Early growth response 1 loops the *CYP2B6* promoter for synergistic activation by the distal and proximal nuclear receptors CAR and HNF4α

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Abstract

Nuclear xenobiotic receptor CAR activates transcription of the *CYP2B6* gene by directly binding to the distal enhancer PBREM. This CAR-mediated activation is synergized by transcription factors EGR1 and HNF4α that bind to the proximal element $OARE_{KI}$ [1]. Two additional EGR1 binding sites have now been found just downstream from PBREM. Internal deletion of EGR 1 sites within the context of the −1.8 kb *CYP2B6* promoter, which contains both PBREM and $OARE_{KI}$, revealed that the distal and proximal EGR1 sites are essential for EGR1 to synergize CAR-mediated transcription. Chromatin conformation capture 3C assays demonstrated that ERG1 may loop the distal PBREM towards the proximal $OARE_{KI}$ so that together, CAR and HNF4α synergistically activate the *CYP2B6* promoter.

INTRODUCTION

Hepatocytes have genes that encode xenobiotic metabolizing enzymes and transporters which are induced following exposure to xenobiotics including therapeutic drugs. Induction of these genes helps to defend the body against the toxicity and carcinogenicity of these xenobiotics, and also affects drug efficacy and can cause drug-drug interactions. Human cytochrome P450 2B6 (CYP2B6), one such enzyme, is clinically important as it metabolizes many of the commonly used therapeutics and activates anti-cancer pro-drugs such as cyclophosphamid and ifosfamide [2]. Numerous drugs, such as phenobarbital, phenytoin and rifampicin, induce transcription of the *CYP2B6* gene, an activity in which the nuclear xenobiotic receptors CAR and PXR perform the essential roles [3]. For example, phenobarbital specifically translocates CAR from the cytoplasm into the nucleus [4], leading the direct binding of CAR to the distal sequence called "phenobarbital responsive enhance module" (PBREM) to trans-activate the *CYP2B6* promoter [5]. While the distal PBREM is the primary CAR binding site required for drug activation of the *CYP2B6* promoter, our recent study has shown that CAR also requires the 53-bp composite regulatory element $OARE_{KI}$ located in the proximal promoter region to activate the *CYP2B6* gene [1,6]. The molecular mechanism by which the *CYP2B6* promoter connects the function of the distal PBREM with that of $OARE_{KI}$ to regulate its activity, however, still remains elusive.

The OARE$_{KI}$ consists of the DR1, E-box and CACCC motifs, but dose not contain a CAR binding sequence. HNF4α, acting as the constitutive factor, always occupies the DR1 site, whereas early growth regulation 1 (EGR1) is the cellular signal-mediated inducible factor that binds to the CACCC site [1]. All of these three transcription factors are essential for the *CYP2B6* promoter to be activated: CAR as the drug activated factor, EGR1 as the signal-induced factor and HNF4α as the constitutive factor. Intriguingly, our previous experiments including ChIP analysis have revealed that CAR indirectly interacts with the OARE$_{KI}$ as binding to CACCC site increases. These observations lead us to hypothesize that EGR1 may facilitate the interaction of CAR with the HNF4α on OARE$_{KI}$ to activate the *CYP2B6* promoter. Here we have identified and characterized two additional EGR1 binding sites in the distal promoter region and performed chromatin conformation capture (3C) assays to investigate the molecular mechanism by which EGR1 facilitates CAR interaction with OARE$_{KI}$.

MATERIALS and METHODS

Reagents

TCPOBOP and DMSO were purchased from Sigma Chemical Co. (St. Lois, MO); poly(dI-dC)·(dI-dC) from GE Healthcare Bio-Science Corp. (Piscataway, NJ). The plasmid – pGL3basic and pcDNA3.1-V5-His-TOPO were obtained from Promega (Madison, WI) and Invitrogen (Carlsbad, CA), respectively. Normal Rabbit IgG and antibodies against EGR1 {(588)X} were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); V5 antibody from Invitrogen.

