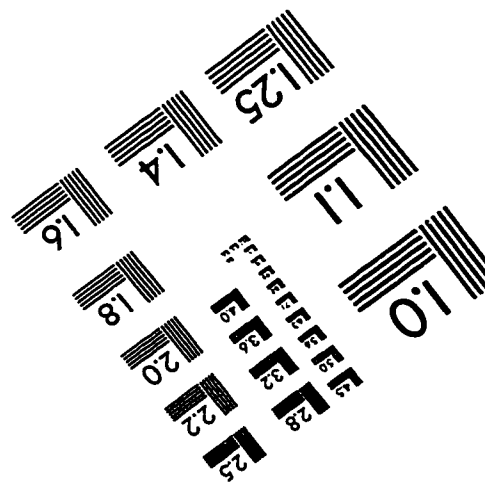
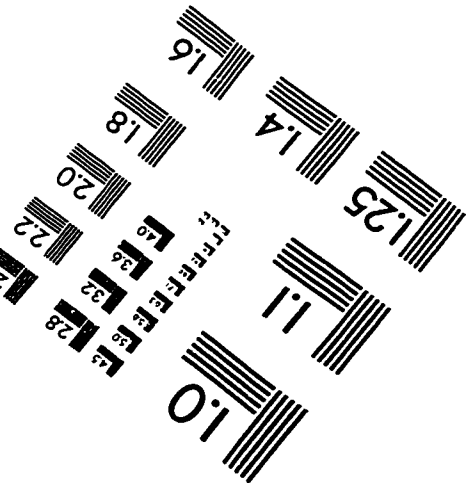
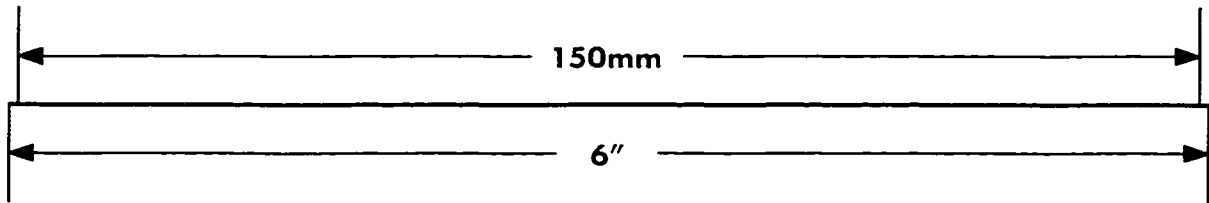
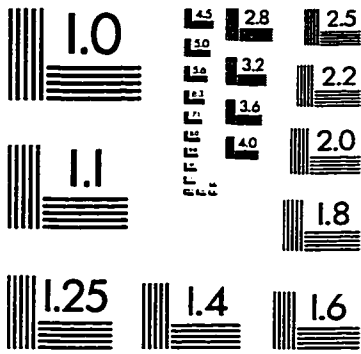
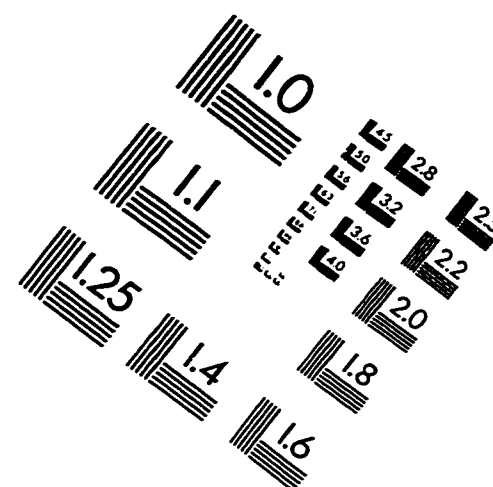
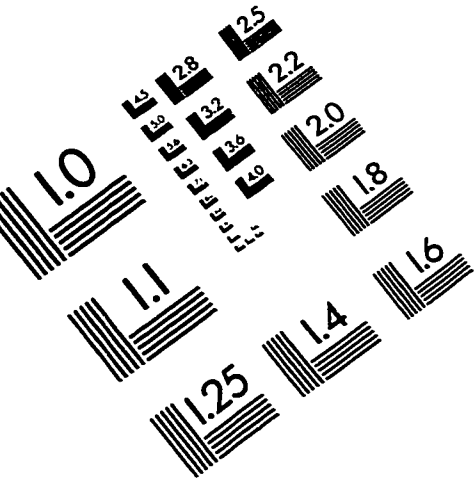
Beck, N.B., Ramsden, R., Sommer, K.M., Omiecinski, C.J. Characterization of the Phenobarbital Responsive Element (PBRE) in the P450 2B2 Gene. Presented at the Pacific Northwest Chapter of the Society of Toxicology, 14th Annual Meeting, Ocean Shores, Washington, September 1997. Platform talk.

Beck, N.B., Omiecinski, C.J. Cooperative Interactions of Nuclear Proteins with the 5' Upstream Region of the Rat Cytochrome CYP2B2 Gene. *International Toxicologist* 7(1) abstr. 1995. Presented at the meeting of the International Congress of Toxicology, Seattle, WA, 1995. Poster Discussion session.

Beck, N.B., Altman, L.C., Koenig, J.Q., Luchtel, D.L., Orsborn, M.T., Costa, L.G., Reilly, M.T., Kavanagh, T.J. Development of a Quantitative Morphometric Approach to Examine the Effects of Ozone on Intercellular Adhesion Molecule-1 on Human Nasal Epithelial Cells. *Toxicologist*. 13(1):298 abstr. 1993. Presented at Society of Toxicology Annual Meeting, New Orleans, LA, 1993. Poster discussion session.

Beck, N.B., Altman, L.C., Luchtel, D.L., Koenig, J.Q., Orsborn, M.T., Kavanagh, T.J., Baker, C., Welch, M., Kinney, J.S. Ozone Stimulates ICAM-1 Expression and Cytokine Release from Cultured Human Nasal Epithelial Cells. *J Allergy Clin Immunol.* 91:254 abstr. 1993. Presented (by L.C. Altman) at Society of Allergy and Clinical Immunology Annual Meeting, Chicago, IL, 1993. Poster presentation.

IMAGE EVALUATION TEST TARGET (QA-3)



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