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Inducer Effect on the Complex Formation between Rat Liver Nuclear Proteins and Cytochrome P450 2B Gene Regulatory Elements

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Abstract—DNA gel retardation assay has been applied to the investigation of complexes between rat liver nuclear proteins and Barbie box positive regulatory element of cytochrome P450 2B (*CYP2B*) genes. The intensities of B1 and B2 bands detected in the absence of an inducer increased after 30 min protein incubation with phenobarbital (PB) or triphenyldioxane (TPD), but not with 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOPOB). In addition, a new complex (B3 band) was for the first time detected under induction by PB, TPD, and TCPOPOB. Increase in the incubation time up to 2 h facilitated the formation of other new complexes (B4 and B5 bands), which were detected only in the presence of TPD. The use of [^{3}H]TPD in hybridization experiments revealed that this inducer, capable of binding to Barbie box DNA, is also present in B4 and B5 complexes. It is probable that the investigated compounds activate the same proteins at the initial induction steps, which correlates with the formation of B1, B2, and B3 complexes. The further induction step might be inducer-specific, as indicated by the formation of B4 and B5 complexes in the presence of TPD only. Thus, the present data suggest the possibility of specific gene activation signaling pathways that are dependent on a particular inducer.

Key words: rat *CYP2B2* gene, triphenyldioxane, phenobarbital, Barbie box, nuclear proteins, gel retardation assay

Cytochromes P450s, products of a gene superfamily, are responsible for the metabolism of a wide range of endogenous and exogenous compounds. Transcription of many cytochrome genes including *CYP2B* is enhanced in the presence of various xenobiotics. However, the mechanism of *CYP2B* induction has remained unclear for a long time. In contrast to cytochrome P450 1A inducers, which interact with the Ah-receptor owing to their planar structure, no structural homology has been found for cytochrome P450 2B inducers. This group (so-called PB-type inducers) includes terpenes, non-planar polychlorinated biphenyls, aliphatic ketones, organochlorine pesticides, and methylenedioxyphenyls [1]. The mechanism of their effect on the target genes has been unknown. It was found only a short time ago that the important step in *CYP2B* and *CYP3A* gene expression includes the activation of a constitutive androstane nuclear receptor (CAR) [2]. This protein was isolated from mouse liver nuclear extracts and characterized according to its ability to bind to *CYP2B* regulatory regions [3, 4]. It has been proved that under PB or TCPOPOB *in vitro* effect CAR is translocated into the nucleus, where it binds to DNA regulatory elements as a heterodimer with a retinoid X receptor (RXR) [5], thus triggering the transcription of various genes including *CYP2B* [6]. Recently it was shown that structurally dissimilar xenobiotics could bind to mouse and human CAR [7]. This fact suggests that CAR as well as pregnane receptor X (PXR) are able to recognize xenobiotic inducers. Species differences in *CYP2B* induction may be explained by heterogeneous affinity of the inducers towards CAR. Thus, clotrimazol activates human but not mouse CAR, whereas TCPOPOB activates mouse but not human CAR [7]. Beside the investigation of nuclear receptors, considerable progress has been achieved in the study of DNA sequences to which these receptors bind. These sequences were found in the proximal as well as in distal sites of *CYP2B* gene regulatory regions. It was found that 5′-flanking region of *CYP2B* contains PB responsive element (PBRE), located within −2318/−2155 bp range [8, 9]. This element acts as a transcription enhancer and

Abbreviations: CYP2B) cytochrome of P450 2B sub-family; CYP1A) cytochrome of P450 1A sub-family; DRE) Dioxin Responsive Element of *CYP1A1* gene; NE) negative element of *CYP2B* gene; PB) phenobarbital; TPD) triphenyldioxane; TCPOPOB) 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene; DMSO) dimethylsulfoxide.

