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Involvement of hepatocyte nuclear factor 4α in the different expression level between *CYP2C9* and *CYP2C19* in the human liver

Sachiyo Kawashima, Kaoru Kobayashi, Kaori Takama, Tomoaki Higuchi, Tomomi Furihata, Masakiyo Hosokawa, Kan Chiba

Laboratory of Pharmacology and Toxicology, Graduate School of Pharmaceutical Sciences, Chiba University, Chiba, Japan.

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Running Title: Effects of HNF4 on the expression of *CYP2C9* and *CYP2C19* genes

*To whom all correspondence should be addressed:

Kaoru Kobayashi, Ph.D.

Laboratory of Pharmacology and Toxicology, Graduate School of Pharmaceutical Sciences, Chiba University, Inohana 1-8-1, Chuo-ku, Chiba 260-8675, Japan

E-mail: kaoruk@p.chiba-u.ac.jp

Tel / Fax: +81-43-226-2895

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Abbreviations: CYP, cytochrome P450; HNF4α, hepatocyte nuclear factor 4α; DR1, direct repeat 1; kbp, kilo base pair; EMSA, electrophoretic mobility shift assay; Luc, luciferase; ChIP, chromatin immunoprecipitation.

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Abstract

Cytochrome P450 2C9 (CYP2C9) and CYP2C19 are clinically important drug-metabolizing enzymes. The expression level of CYP2C9 is much higher than that of CYP2C19, though the factor(s) responsible for the difference between the expression levels of these genes is still unclear. It has been reported that hepatocyte nuclear factor 4α (HNF4α) plays an important role in regulation of the expression of liver-enriched genes, including *CYP* genes. Thus, we hypothesized that HNF4α contributes to the difference between the expression levels of these genes. Two direct repeat 1 (DR1) elements were located in both the *CYP2C9* and *CYP2C19* promoters. The upstream and downstream elements in these promoters had the same sequences, and HNF4α could bind to both elements *in vitro*. The transactivation levels of constructs containing two DR1 elements of the *CYP2C9* promoter were increased by HNF4α, whereas those of the *CYP2C19* promoter were not increased. The introduction of mutations into either the upstream or downstream element in the *CYP2C9* gene abolished the responsiveness to HNF4α. We also examined whether HNF4α could bind to the promoter regions of the *CYP2C9* and the *CYP2C19* genes *in vivo*. The results of chromatin immunoprecipitation assays showed that HNF4α could bind to the promoter region of the *CYP2C9* gene but not to that of the *CYP2C19* promoter in the human liver. Taken together, our results suggest that HNF4α is a factor responsible for the difference between the expression levels of CYP2C9 and CYP2C19 in the human liver.

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Cytochrome P450s (CYPs) comprise a superfamily of metabolic enzymes that play important roles in the oxidative metabolism of xenobiotics and endogenous substrates (Gonzalez et al., 1994). The human CYP2C subfamily is comprised of four isoforms (CYP2C8, CYP2C9, CYP2C18, and CYP2C19) that account for about 20% of the total human adult liver P450 contents (Shimada et al., 1994). Among the CYP2C subfamily isoforms, CYP2C9 and CYP2C19 play critical roles in the metabolism of clinically used drugs (Goldstein and de Morais, 1994). It has been reported that the expression level of the *CYP2C9* gene in the human liver is about 20-times higher than that of the *CYP2C19* gene (Furuya et al., 1991; Romkes et al., 1991; Inoue et al., 1997), indicating that there are some differences between the regulatory mechanisms of *CYP2C9* and *CYP2C19* gene transcriptions. It has been reported that pregnane X receptor, constitutive androstane receptor, glucocorticoid receptor, and hepatocyte nuclear factor 3γ participate in the basal expression of *CYP2C9* and *CYP2C19* genes (Gerbal-Chaloin et al., 2002; Ferguson et al., 2002; Chen et al., 2003; Bort et al., 2004). However, the factor(s) responsible for the difference between the expression levels of *CYP2C9* and *CYP2C19* genes is still unclear.

