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Dynamics of DNA–Protein Complex Formation in Rat Liver during Induction by Phenobarbital and Triphenyldioxane

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Abstract—*CYP2B* gene expression in liver of rats treated with phenobarbital and triphenyldioxane at early stage of induction (40 min-18 h) was studied using electrophoretic mobility shift assay (EMSA) and RT-PCR. During first 6 h after induction, differences in the dynamics of formation of DNA–protein complexes were shown for each inducer. Later (18 h after induction), the intensity pattern of these complexes became the same for both phenobarbital and triphenyldioxane treated animals. This suggests the existence of specific signaling for each inducer only in early stages of *CYP2B* activation. Increase in nuclear protein (possible transcription factor) binding to Barbie-box regulatory sequence of *CYP2B* genes was accompanied by their increased expression. Thus, we have demonstrated for the first time that early stages of induction (40 min and 3 h after administration of phenobarbital and triphenyldioxane, respectively) are accompanied by activation of nuclear proteins that can bind to Barbie-box element of *CYP2B*. Although various chemical inducers cause distinct activation of such binding, this process involves activation of gene transcription.

Key words: *CYP2B* genes, triphenyldioxane, Barbie-box sequence, nuclear extract proteins, electrophoretic mobility shift assay (EMSA), RT-PCR

Enzymes known as cytochrome P450 are now classified as a large superfamily of proteins encoded by many genes. The main function of these enzymes consists of metabolism of various exogenous and endogenous substances. Many cytochromes P450s are readily inducible: various stimuli increase their mRNA synthesis and enzymatic activity. However, our knowledge of mechanisms underlying activation of these enzymes is rather fragmentary. For example, the mechanism responsible for induction of cytochrome P450 2B subfamily (CYP2B) is not well understood. Enzymes of this subfamily are induced by structurally diverse xenobiotics; they are known as phenobarbital-type inducers [1]. Promoter of *CYP2B* genes, which consists of more than 2000 base pairs (bp), contains enhancer elements localized in proximal and distal parts. The distal part contains the enhancer element known as PBREM, which consists of two nuclear receptor binding sites (NR1 and NR2 sites) and also nuclear factor 1 binding site (NF1 site) [2]. According to Negishi et al. only the NR sites are responsible for sensitivity to phenobarbital; the NF site is possibly required for full activity of PBREM [3]. The search for a receptor that might bind to this element resulted in the identification of CAR protein, the constitutively active receptor, which responds to phenobarbital (PB) by translocation into the nucleus where it forms a heterodimer with retinoid X receptor (RXR) and interacts with the PBREM-element. The mechanism responsible for phenobarbital-induced translocation of CAR into the nucleus and subsequent activation is not yet clear.

The proximal part of the promoter contains nucleotide sequences that are homologous to various genes encoding CYP2B: a positive element and a negative element, which includes such constitutive region as Barbie-box, C/EBP-binding site, and CCAAT box. Induction of *CYP2B* by phenobarbital involves the promoter site −179/+1 bp. This site also known as the minimal promoter includes the negative element (−160/−127 bp) and the positive element containing the Barbie-box sequence. Padmanaban's group demonstrated phenobarbital-induced increase in protein factor binding with these regulatory sites [4, 5]. It was also demonstrated that

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phosphorylation status of these proteins influences their interaction with the promoter region and activation of *CYP2B* transcription [6, 7]. Recently we found an effective species-specific inducer of CYP2B, triphenyldioxane (TPD). Being administered at a dose of 10 times less than the classic inducer—phenobarbital, this compound increased CYP2B activity in rat but not in mouse liver [8]. The mechanism responsible for the effect of this compound is not fully understood. Previously we demonstrated that incubation of hepatic nuclear proteins from intact rats for 30 min with *CYP2B* positive regulator promoter element, Barbie-box, in the presence of PB and TPD *in vitro* resulted in formation of three DNA–protein complexes; two of them were also found in the absence of inducers. Prolonged incubation for 2 h with TPD resulted in appearance of two additional complexes [9]. This suggests the existence of TPD-specific pathways of *CYP2B* gene activation. *In vivo* experiments also demonstrated increased binding of nuclear protein extracts (isolated 18-48 h after inducer administration) with Barbie-box sequence [10]. However, according to literature data, the time interval 18-48 h corresponds to maximal mRNA synthesis, i.e., transcription complexes should be formed earlier. So, study of earlier stages of induction may elucidate the dynamics of complex formation between Barbie-box and nuclear proteins and their relation to gene activation.