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is involved in the formation of a multi-component complex with specific regulatory proteins that bind to the DNA recognition sites. PBRE contains a central binding site for the NF1 nuclear factor, flanked with two other receptor binding sites, denoted as NR1 and NR2. CAR receptor binds to each PBRE site as a heterodimer with RXR, thus resulting in the transcription of PB-inducible genes [10]. The PBREM element was also found in mouse *Cyp2b9* and *Cyp2b10* genes and human *CYP2B6* gene [6, 11]. Numerous studies showed that minimal *CYP2B2* promoter (−179/+1 bp) is also involved in the transcription of this gene affected by PB [12-14]. This region consists of a positive element (PE, −98/−69 bp) also including the Barbie box element (−89/−73 bp), and a negative element (NE, −160/−127 bp) [12, 15]. It was demonstrated that nuclear proteins interact with Barbie box sequence according to their phosphorylation status [16]. The supposition was made that the inducers initiate transcription of the corresponding genes, thus triggering the nuclear protein phosphorylation [12].

During the investigation of *CYP2B* induction mechanisms in rat liver, we discovered an effective interspecies specific *CYP2B* inducer, triphenyldioxane (TPD). This compound results in a substantial increase in *CYP2B* activity in rat but not mouse liver [17]. However, the mechanism of its action remained unknown. Our experiments on the DNA gel retardation assay with Barbie box element has shown that rat treatment with TPD or PB causes the increase in band intensities corresponding to B1 and B2 DNA–protein complexes [18]. These results give evidence for similar mechanisms of these inducers when injected into animals. For the evaluation of the earlier steps in the gene activation process, we have investigated the formation of these complexes using untreated rat liver nuclear protein extracts to which TPD or other *CYP2B* inducers (PB or TCPOPOB) were added.

MATERIALS AND METHODS

TPD and TCPOPOB used as inducers were synthesized in the Institute of Biochemistry, Russian Academy of Sciences (Moscow). PB was obtained from Merck (Germany). The [γ-^{32}P]ATP (~6000 Ci/mmol) was purchased through Amersham (USA). [^{3}H]TPD (~50 Ci/mmol) was prepared and purified chromatographically at the Institute of Bioorganic Chemistry (Novosibirsk, Russia). Pepstatin, aprotinin, and leupeptin were obtained from Sigma (USA). Oligonucleotides used in this work were synthesized at the Institute of Cytology and Genetics (Novosibirsk, Russia): Barbie box (5′-ATAGCCAAAGCAGGAGG-3′, covering −89 to −73 bp of the *CYP2B2* gene and its complementary sequence); negative element (5′-TGTCCTGTGATCTGTTTCG-TGGTGTCCTTGCCAAC-3′, covering −160 to −127 bp of the *CYP2B2* gene and its complementary sequence); dioxin responsive element (5′-GATCTGGCTCTTCT-CACGCAACTCCCG-3′, and its complementary sequence).

Treatment of animals. Male Wistar rats (4-6-week-old) were used in this work. Animals were injected with a single dose of TPD, TCPOPOB (10 or 3 mg/kg body weight in corn oil, respectively), or PB in physiological saline (80 mg/kg body weight). Control animals were injected with an equal volume of corn oil or physiological saline. Animals were killed 16 h after inducer injection.

Preparation of nuclear protein extracts. Liver samples of 2 g from two adult rats were homogenized separately in a threefold excess of buffer containing 10 mM HEPES (pH 7.4), 1.5 mM $MgCl_2$, 10 mM KCl, 7.8 mU/ml trypsin inhibitor, and aprotinin, leupeptin, and pepstatin (2.25 mg/ml each). The homogenates were centrifuged at 12,000*g* for 20 min at 4°C. Nuclei precipitate was used to produce nuclear extracts as described earlier [19].

