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From Transcription to Drug Resistance—The Cytochrome P-450 Link

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The cytochrome P-450 levels in the chloroquine-resistant malarial parasite were higher than those of the drug sensitive strains. The messenger RNA was also induced in the parasite by phenobarbitone. Pretreatment of the parasite culture with phenobarbitone affords partial protection to chloroquine toxicity, indicating a possible role for cytochrome P-450 in chloroquine resistance.

Key Words: Drug resistance, Transcriptional regulation, Cytochrome P-450, Chloroquine resistance

Introduction

This laboratory has been concerned with studies on the transcriptional regulation of the cytochrome P-450 gene superfamily in rat liver. This system offers several advantages to be used as a model to understand eukaryotic gene transcription. Many members of this family are inducible. They show developmental regulation and are widely distributed across species and tissues. The proteins are basically hydroxylases catalyzing crucial steps in a variety of metabolic pathways of endogenous chemicals, besides being involved in the biotransformation of a variety of foreign chemicals. This brief review summarizes the conceptual framework that is emerging to explain the transcriptional regulation of CYP2B1/B2 subfamily, by the prototype inducer, phenobarbitone (PB), in rat liver and its implication for eukaryotic gene transcriptional regulation in general.

CYPIA1 Vs CYP2B1/B2 Families

The cytochrome P-450 superfamily is now divided into 27 families and subfamilies and the list is growing (Oritzde Montellano 1986). However, detailed studies on transcriptional regulation are mostly available only with the CYPIA1 subfamily, inducible by polycyclic aromatic hydrocarbons. Data from several groups of workers indicate that transcriptional regulation of the CYPIA1 subfamily by the prototype inducer, 3-methylcholanthrene, follows the 'steroid' pathway. A specific receptor for the hydrocarbon referred to as Ah receptor translocates the ligand from the cytoplasm to the nucleus. Specific cis-acting elements have been identified upstream of the CYPIA1 gene, functioning as xenobiotic response elements and interacting with the Ah receptor. Besides, a basal transcription element has also been identified (Nebert 1989, Gonzalez 1988, Waxman & Azaroff 1992).

The situation with the PB-inducible CYP2B1/B2 gene is far from clear. Although, the B1 and B2 genes are distinct, they show 97% homology having 9 exons and at this stage are treated as a single unit, although differences do exist between the two genes in terms of expression. Efforts to identify a receptor for PB have not been successful. Unlike the polyaromatic hydrocarbons, PB is a broad-based inducer. It also induces other genes such as NADPH - cytochrome P-450 reductase, UDP-glucuronyl transferase, epoxide hydrolase, aldehyde dehydrogenase and is a hypertrophic agent. At the same time, the specific action of PB is indicated by the fact that CYPB1/B2 is the major cytochrome P-450 species induced and the response in terms of transcriptional activation of the gene is seen within an hour of administration of the drug to the rat. Finally, CYP2B1/B2 is induced by apparent structurally diverse set of compounds, although the site of action is likey to be at the level of transcription (Waxman & Azaroff 1992).

In view of these interesting characteristics of the CTP2B1/B2 gene, this laboratory has focused on the regulatory features of transcription of this gene. Since, earlier studies clearly indicated that transcriptional activation is the primary mechanism of induction of this gene (Atchisonm & Adesnik 1983, Ravishankar & Padmanaban 1985), studies were initiated to identify cis-acting regulatory elements on the 5'-flanking region.

Major Promoters are Located in the Near Upstream of the CYP2B1/B2 Gene

The DNA sequence upto −800nt of the upstream revealed several potential regulatory sites such as direct and inverted repeats, stretch of alternating purines and pyrimidines capable of assuming a Z conformation and putative NF1 and glucocorticoid binding sites (Rangarajan et al. 1987). A novel whole nuclear transcription assay was developed to transcribe cloned minigene fragments using S1 nuclease protection assay. It was shown that a cloned DNA containing −178nt of the 5'-upstream and the first exon (pP450e178) can be transcribed in whole nuclei reflecting the transcription status of the CYP2B1/B2 gene under different treatment conditions. Thus, the pP450e178 transcripts were hardly detectable in uninduced nuclei but significant amounts could be detected in nuclei isolated from PB-treated rats (PB-nuclei). Again, nuclei from PB + $CoCl_2$ treated rats gave a low level of pP450e178 transcripts and this could be counteracted by adding heme *in vitro* (Rangarajan & Padmanaban 1989). These results are in conformity with the proposal that an optimal amount of heme is required for the transcription of the CYP2B1/B2 gene (Ravishankar & Padmanaban 1983, 1985, Rangarajan & Padmanaban 1989). Thus, these results clearly indicate that the near upstream of the 5'-flanking region (−178 nt) is able to confer the regulatory features of the gene manifested under a variety of treatment conditions *in vivo*. This does not, however, preclude the existence of further upstream and far upstream enhancers.