Plasmids

The −1.8-kb *CYP2B6* promoter was previously cloned into a basic firefly luciferase reporter plasmid pGL3-basic [6]. Deletions were constructed in the context of the −1.8-kb *CYP2B6* promoter using the Quick Change® site-directed mutagenesis kit (Stratagene, TX) and proper primers: ΔCA1 (−269/−244), ΔCA2 (−1477/−1468), ΔCA3 (−1519/−1511) and ΔCA123 (−269/−244 and −1519/−1468). The promoters used for 3C assays were amplified to add a XhoI site at both the 5′ and 3′ ends and were cloned into a pGL3-basic plasmid. Human EGR1 cDNA was amplified from Caco2 RNA and was cloned into pcDNA3.1-V5-His-TOPO plasmids (Invitrogen). Various deletion mutants of EGR1 were likewise constructed using the Quick Change® site-directed mutagenesis kit: pcDNA3.1-EGR1$_{\Delta 1-283}$, pcDNA3.1- EGR1$_{\Delta 3-283}$, pcDNA3.1-EGR1$_{\Delta 270-306}$ pcDNA3.1-EGR1$_{\Delta 324-377}$, pcDNA3.1- EGR1$_{\Delta 367-393}$, pcDNA3.1- EGR1$_{\Delta 422-543}$. All other plasmids used were previously described in our laboratory.

Cells and transfection

HepG2 and Ym17 cells were cultured in minimal essential medium supplemented with 10% (v/v) fetal bovine serum, antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin) and 2 mM glutamine. Ym17 cells are a mCAR-expressing stable cell line derived from HepG2 cells (6). For Luc reporter assays, cells were cultured on a 24-well plate at a density of 3.5×10^5 cells per well for 24 h prior to transfection with a given plasmid or a combination of multiple plasmids using FuGENE6 (Roche Diagnostics, Indianapolis, IN). After 24 h transfection, the cells were treated with TCPOBOP (250 nM), OA (10 nM) or CITCO (250 nM) for an additional 48h and were harvested for Luciferase assays using the Dual-Luciferase reporter assay system (Promega).

Chromosome conformation capture (3C)

3C assays were performed as described previously with some modifications [7]. Briefly, 100 μg of nuclear extract prepared from Ym17 cells were shaken with 50 ng of a given promoter at room temperature for 45 min and were incubated with formaldehyde to crosslink protein-DNA complexes. After glycines being added to stop cross linking, complexes were precipitate by ethanol at −20 °C., Precipitates were dissolved in 80 μl of TE, digested by XhoI at 37 °C for 2h and were incubated for 20 min at 70 °C to inactivate the enzyme activity of XhoI. Digests were diluted in ligation buffer not to exceed DNA concentration of over 0.5 ng/μl, from which 20 ng of DNA were ligated by T4 DNA ligase (2000 units, Promega) for 4h at 16 °C or by Quick ligase (Roche) for 5 min at room temperature. After ligation, protein DNA complexes were de-cross linked by adding SDS and NaCl up to 1% and 0.3 M, respectively and treated overnight at 55 °C and then treated with proteinase K for additional 1 hr at 65 °C, from which DNA was purified by PCR purification kit (QIAGEN). Purified DNA was subjected to amplification: the −1769/−31 and −682/−618 regions for the looped and linear DNA structures, respectively. For analysis by regular PCR, DNA was amplified by LA Taq (Takara), resolved by 1.5% agarose gel and visualized by ethylene bromide. For real-time PCR analysis using the 7900HT Fast Real-Time PCR System (Applied Biosystems) using two primer sets (forward: AGGATAAAAGGCCCAGTTGGA, reverse: CTAAGATTGGGTGCTCATTGCA; for circulating, forward: GGCCAGGATGGTCTCGAA, reverse: CCAGCACGTTGGGAAGCT for internal control).

Gel shift

Gel shift assays were performed with nuclear extracts prepared from cells harvested from 4 confluent 145 cm^2 dishes for each drug treatment using the procedures described previously [1,8].