DNA gel retardation assay. Double-stranded oligonucleotides labeled at the 5′-end using T4-polynucleotide kinase and [γ-^{32}P]ATP [19] were employed for the investigation of nuclear protein binding with DNA regulatory elements. Standard conditions for the binding reactions involved the incubation of 3-5 μg of nuclear protein (in a final volume of 25 μl), 0.5 μg of poly(dI)/poly(dC), and γ-^{32}P-labeled probe (0.5-1.0 ng) in the binding buffer (25 mM HEPES, pH 7.5, 50 mM NaCl, 2 mM $MgCl_2$, 4% glycerol). All the components except for the oligonucleotide probe were mixed together and preincubated at 37°C for 15 min. The same reaction was performed with the addition of 4 mM (or 0.4 mM) PB, TPD, and/or TCPOPOB. After the addition of γ-^{32}P-labeled oligonucleotide probe, the incubation was allowed to proceed for 30 min at room temperature.

In vitro effect of the inducers was studies under three different conditions. First, the hybridization reaction was performed under standard conditions (see above) in the presence of PB (solved in 25 mM HEPES, pH 7.5) or TPD or TCPOPOB (solved in DMSO). Second, the mixture was preincubated for 15 min, after which the binding reaction was carried out for 90 min at room temperature. Third, the nuclear proteins were incubated in the presence of inducer for 90 min at room tempeature and then dialyzed for 2 or 12 h to remove the inducer, as recommended by He and Fulco [16].

[^{3}H]TPD *in vitro* effect on nuclear protein binding to DNA was studied under the same conditions as for PB analyzed as described above for PB, TPD, and TCPOPOB. Incubation of [^{3}H]TPD (21 pmol/μl) and non-labeled compounds (PB, TPD, and TCPOPOB) with DNA probes was carried out for 30 or 120 min at room temperature in the absence of nuclear proteins.

Each experiment with the use of the labeled probe was accomplished by a competitive assay with addition of a 100-fold excess of non-labeled oligonucleotide analog

into the reaction mixture at the preincubation step. After the binding reaction was complete, the probes were analyzed in 10% polyacrylamide gel in TBE buffer, pH 7.5, followed by radioautography using GBX2 film (Kodak, USA) [20].

RESULTS

Effect of PB, TPD, and TCPOPOB on the formation of nuclear protein complexes with *CYP2B* gene regulatory regions. The effects of chemical compounds with various structures on the formation of nuclear protein complexes with Barbie box element was investigated in a number of DNA gel retardation experiments using liver nuclear extracts from treated and untreated rats. The results shown in Fig. 1 represent a characteristic shift pattern with B1 and B2 protein complexes, registered under incubation of nuclear proteins with Barbie box in the absence of inducers (lanes *2* and *9*). Animal treatment with PB or TPD (lanes *4* and *6*) but not with TCPOPOB (lane *8*) resulted in the increase of B2 band intensity. Similar experiments were performed using nuclear extracts from the control rats, where various chemical compounds were added to the incubation mixture. As seen from Fig. 1, a new B3 complex formed after 30 min incubation appears in this case (lanes *3*, *5*, *7*), beside B1 and B2 complexes. A 10-fold decrease in inducer amount in the analyzed probe did not result in the extinction of these complexes (data not shown).

To show that only Barbie box is involved in B3 complex formation, we performed competitive experiments with the addition of other regulatory elements. It is seen that neither DRE nor NE influence the formation of this complex (Fig. 2, lanes *2*, *4*, *5*, *7*), whereas the addition of 100-fold excess of Barbie box oligonucleotide resulted in the disappearance of the complex (Fig. 2, lanes *3* and *6*). The addition of a 100-fold excess of non-labeled NE (but