In this study, we investigated the effects of PB and TPD on DNA–protein complex formation at early stages of induction after administration of these compounds to rats (from 40 min to 18 h). We also investigated CYP2B mRNA level during these time intervals.

MATERIALS AND METHODS

Chemicals. TPD was synthesized at the Institute of Cytology and Genetics, Siberian Branch of the Russian Academy of Sciences (Novosibirsk); phenobarbital was purchased from Merck (Germany). [γ-^{32}P]ATP (6000 Ci/mol) and oligonucleotides were synthesized at the Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the Russian Academy of Sciences (Novosibirsk). Pepstatin, leupeptin, aprotinin, and poly(dI-dC) were produced by Sigma (USA). Kodak GBX2 film (USA) was used for autoradiography.

Treatment of animals. Male Wister rats (150-170 g) were obtained from the vivarium of the Institute of Cytology and Genetics. Inducers were administered intraperitoneally: TPD (10 mg/kg) in sunflower oil, PB (80 mg/kg) in 0.9% NaCl. Control rats received equal volume of vehicle. Three rats were used for each experimental point. All experiments were repeated in triplicate.

Nuclei were isolated from rat liver cells 40 min, 3, 6, and 18 h after administration of the inducers as described earlier [11]. Livers were homogenized in buffer I (50 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$, 0.25 mM sucrose, 0.1 mM PMSF) using a Teflon homogenizer. Homogenate was layered onto buffer I containing 2.1 M sucrose (ratio 4 : 1) and then centrifuged at 4°C using a Beckman centrifuge (SW28 rotor) at 50,000*g* for 50 min. The fraction containing nuclei was washed with saline and used for subsequent isolation of nuclear proteins.

Nuclear extract proteins were prepared as described [12]. Briefly, nuclei were suspended in the lysing buffer (10 mM HEPES, pH 7.4, 3 mM $MgCl_2$, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 10% glycerol) with addition of 1/10 volume of saturated ammonium sulfate solution. The suspension was centrifuged at 4°C using a Beckman centrifuge (rotor Ti60) at 105,000*g* for 90 min. The supernatant was mixed with ammonium sulfate (320 mg/ml) and centrifuged at 4°C using a Beckman centrifuge (rotor Ti60) at 105,000*g* for 50 min. The pellet containing nuclear proteins was dissolved in buffer containing 25 mM HEPES, pH 7.5, 50 mM NaCl, 2 mM $MgCl_2$, and 4% glycerol and dialyzed against the same buffer for 3 h. In all samples, protease inhibitors were added.

Analysis of DNA–protein complexes by electrophoretic mobility shift assay (EMSA). Oligonucleotides corresponding the *CYP2B* region from –89 to –73 bp, Barbie-box sequence 5′-ATAGCCAAAGCAGGAGG-3′ B and its complementary sequence, were used. The nucleotide was labeled at the 5′-end using T4-polynucleotide kinase and [γ-^{32}P]ATP. Unincorporated [γ-^{32}P]ATP was removed by gel filtration on Sephadex G-25. Nuclear extract proteins (5 mg) were incubated with 0.3 μg of poly(dI-dC) and γ-^{32}P-labeled oligonucleotide in buffer containing 25 mM HEPES, pH 7.5, 50 mM NaCl, 2 mM $MgCl_2$, and 4% glycerol for 2 h. For analysis of specificity of DNA–protein complexes, 100-fold excess of the cold oligonucleotide was added to the incubation mixture. After the incubation, samples were analyzed in 10% polyacrylamide gel in TBE-buffer (pH 7.5) followed by autoradiography.

Total RNA was isolated from rat liver using an RNAeasy Mini Kit (Qiagen, USA).

***CYP2B* gene expression was analyzed** by semi-quantitative RT-PCR. Besides *CYP2B*, expression of *β-actin* (the housekeeper gene) was determined in each sample. The following primers were used: forward 5′- GGA GAG CGC TTT GAC TAC-3′ and reversed 5′-CTC GTG GAT AAC TGC ATC-3′ corresponding to exons 6-7 of *CYP2B*; forward 5′-ACC CAC ACT GTG CCC ATC TA-3′, and reversed 5′-CGG AAC CGC TCA TTG CC-3′ corresponding to *β-actin* gene. PCR-products were separated in 1.5% agarose gel, stained with ethidium bromide, and scanned in the UV using a video system DNA Analyzer (Moscow, Russia).