RESULTS AND DISCUSSION

Requirement of both DNA binding and activation domain

EGR1 molecule consists of multiple domains such as the activation, repression and DNA binding domains (Fig. 1A). Domain-based deletion mutants were constructed to delineate the region of EGR1 responsible for the synergistic activation of the *CYP2B6* promoter. Deletion of either the activation (m1)[9], repression (m2)[10] or C-terminal (m6)[11] domain did not affect EGR1 binding to the CACCC motif of $OARE_{KI}$ in gel shift assays (Fig. 1B). As expected, two different deletions (m3 and m5) of the DNA binding domain [11] resulted in the loss of binding ability. These mutants were co-transfected into COS1 cells to examine their capability to synergize the CAR-HNF4α mediated activation of the *CYP2B6* promoter. The m1, m3 and m5 mutants lost this capability, while the m2 and m6 mutants fully retained it as observed with wild type EGR1 (Fig. 1C). Thus, these results indicated that EGR1 requires its activation and DNA binding domains to synergize the CAR-HNF4α mediated transcription.

Requirement of both distal and proximal CACC motifs

The *CYP2B6* promoter is regulated by the two essential composite DNA sequences: the distal PBREM and the proximal $OARE_{KI}$ (Fig. 2). Our previous study showed that EGR1 depends on its direct interaction with the proximal CACCC motif (−269/−244) to synergistically activate the −1.8 kb *CYP2B6* promoter [1]. This proximal CACCC motif (now designated CA1) is located at the 5′-end of the 53 bp $OARE_{KI}$ (−269/−217) of the *CYP2B6* promoter. Sequence-based motif search of the −1.8 kb *CYP2B6* promoter found two additional CACCC motifs (CA2 and CA3) at just down stream of the proximal PBREM (−1733/−1683): $^{-1519}$CACACATTCACACCCAC^{-1511} and $^{-1477}$TGTGTGGGTG^{-1468},

respectively. Given this finding, the role of these distal CA2 and CA3 motifs in the EGR1 mediated synergistic activation was investigated. Gel shift and super-shift assays were performed using the CA1, CA2 and CA3 sequences as probes, confirming that both CA2 and CA3 bound to EGR1 in a similar manner to the CA1 (Fig. 3A). EGR1 appeared to bind to all three probes: CA1, CA2 and CA3. Within the context of the −1.8 kb *CYP2B6* promoter, the CA1, CA2 and CA3 were internally deleted singularly or in combinations. Subsequently, these deletion promoters were co-transfected with an EGR1 expression plasmid into Ym17 cells so that their ability to be activated by EGR1 could be examined. The CA1 deletion decreased activation by EGR1 approximately 5-fold. Although the single deletion of either CA2 or CA3 resulted in the fully retention of the EGR1 activation, the simultaneous deletion of both CA2 and CA3 decreased the promoter activity to the same level as observed with the CA1 deletion (Fig. 3B). The triple deletion of CA1, CA2 and CA3 completely abrogated the ability of EGR1 to activate the −1.8 kb *CYP2B6* promoter. These results suggested that both the proximal and distal CACC motifs are required for EGR1 to activate the −1.8 kb *CYP2B6* promoter. With respect to the two distal motifs, only one of the distal CA2 and CA3 motifs was necessary for EGR1 activation in the presence of the proximal CA1 motif.