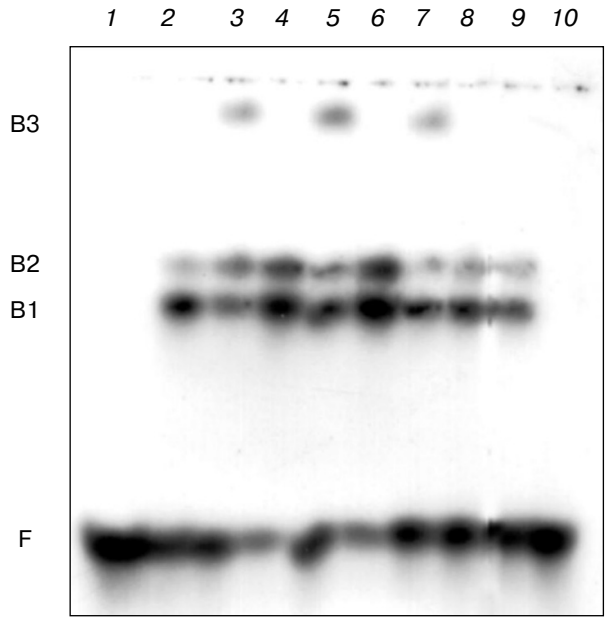

Fig. 1. Binding of nuclear proteins to γ-^{32}P-labeled Barbie box element affected by inducers of PB-type. Results of DNA gel retardation assay of the complexes between γ-^{32}P-labeled Barbie box and nuclear proteins from untreated rat liver (C) and induced rats (I) are presented. Incubation mixture contained nuclear extracts from the liver of untreated rats (C) with addition of an inducer (up to concentration of 4 mM) or rats treated with PB (I_{PB}), TPD (I_{TPD}), or TCPOPOB ($I_{TCPOPOB}$). Incubation time was 30 min. γ-^{32}P-labeled Barbie box is present in each lane. Positions of B1, B2, and B3 bands are indicated. F shows migration of unbound probe. Lanes: *1*, *10*) γ-^{32}P-labeled Barbie box alone; *2*, *9*) C; *3*) C in the presence of PB; *4*) I_{PB} nuclear proteins; *5*) C in the presence of TPD; *6*) I_{TPD} nuclear proteins; *7*) C in the presence of TCPOPOB; *8*) $I_{TCPOPOB}$ nuclear proteins.

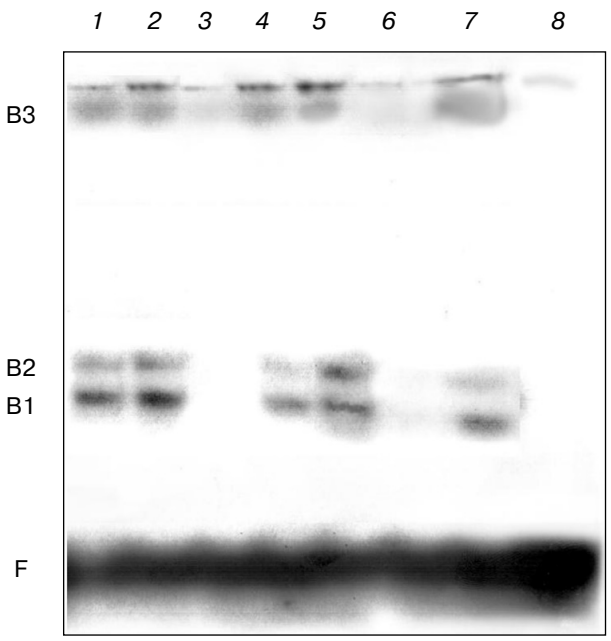

Fig. 2. Competitive effect of NE and DRE oligonucleotides on nuclear protein binding to γ-^{32}P-labeled Barbie box. Incubation mixtures contained nuclear extracts from untreated rat liver (C), γ-^{32}P-labeled Barbie box oligonucleotide, either PB or TPD (4 mM), and a 100-fold excess of non-labeled competing oligonucleotide. Positions of B1, B2, and B3 bands are indicated. F shows the unbound probe. Incubation time was 90 min. Lanes: *1*) C in the presence of PB without a competitor; *2*) C in the presence of PB and 100-fold excess of DRE; *3*) C in the presence of PB and 100-fold excess of non-labeled Barbie box; *4*) C in the presence of PB and 100-fold excess of NE; *5*) C in the presence of TPD and 100-fold excess of DRE; *6*) C in the presence of TPD and 100-fold excess of unlabeled Barbie box; *7*) C in the presence of TPD and 100-fold excess of NE; *8*) γ-^{32}P-labeled Barbie box alone.