Identification of a Positive Cis-acting Element in the Near Upstream of the 5'-Flanking Region

DNase-foot print analysis of pP450e178 DNA with nuclear extracts from PB-treated rats indicated protection of the region −54 to −89 nt. Whole nuclear transcription analysis of Bal 31 mutants generated from pP450e178 revealed that the mutant pP450e116 (containing −116 of the upstream) is well transcribed, but the mutant pP450e75 (containing −75 nt of the upstream) is poorly transcribed. Gel shift assays with the −178 nt upstream and oligonucleotides spanning the region have

revealed that oligonucleotide-1 (−69 to −98 nt) gives a pattern similar to that of the −178 nt fragment. Essentially, two major complexes are seen and the bottom complex intensifies with nuclear extracts from PB-treated rats. The transcription of pP450e178 DNA in cell-free extracts is inhibited by the addition of oligonucleotide-1 indicating that it competes for an essential transcription factor(s). All these results have indicated that the region −69 to −98 nt acts as a positive cis-acting element in the transcription of the CYP2B1/B2 gene (Upadhya et al. 1992).

Identification of a Negative Cis-acting Element in the Near Upstream of the 5′-Flanking Region

Preliminary unpublished results have indicated that the region—178 to −116 nt may harbour a negative element. This is on the basis that the Bal 31 mutant pP450e116 is transcribed more efficiently than pP450e178 in whole nuclei, although pP450e75 is poorly transcribed. Besides, the gel retardation and DNase-1 foot analysis data indicate factor binding to this region in the uninduced state, that is inhibited under conditions of PB-treatment. Further, the region also contains altered glucocorticoid response element, the core sequence TGTCCT being prsent twice. This region may mediate the repressive effect of dexamethasone on PB-dependent activation of CYP2B1/B2 gene transcription (Venkateswara Rao 1990). Thus, it appears likely that the region −178 to −116 nt may harbour a negative element accounting for the repressed state of the gene in the uninduced animal and the repressive effect of the glucocorticoid when given along with PB.

Characterization of the Transacting Factors Binding to the Positive Element

Cross-linking analysis of the gel retarded complexes have revealed two proteins in the range 38—42 Kd, which includes the size of the oligonucleotide present in the complex. Preliminary studies have yielded similar proteins, when the nuclear extract is fractionated on oligonucleotide-affinity columns. The purified preparations are capable of stimulating pP450e178 DNA transcription in cell-free extracts. Further studies are needed to characterize these factors in detail.

Phosphorylation of Transcription Factor(s) as a Possible Regulatory Step

Recently, He and Fulco (1991) have reported that a 17nt fragment within the positive element identified in this laboratory, forms a strong complex with nuclear extracts from PB-treated rats. Interestingly, they have also shown that addition of PB *in vitro* to control extract (uninduced) has a similar effect. Preliminary studies now reveal that this effect of PB may be due to its interference with the phosphorylation machinery. It appears that the transcriptional regulation of the CYP2B1/B2 gene will be governed by the phosphorylation status of the transcription factors concerned.

Phenobarbitone-Response Element to Express Heterologous Genes *in vivo*

The −178 nt of the 5′-flanking region of the CYP2B1/B2 gene has been fused into a pUC based vector with human growth hormone gene serving as the reporter. This DNA has been injected into rats as polylysine — asialoglycoprotein protein complex in short term (3 days) and long term (1 month) experiments. It has been found that expression of growth hormone mRNA can be demonstrated only in animals receiving phenobarbitone. This indicates the exciting possibility that a simple approach can be used to target genes into liver and express the same under appropriate conditions.