Densitometry was carried out using Total Lab software. Expression of each gene was presented in relative

units as the ratio of intensity of staining of the specific bands of *CYP2B* to *β-actin* gene.

Statistics. Results were treated statistically using STATISTICA software. Statistical significance of differences between various groups was evaluated Student's *t*-test.

RESULTS

The effect of phenobarbital type inducers on the complex formation between nuclear proteins and Barbie-box regulatory element was investigated by electrophoretic mobility shift assay (EMSA). Nuclear proteins isolated from control rats and rats pretreated with inducers for 40 min, 3, 6, and 18 h were used. Figures 1 and 2 show characteristic patterns of DNA–protein complex formation. Incubation of Barbie-box with nuclear proteins isolated from liver of control rats resulted in formation of two complexes, I and II (Fig. 1, lane *2*; Fig. 2, lane *2*).

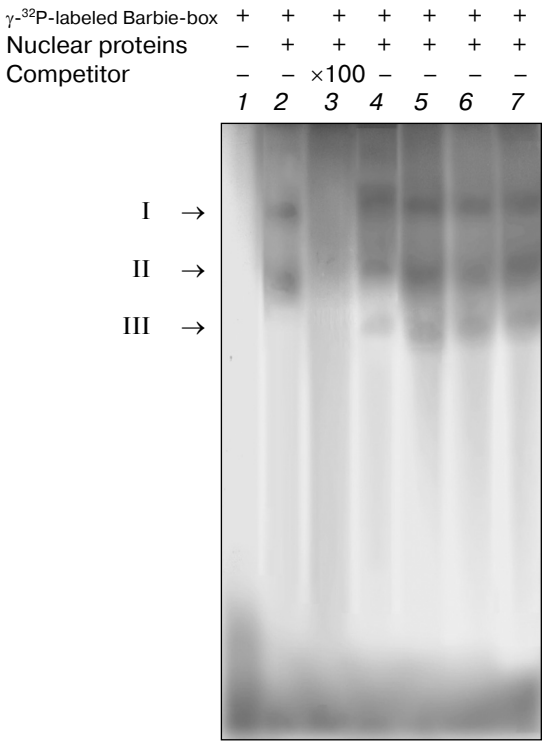

Fig. 1. Effect of time interval after inducer administration to rats on dynamics of complex (I, II, III) formation between nuclear proteins and γ-^{32}P-labeled Barbie-box sequence. Results of electrophoretic mobility shift assay of DNA–protein complexes formed by γ-^{32}P-labeled Barbie-box and hepatic nuclear proteins from control and phenobarbital (PB)-induced (I_{PB}) rats are shown. Electrophoretic mobility was assayed in 10% polyacrylamide gel. The incubation time was 30 min. Unlabeled Barbie-box oligonucleotide was used as the competitor. Lanes: *2*) control; *3*) I_{PB} (3 h) + unlabeled Barbie box; *4*) I_{PB} (40 min); *5*) I_{PB} (3 h); *6*) I_{PB} (6 h); *7*) I_{PB} (18 h).

The same experiment with nuclear proteins isolated from livers of rats pretreated with PB or TPD revealed both change in binding intensity (of complexes I and II) and formation of a new complex. The intensity of binding signal of complexes between nuclear proteins and γ-^{32}P-labeled Barbie-box was evaluated using densitometry of radioautographs (data not shown).

Use of hepatic nuclear extracts from PB treated rats revealed that *in vivo* induction for 40 min was accompanied by reduction of binding intensity of nuclear proteins during formation of complexes I and II and formation of a new complex (III) of lower molecular mass (Fig. 1, lane *4*). However, longer time interval after inducer administration (3 h) was characterized by increased binding intensity of all three complexes, which became the same at 18 h after inducer administration (Fig. 1, lane *7*). It should be noted that formation of these complexes was sensitive to competitive inhibition by cold DNA probe containing non-labeled Barbie-box sequence (Fig. 1, lane *3*).