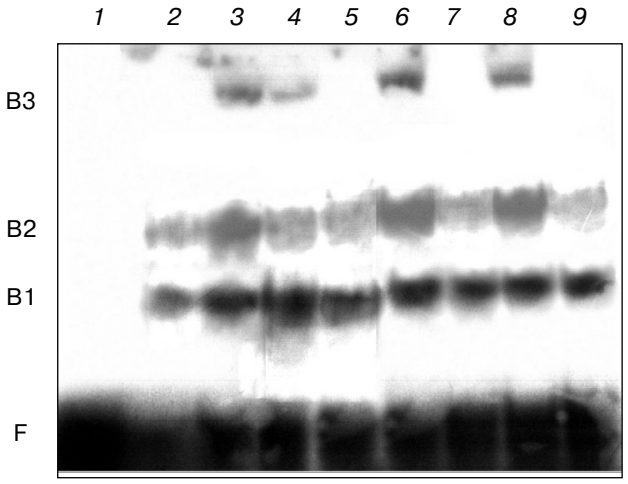

Fig. 3. Binding of nuclear proteins to the Barbie box element in the presence of inducers and after dialysis. In all cases the γ-^{32}P-labeled Barbie box was used. Nuclear proteins from untreated rat liver (C) were incubated with PB, TPD, or TCPOPOB in the absence of oligonucleotide probe and then the inducer was removed by dialysis for 2 or 12 h prior the DNA binding reaction. Incubation time was 90 min. Positions of B1, B2, and B3 bands are indicated. F shows the migration of the unbound probe. Lanes: *1*) γ-^{32}P-labeled Barbie box alone; *2*) C in the presence of Barbie box; *3*) C in the presence of Barbie box and 4 mM TPD; *4*) C in the presence of Barbie box and 4 mM TPD after 2 h dialysis; *5*) C in the presence of Barbie box and 4 mM TPD after 12 h dialysis; *6*) C in the presence of Barbie box and 4 mM PB; *7*) C in the presence of Barbie box and 4 mM PB after 2 h dialysis; *8*) C in the presence of Barbie box and 4 mM TCPOPOB; *9*) C in the presence of Barbie box and 4 mM TCPOPOB after 2 h dialysis.

nor DRE) oligonucleotide inhibited the formation of B2 complex in the analogous competitive experiments (Fig. 2, lanes *4* and *7*). We also performed the experiments with inducers removed from the incubation mixture by dialysis. As seen in Fig. 3, formation of B3 complex is dependent only on the presence of analyzed PB-type inducers (lanes *3*, *6*, *8*). This complex was not found, however, when PB or TCPOPOB was removed from the nuclear extracts by 2 or 12 h dialysis (Fig. 3, lanes *5*, *7*, *9*). No detectable variations were found for the intensities of B1 and B2 bands.