Possible involvement of Cytochrome P-450 in Chloroquine Resistance in the Malarial Parasite, *Plasmodium falciparum*

The resurgence of malaria and the spread of chloroquine resistance have become a major public health problem in developing countries. The proposal that chloroquine resistance is due to high rates of efflux of the drug, mediated by P-glycoprotein and reversed by verapamil as is the case with multi drug resistant (Mdr) tumour cell lines, has attracted considerable attention (Krogstad et al. 1987, Martin et al. 1987). Two Mdr genes, Mdr1 and Mdr2 have been identified in *Plasmodium falciparum* and Mdr1 was implicated in chloroquine resistance (Foote et al. 1989, Higgins 1989). However, recent studies have raised several challenging questions. The time course of chloroquine uptake in resistant and sensitive strains has been interpreted to be consistent with weakened vacuolar proton pump rather than with an Mdr pump (Ginsburge & Stein 1991, Ginsburge & Krugliak 1992), although arguments have been advanced against this hypothesis (Krogstod et al. 1992). Results from genetic crosses between chloroquine-resistant and chloroquine-sensitive strains of *P. falciparum* have shown that the genetic locus governing chloroquine efflux and resistance is independent of the known Mdr-like genes (Wellems et al. 1990), but can be traced to a 400 kb region on chromosome 7 (Willems et al. 1991). While some moderately chloroquine-resistant clones of *P. falciparum* show amplification of Mdr gene (Foote 1989), highly resistant clones manifest deamplification of Mdr1 gene (Barnes et al. 1992). All these results would indicate that the known Mdr genes in *P. falciparum* are not directly linked to chloroquine resistance. An indirect involvement is perhaps indicated by the detection of mutations in the Mdr1 gene in chloroquine resistant isolates (Karez & Cowman 1991).

In this context, the reports that the cytochrome P-450 (P-450) dependent hydroxylase activities are higher in chloroquine-resistant than sensitive *P. berghei* (Salganik et al. 1987) and *P. falciparum* (Ndifor et al. 1990) assume significance. These workers have also shown that inhibitors of monooxygenase activities are functional with respect to the parasite enzyme activities and phenobarbitone treatment of the *P. falciparum* culture induces the monooxygenase activities. It is, therefore, of interest to examine the basis for the higher levels of P-450 and P-450 dependent enzyme activities in the resistant than sensitive strains and their inducibility by phenobarbitone. In the present study we have quantified P-450 mRNA in *P. falciparum* using a rat phenobarbitone - inducible CYPIIB1/B2 cDNA probe. The heterologous probe hybridizes to the parasite mRNA and it is clear that the differences in enzyme activities are due to differences in P-450 mRNA levels.

We have earlier shown that atleast one of the biochemical mechanisms of chloroquine action is to inhibit heme dependent protein synthesis in the parasite (Surolia & Padmanaban 1991) by virtue of the ability of chloroquine to chelate with heme (Fitch & Kanjananggulpan 1987). We have also shown that *P. falciparum* is capable of heme biosynthesis *de novo* and inhibition of a step in the pathway results in inhibition of parasite protein synthesis leading to its death (Surolia & Padmanaban 1992). Cytochrome P-450 synthesis would demand the availability of heme and, therefore, inhibition of heme synthesis will lead to inhibition of P-450 synthesis. Therefore, inhibitors of heme synthesis can also be expected to help in overcoming chloroquine resistance along with the monooxygenase inhibitors.

The basis for P-450 involvement would obviously be a detoxification pathway for

chloroquine in the malarial parasite. The *P. falciparum* strains and field isolates from different geographic regions have been analysed and the chloroquine resistant strains manifest higher P-450 dependent enzyme activities than the sensitive strains (Karcz & Cowman 1991, Salganik et al. 1987, Ndifor et al. 1993). We have been able to confirm these results with our strains. The recent study of Ndifor et al. (1993) has shown that inhibitors of mono-oxygenase enzyme such as cimetidine can enhance chloroquine sensitivity of the resistant field isolates. The present study reveals that the increase in P-450 dependent enzyme activities in the resistant strains or phenobarbitone treated sensitive strains is due to an increase in P-450 mRNA levels and, therefore, most likely due to differential P-450 gene expression. The basis for phenobarbitone protection against chloroquine toxicity to the parasite is likely due to the activation of the P-450 gene in *P. falciparum* and the results are not due to a differential accumulation of the drug. Studies are in progress to isolate the P-450 gene(s) from the parasite and also to analyse the potential of the parasite to metabolize chloroquine. It would be of interest to examine whether the P-450 gene locus maps within the 400 kb region of chromosome 7 implicated in chloroquine resistance (Wellems et al. 1991). If enhanced efflux of the antimalarial in the resistant strain is the real mechanism involved in conferring chloroquine resistance, then it would be of interest to examine the nature of the substrate for the efflux pump, although it was indicated that the effluxed species is unaltered chloroquine (Krogstad et al. 1987). The involvement of Mdr and P-450 in the reversal of chloroquine toxicity brought about by a variety of chemicals needs to be analysed carefully.

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