Looping distal PBREM close to proximal OARE$_{KI}$

Requiring both the distal and proximal CA sites for EGR1 to synergize CAR-mediated transcription of CYP2B6 promoter led us to hypothesize the presence of a looping mechanism that enables physical access of CAR on the distal PBREM to HNF4α on the proximal OARE$_{KI}$. Chromosome conformation capture (3C) assays [12,13] were employed to explore this looping mechanism. A recently developed *in vitro* version of 3C technique is an assay for demonstrating DNA looping, in which two distant cis-acting elements in a head-to-head orientation are designed to be PCR amplified as a single strand DNA in a head-to-tail orientation after ligation when protein bindings enable them to come close one to another [7]. 3C technique has already been employed to successfully show that the glucocorticoid receptor activates two promoters that are looped close to the single glucocoroticoid response element [12]. In our 3C assay with the −1.8 kb *CYP2B6* promoter, if looping occurs, PCR reaction should produce the 80 bp fragment that contains a region of the sequence connecting PBREM and OAREKI in head-to-tail orientation (Fig. 4A). When the −1.8 kb CYP2B6 promoter was incubated with nuclear proteins prepared from Ym17 cells co-treated with TCPOBOP and PMA, a dramatic amplification of the 80 bp fragments was observed and compared with the corresponding assay with nuclear proteins from DMSO-treated Ym17 cells (Fig. 4B). No such increase of the amplification was detected when all three CA sites were deleted from the −1.8 kb *CYP2B6* promoter. In addition to the regular PCR reaction, real time PCR was also utilized in our 3C assays, showing approximately 8-fold increase of amplification with the −1.8 kb *CYP2B6* promoter by the nuclear extracts from the co-treated Ym17 cells and no increase with the promoter lacking all three CA site (Fig. 4C). These results are consistent with the hypothesis that EGR1, by binding to the CA sites, induces the looping of the distal PBREM closer to the proximal OARE$_{KI}$ within the *CYP2B6* promoter.

In conclusion, we have now uncovered a novel looping mechanism, whereby the distal PBREM close to the proximal OARE$_{KI}$, EGR1 enables CAR to cross talk with HNF4α to synergistically activate *CYP2B6* promoter. Multiple steps of protein-DNA and -protein interactions expected to occur during this DNA looping still remain a future subject of intense investigations. Functional cross talk of CAR with HNF4α is well known its role in the transcriptional regulation of *SULT2A1* and various *CYP* genes [14–16], for one of which indirect interactions of CAR with HNF4α are suggested [15]. Although the molecular mechanism by which EGR1 bends DNA to loop the *CYP2B6* promoter is not yet

understood, it is reasonable to think that ERG1 proteins at the distal and proximal CA sites may directly or indirectly interact to loop PBREM close to OARE$_{KI}$ within the *CYP2B6* promoter. A presumed interaction of ERG1 with CAR and/or HNF4α and/or the CAR interaction with HNF4α may simultaneously undergo in order for EGR1 to bend DNA. The concept of DNA looping is widely accepted as a principle mechanism by which two distantly separated cis-acting elements interact to regulate transcription, another example of which is a possible EGR1 involvement in looping the RUSH-1α gene promoter for activation [17]. CYP2B6 is one of the human P450 enzymes that metabolize numerous therapeutic drugs. CAR mediates induction of *CYP* genes by therapeutics, affecting drug efficacy and causing drug-drug interactions. Since CAR is not able to activate the *CYP2B6* gene without HNF4α and EGR1, understanding the molecular mechanism of their cross talk should provide us with various ways to control CYP2B6 activities with clinical implications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations used

CAR	nuclear constitutive active/androstane receptor
PXR	pregnane X receptor
CYP	cytochrome P450
EGR1	early growth response 1
HNF4α	hepatocyte-enriched nuclear factor 4α
OAREKI	OA response element KI
PB	phenobarbital
PBREM	PB responsive enhancer module
TCPOBOP	1,4-bis-[2-(3,5-dichloropyridyloxy)]benzen
PMA	phorbol 12-myristate 13-acetate
PKC	(PMA)-activated protein kinase C
DMSO	dimethylsulfoxide