Incubation of hepatic nuclear extract proteins isolated from TPD-pretreated rats with Barbie-box sequence also resulted in formation of various DNA–protein complexes. Their number and intensity depended on time interval after administration of this inducer. During the first hour after its administration, only complex I (Fig. 2, lane *3*) corresponding to one of two complexes registered using control nuclear proteins (Fig. 2, lane *2*) was recognized. Incubation of *CYP2B* Barbie-box element with hepatic nuclear proteins isolated from rats pretreated with TPD for 3, 6, and 18 h resulted in formation of three complexes (Fig. 2, lanes *4*, *5*, *6*). Addition of 100-fold excess of cold Barbie-box nucleotide sequence also resulted in almost complete disappearance of complexes I, II, and III (Fig. 2, lane *6*). This also suggests specific complex formation.

For further verification of the fact that the inducible activation of nuclear proteins is accompanied by increased expression of *CYP2B*, we also determined level of expression of these genes. The content of mRNA was evaluated by the method of semi-quantitative RT-PCR. Total RNA was isolated from livers of rats pretreated with PB and TPD for 40 min, 3, 6, and 18 h. Figure 3a shows results of RT-PCR. For quantitative evaluation of gene expression, the electrophoregram was subjected to densitometry, and results in Fig. 3b are presented as a ratio of intensities of specific band corresponding to *CYP2B* and *β-actin* genes. Control samples are characterized by weak constitutive expression of *CYP2B*. During the first 40 min after administration of PB but not TPD there was insignificant increase in mRNA level, which coincided with reduction of number of DNA–protein complexes under TPD induction (Fig. 2, lane *3*). Insignificant changes in the gene expression were also observed in the second time interval studied (3 h after inducer administration). A marked tendency toward increase in gene

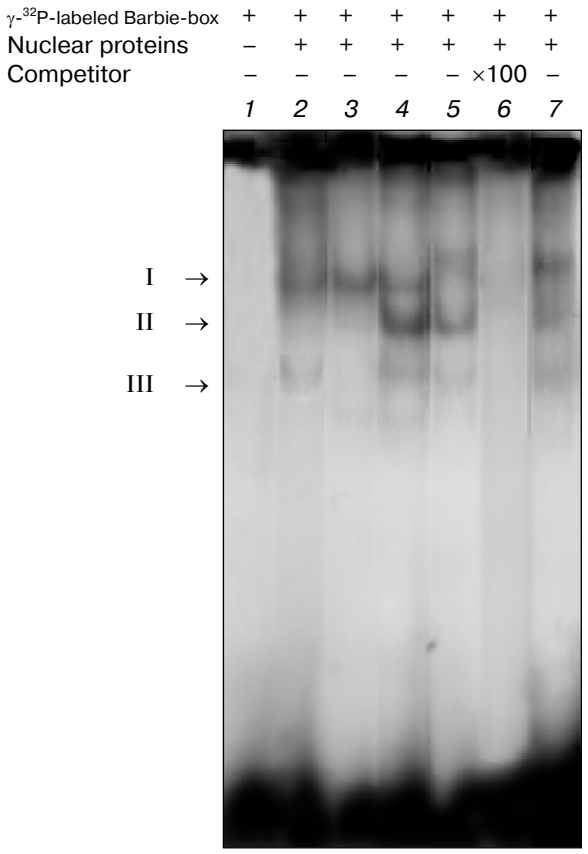

Fig. 2. Effect of time interval after inducer administration to rats on dynamics of complex (I, II, III) formation between nuclear proteins and γ-^{32}P-labeled Barbie-box sequence. Results of electrophoretic mobility shift assay of DNA–protein complexes formed by γ-^{32}P-labeled Barbie-box and hepatic nuclear proteins from control and triphenyldioxane (TPD)-induced (I_{TPD}) rats are shown. Electrophoretic mobility was assayed in 10% polyacrylamide gel. The incubation time was 30 min. Unlabeled Barbie-box oligonucleotide was used as the competitor. Lanes: *2*) control; *3*) I_{TPD} (40 min); *4*) I_{TPD} (3 h); *5*) I_{TPD} (6 h); *6*) I_{TPD} (6 h) + unlabeled Barbie box; *7*) I_{TPD} (18 h).

expression was noted at 6 and 18 h after administration of both inducers. It should be noted that at 6 and 18 h after administration of both PB and TPD the content of CYP2B mRNA was significantly higher than that in control animals.