Use of [^{3}H]TPD in EMSA. To elucidate the inducer role in the formation of DNA–protein complexes, we carried out a series of experiments using [^{3}H]TPD. For this purpose the radioactively labeled inducer was incubated with three different oligonucleotides: Barbie box, NE, and DRE. As seen in Fig. 4, [^{3}H]TPD is complexed with Barbie box oligonucleotide (lane *3*). Maximal intensity of the radioactive signal was observed after 2 h incubation with an inducer and remained constant for 8 h (data not shown). As evident from the electrophoresis, the mobility of [^{3}H]TPD–DNA complex was consistent with that of γ-^{32}P-labeled Barbie box (Fig. 4, lanes *1* and *3*, respectively). Thus, [^{3}H]TPD interacts with a double-stranded Barbie box sequence. In this connection the question of whether PB is able to compete for binding with Barbie box was addressed. We have demonstrated that a 100-fold PB excess does not affect [^{3}H]TPD interaction with Barbie box element (Fig. 4, lane *4*). [^{3}H]TPD did not bind to NE or DRE elements, as evidenced by the absence of radioactive signal (Fig. 4, lanes *6* and *8*). When nuclear proteins were added to the reaction mixture, [^{3}H]TPD formed two high molecular weight complexes with Barbie box (B4 and B5; Fig. 4, lane *5*), which were not affected by a 100-fold excess of PB (Fig. 4, lane *7*). New B4 and B5 complexes were detected after 2 h incubation. When analogous experiments were performed using γ-^{32}P-labeled Barbie box, a whole range of B1-B5 complexes was observed (Fig. 5, lane *2*). However, the corresponding B4 and B5 bands were not detected under 30-min incubation, and B3 band was of lower intensity (Fig. 4, lane *2*, and Fig. 5, lane *3*).

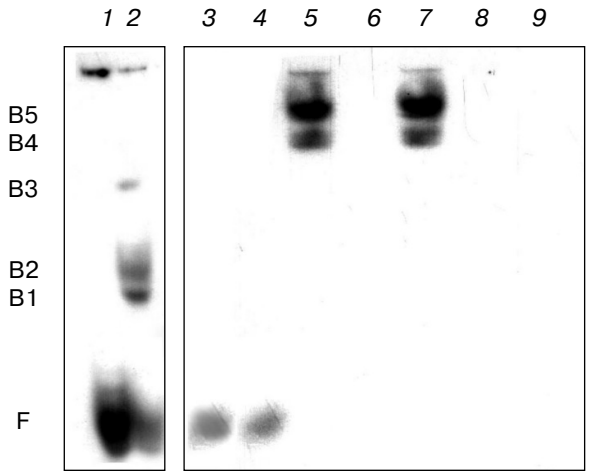

Fig. 4. TPD effect on the formation of specific complexes between nuclear proteins and Barbie-box element. The results of the gel retardation assay of the complexes between either γ-^{32}P-labeled Barbie box alone or [^{3}H]TPD with Barbie box and nuclear proteins from untreated rat liver (C). Position of B1-B5 complexes are indicated, F is unbound probe. Lanes: *1*) γ-^{32}P-labeled Barbie box alone; *2*) C incubated with γ-^{32}P-labeled Barbie box in the presence of 4 mM TPD for 30 min at room temperature; *3*) [^{3}H]TPD in the presence of Barbie box; *4*) [^{3}H]TPD in the presence of Barbie box and 100-fold excess of PB; *5*) non-labeled Barbie box incubated with C and [^{3}H]TPD for 2 h; *6*) non-labeled DRE incubated with [^{3}H]TPD; *7*) non-labeled Barbie box incubated with C, [^{3}H]TPD, and 100-fold excess of PB for 2 h; *8*) non-labeled NE incubated with [^{3}H]TPD for 30 min; *9*) non-labeled NE incubated with C and [^{3}H]TPD for 30 min.

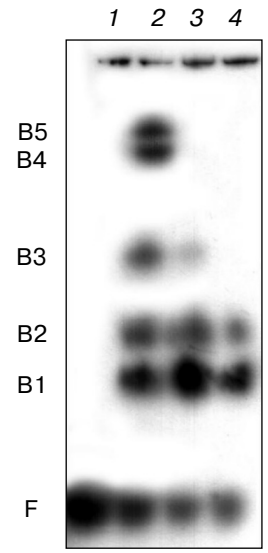

Fig. 5. Binding of nuclear extract proteins from untreated rat liver to γ-^{32}P-labeled Barbie box oligonucleotide with TPD after different incubation time. Reaction mixture was analyzed 30 min or 2 h after incubation at room temperature. Positions of B1-B5 bands are shown. F corresponds to inbound probe. Lanes: *1*) γ-^{32}P-labeled Barbie box alone; *2*) C + γ-^{32}P-labeled Barbie box + TPD after 2 h incubation; *3*) C + γ-^{32}P-labeled Barbie box + TPD after 30 min incubation; *4*) C + γ-^{32}P-labeled Barbie box after 30 min incubation.