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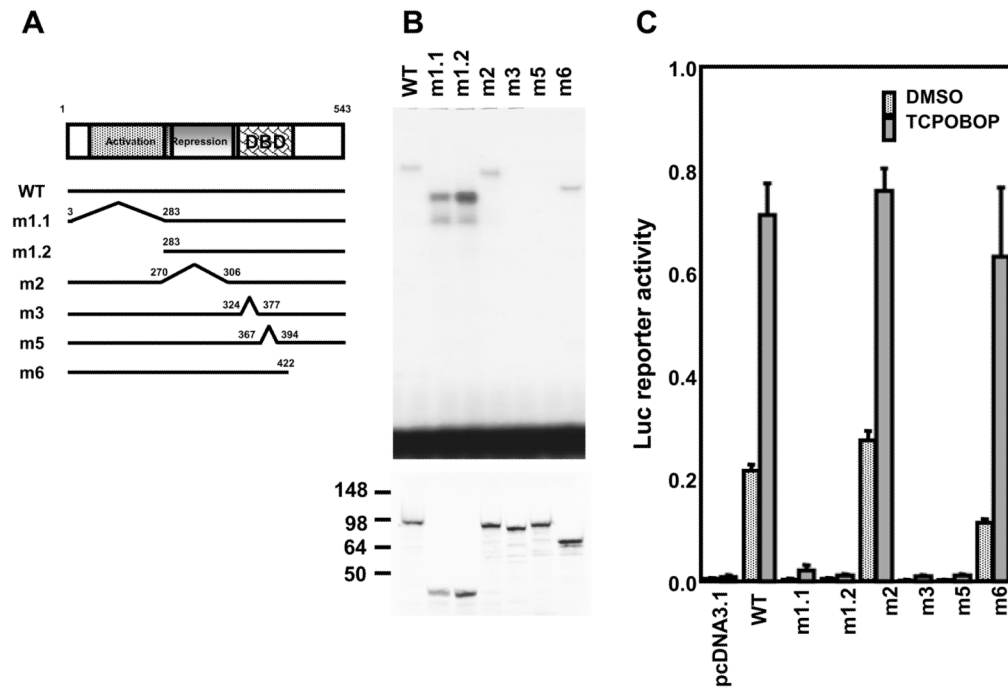
Figure 1. Structural domain-based function of EGR1