DISCUSSION

In this study, we have compared effects of TPD and PB on complex formation between rat liver nuclear proteins and Barbie-box element of *CYP2B* genes at early stages of induction. Study of the interaction of nuclear proteins with Barbie-box regulatory element of this gene revealed that both treatments of rats with PB and TPD effectively stimulated DNA–protein complex formation at various time intervals after administration of these compounds. In the case of phenobarbital induction three DNA–protein complexes were registered and the pattern of their formation was almost the same at all time intervals studied. The only exception was the first time interval, 40 min after administration when intensity of protein binding during formation of complexes I and II reduced (Fig. 1). This is consistent with observations from Padmanaban's laboratory [4-6]. These authors demonstrated formation of three complexes at 6 and 24 h time intervals after phenobarbital induction. On incubation of hepatic nuclear proteins from TPD-treated rats with Barbie-box sequence, we also registered dynamics of intensity changes during DNA–protein complex formation (Fig. 2).

Differences in dynamics of DNA–protein complex formation seen during induction with PB and TPD suggest the existence of specific nuclear receptors for each inducer. Our previous results indicating that *in vitro* incubation of nuclear proteins with Barbie-box in the presence of PB caused formation of three complexes, whereas in the presence of TPD five complexes were detected [13] seem to support this suggestion.

The distinct pattern of complex formation between nuclear proteins and Barbie-box sequences seen during induction with PB and TPD may reflect processes of activation of nuclear and cytosolic proteins followed by subsequent translocation of cytosolic proteins into the nucle-

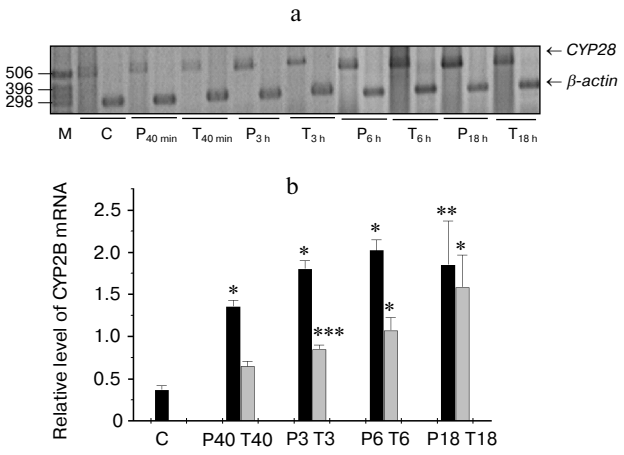

Fig. 3. Effect of time interval after inducer administration to rats on dynamics of *CYP2B* gene expression. a) Electrophoretic separation of RT-PCR products obtained by amplification of *CYP2B* and *β-actin* genes. b) Data of densitometric analysis expressed as the ratio between intensities of staining of *CYP2B* specific band to *β-actin* gene band. Designations: C) control; $P_{40\ min,\ 3\ h,\ 6\ h,\ 18\ h}$) phenobarbital-induced rats; $T_{40\ min,\ 3\ h,\ 6\ h,\ 18\ h}$) triphenyldioxane-induced rats. RNA was isolated from rat liver at 40 min, 3, 6, and 18 h time intervals after inducer administration. Results represent mean ± SEM ($n = 3$). Asterisks show statistical significance of differences compared to corresponding controls: * $p < 0.001$, ** $p < 0.01$, *** $p < 0.05$.

us. It is also possible that during a particular time interval inducers may influence the conformation of specific nuclear protein receptors. Increase in intensity of DNA–protein complexes detected at 18 h may be related to their synthesis or activation through phosphorylation/dephosphorylation processes. At later stages of induction (18 h) the intensity of complexes was the same for both inducers. This suggests that for each inducer specific signaling pathways exist only at early stage of *CYP2B* gene activation.

Formation of new DNA–protein complexes was accompanied by increased transcription of *CYP2B*. In fact, in control samples only two complexes were registered, in the case of short term (40 min) TPD induction when only one complex was registered, mRNA content was very low. Maximal mRNA coincided with the appearance of new complexes seen on the induction with both PB and TPD for 18 h.

Thus, in the present study we have demonstrated for the first time that early stages of induction (40 min after PB administration or 3 h after TPD administration to rats) are characterized by activation of nuclear proteins that can bind to *CYP2B* Barbie-box element. Although these inducers have different chemical structures, which obviously underline different pathways of activation of such binding, our experiments clearly demonstrate that this process involves activation of gene transcription.

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