DISCUSSION

DNA gel retardation assay is a sufficiently simple and convenient tool for the investigation of early events in gene induction. It allows examining the interaction of nuclear proteins (putative transcription factors) with the transcriptional gene regulatory elements. In the present study we have investigated the effect of TPD on complex formation between nuclear proteins and Barbie box. DNA gel retardation experiments performed in the Fulco [16] and Pabmanaban [12] laboratories have shown that Barbie box forms two types of complexes with nuclear proteins. Numerous studies demonstrated that minimal *CYP2B2* promoter (–179/+1 bp), which includes the Barbie box, is an important regulatory region, involved in PB induced transcription [7-9]. As we reported previously, the nuclear extracts from PB- or TPD-treated rat liver form complexes with Barbie box sequence, and the intensity of these complexes substantially increased under induction [18]. In this work we have detected two Barbie box complexes with nuclear proteins from untreated rat liver after 30 min incubation (Fig. 1). In addition, we have registered for the first time a new B3 complex, which is formed in the presence of PB, TPD, or TCPOPOB. Moreover, the experiments demonstrating that this complex is formed in the presence of Barbie box element and inducers of PB-type in the incubation mixture were performed (Figs. 2 and 3). It is probable that each of the examined compounds activate one and the same transcription factors, also including CAR-receptor. This assumption is confirmed by the fact that structurally dissimilar compounds can bind to this protein [7]. The increase of incubation time with an inducer up to 2 h resulted in the formation of two new B4 and B5 complexes, detected in the presence of TPD only. [^{3}H]TPD involved in the experiments was bound to Barbie box within B4 and B5 complexes (Fig. 4), that in our opinion provides further evidence of TPD effect for the formation of those. It is interesting to note that B1-B3 complexes were not detected in this case, which may account for low affinity of the certain nuclear proteins toward Barbie box. It is rather difficult to explain the mechanism of *in vitro* xenobiotic effect on the nuclear proteins based on up-to-date knowledge. A comparison of the results obtained under *in vitro* inducer addition and its injection into animals, displays similar shift patterns (Fig. 1, lanes *2* and *3*). It should be mentioned that B3 complex, which appeared after the addition of an inducer to nuclear proteins from untreated animals, was detected only at the early induction steps, as well as B4 and B5 complexes. They disappear after 6 h incubation (data not shown). It can be assumed that a xenobiotic inducer changes the protein conformation upon binding, thus facilitating the formation of heterodimers with other proteins. Another possibility is that inducer binding to nuclear proteins may trigger their phosphorylation/dephosphorylation processes.

Summarizing, the results provide evidence for specific signaling pathways for each inducer. Chemical compounds involved in the present investigation activate the same nuclear proteins at the initial induction steps, as proved by the formation of B1, B2, and B3 complexes. Further induction events may differ for various inducers and thus appear to be inducer-dependent. This supposition is confirmed by the formation of B4 and B5 complexes specific for TPD only. The use of radioactively labeled TPD elucidated its role in the formation of DNA–protein complexes and even its ability to bind to Barbie box element. The lack of the radioactive signal upon [^{3}H]TPD incubation with NE or DRE elements may be an indication of a regulatory significance of inducer binding to Barbie box for *CYP2B* gene expression. Nevertheless, particular details of the molecular mechanism of TPD induction remain unclear. Further study of these events, also including the investigation of CAR activation, interaction of nuclear proteins with *CYP2B* PBRE elements, as well as identification of nuclear proteins within new complexes may help to establish the mechanisms of *CYP2B* gene activation.

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