(A) Deletion map of EGR1 molecule. Domains of activation, repression and DNA binding (DBD) are shown in boxes. Numeric numbers indicate residues beginning with and ending with for each deletion. (B) Gel shift assays to show binding of EGR1 and its deletion mutants to CA rich region within CYP2B6 $OARE_{KI}$. Double-stranded ^{32}P-end-labeled CA rich probe ($-253/-242$) was incubated with *in vitro* translated EGR1 (WT), its mutants (m1-m6). m1-1, 1–2, 2, 3, 5, and 6: EGR1 deletion mutants lacking the activation domain (1–283) (3–283)[9], repression domain (270–306)[10], DNA binding domain (324–377)[11], DNA binding domain (367–393)[11] and C-terminal region, respectively (422/543)[11]. Numbers indicate amino acid residues. As a negative control, empty vector (pcDNA3.1) was mixed with an *in vitro* translation cocktail and incubated with labeled probe. Autoradiography below is to show an equality of ERG1 proteins used for gel shift assays. EGR1 and its mutants were produced *in vitro* translated proteins in TNT coupled reticulocyte lysate system (Promega) and [^{35}S] Methionine (Amersham Biosciences, Piscataway, NJ), were separated on a 11% SDS-PAGE gel and were subjected to autoradiography. Numeric numbers indicate sizes of molecular markers (C) Transient transfection assays to show EGR1 activity. Ym17 cells were co-transfected by the -1.8 kb promoter of *CYP2B6* or a given deletion promoter with phRL-SV40 cells and were subsequently treated with either DMSO, or 250 nM TCPOBOP. Reporter activity was calculated by dividing firefly luciferase activity with renilla luciferase activity.
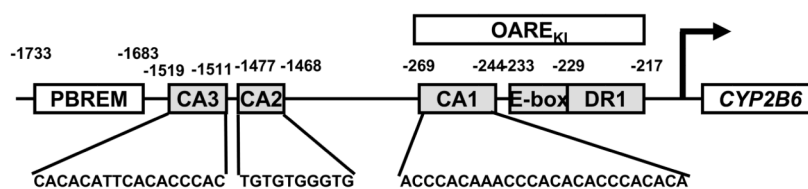
Figure 2. Motif map of *CYP2B6* promoter
The −1.8 kb *CYP2B6* promoter contains three CA rich regions: promoter of *CYP2B6*: CA1 (−269/−244), CA2 (−1477/1468) and CA3 (−1519/−1511), which are positioned relative to PBREM (−1733/−1683) and OARE$_{KI}$ (−269/−217). The OARE$_{KI}$ sequence can be further divided into CA1, E-box (−233/−228) and DR1 (−229/−217) regions.
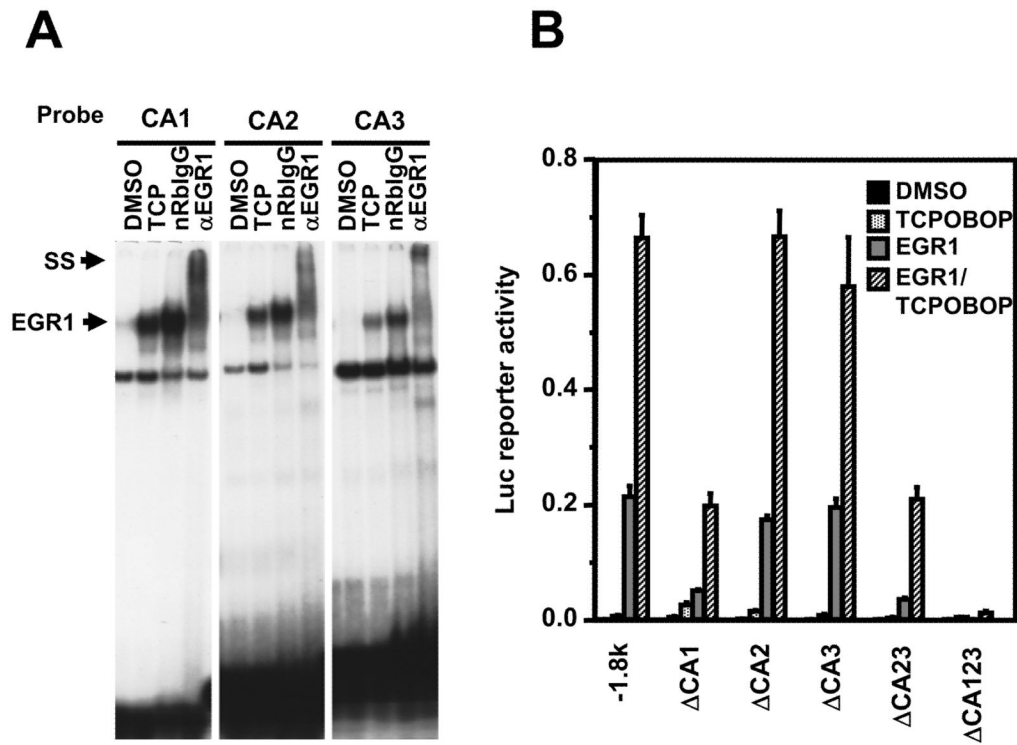
Figure 3. Role of CA motifs in trans-activation activity of ERG1
(A) Gel shift assays to show binding of EGR1 to three CA rich regions within *CYP2B6* promoter. A given double-stranded ^{32}P-end-labeled CA rich probes (CA1, −253/−242; CA2, −1477/1468; CA3, −1519/−1511) was incubated with nuclear extracts prepared from Ym17 cells treated with DMSO or TCPOBOP plus PMA (TCP). For supershifts, anti-EGR1 antibody and normal rabbit IgG (nRbIgG) were co-incubated. (B) Transient transfections to show that the three CA-rich regions are critical to activate *CYP2B6* promoter by EGR1. Ym17cells were co-transfected by −1.8 kb promoter construct or were its deletion mutants (−1.8 kb ΔCA1, −1.8 kb ΔCA2, −1.8 kb ΔCA3, −1.8 kb ΔCA23, −1.8 kb ΔCA123) with phRL-SV40, pcDNA3.1or pcDNA3.1-EGR1. The cells were first treated with DMSO or 250 mM TCPOBOP and were harvested for measuring luciferase activity. Reporter activity was calculated by dividing firefly luciferase with *Renilla* luciferase activities.
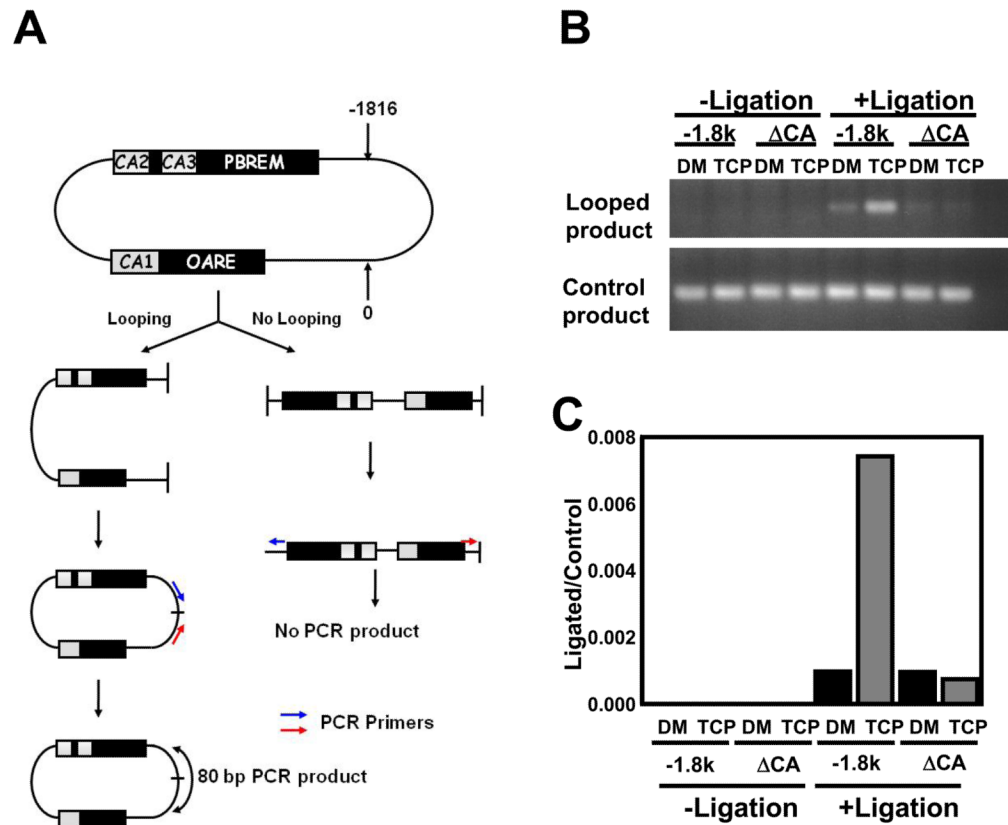
Figure 4. Chromatin conformation capture
(A) Schematic representation of the 3C experimental procedure. The numeric numbers 0 and
−1816 indicate the XhoI cutting sites. Arrows indicate the position of PCR primers and
double arrow indicates the position of the PCR amplicon. (B) The −1.8kb *CYP2B6* (−1.8kb)
and ΔCA123 CYP2B6 (ΔCAs) promoters were incubated with nuclear extracts prepared
from Ym17 cells treated with DMSO (DM) or TCPOBOP plus PMA (TCP). After
incubation, the reaction mixture was divided into two parts, of which one was ligated, while
the other was unligated. Degrees of ligation were estimated by PCR amplification using two
sets of primers for looped and linear products, respectively on an ethydium bromide-stained
1.5% agarose gel. (C) The same ligated and unligated samples used in Fig. 4A were
subjected for real time PCR.