Hepatocyte nuclear factor 4α (HNF4α) is a member of the nuclear receptor superfamily (Sladek, 1990) and is expressed at high levels in the liver, kidney, pancreas, and small intestine (Sladek, 1990; Thomas et al., 2001). HNF4α appears to be an important factor for liver differentiation and function since it is involved in regulation of the expression of numerous liver-enriched genes, such as those related to glucose or lipid metabolism (Watt et al., 2001), those related to synthesis of blood

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coagulation factors (Sladek and Seidel, 2001), and drug-metabolizing enzymes, including CYP3A4, CYP2A6, CYP2C9 and CYP2D6 (Jover et al., 2001). It is thought that HNF4α binds to a specific DNA sequence called a direct repeat (DR) 1 element as a homodimer to stimulate transcription of these genes (Tirona et al., 2003; Pitarque et al., 2005; Cairns et al., 1996). However, HNF4α does not always transactivate all genes that have a DR1 element. For example, it has been reported that a DR1 element exists in the *CYP2C18* promoter but that HNF4α does not bind to the DR1 element of the *CYP2C18* gene and dose not transactivate this promoter (Ibeanu and Goldstein, 1995).

It has been reported that there are two DR1 elements in the promoter region of the *CYP2C9* gene and HNF4α can activate the transcription of this gene via the DR1 element (Ibeanu and Goldstein, 1995; Chen et al., 2005). We also identified two DR1 elements in the *CYP2C19* promoter, but it is not clear whether these elements are functional. Therefore, to clarify the mechanism determining the difference between the expression levels of *CYP2C9* and *CYP2C19* genes, we hypothesized that HNF4α contributes to the difference between the expression levels of CYP2C9 and CYP2C19 in the human liver. The 5'-flanking regions from -2 kbp to the translation start site of these genes were analyzed by electrophoretic mobility shift assays, co-transfection assays, mutagenesis, and chromatin immunoprecipitation assays. Our results suggest that HNF4α participates in the regulation of *CYP2C9* gene transcription but not in that of the *CYP2C19* gene despite the fact that the same DR1 elements exist in both gene promoters.

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Materials and Methods

Electrophoretic mobility shift assays (EMSAs) and supershift assays

EMSAs were performed using dsDNA (double-stranded DNA) labeled with [γ-^{32}P]dATP (Amersham Biosciences, Piscataway, NJ) and 10 µg of the nuclear extracts as previously described (Furihata et al., 2004). The following is the sequences of the oligonucleotides used as probes, wild-type or mutated specific cold competitors: 5'-ACAAGACCAAAGGACATTT-3' for the DR1-A WT, 5'-ACACCCCCAAAGGACATTT-3' for the DR1-A MT, 5'-AGTGGGTCAAAGTCCTTTC-3' for the DR1-B WT, 5'-AGTCCCTCAAAGTCCTTTC-3' for the DR1-B MT, 5'-TCGAGCGCTGGGCAAAGGTCACCTGC-3' for the HNF4 WT, and 5'-TCGAGCGCTAGGCACCGGTCACCTGC-3' for the HNF4 MT. Only the sequences of the sense strands are displayed above, and mutated nucleotides are underlined. Nuclear extracts were prepared from HepG2 cells by using a CelLytic Nuclear Extraction Kit (Sigma, St Louis, MO) according to the manufacturer's protocol. After extracting nuclear contents, the protein concentration was determined by using a Bio-Rad *Dc* Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA). The nuclear extracts were stored at -80 °C until used.

For competition experiments, unlabeled competitive dsDNA was added to the binding reaction mixture at a 50-fold excess of the probe amount before addition of the probe. For supershift assays, either 2 µg of IgG against HNF4α (2ZK9218H, Perseus Proteomics, Tokyo, Japan) or control mouse

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IgG (sc-2025, Santa Cruz Biotech, CA) was added to the binding reaction mixture at room temperature for 30 min before addition of the probe.

Plasmids

The 5'-flanking regions of the *CYP2C9* and *CYP2C19* genes were isolated by PCR with the common sense primer 5'-ACCTCTAGATTGCTTTTCTTTGCCCTGTAT-3' (for *CYP2C9* and *CYP2C19*) and the antisense primer 5'-GAGGACCTGAAGCCTTCTCTTCTTGTTA-3' (for *CYP2C9*) or 5'-GGGGACCTGAAGCCTTCTCCTCTTGTTA-3' (for *CYP2C19*) using human genomic DNA as a template. The amplicons were subcloned into a pGEM-T easy vector (Promega, Madison, WI). After *Xba*I and *BamH*I digestion, the fragment was ligated into a pGL3-Basic vector (Promega). These constructs are hereafter referred to as 2C9 -2k and 2C19 -2k, respectively. The nucleotide sequences were determined using a Dye Terminator Cycle Sequencing-Quick Start Kit (Beckman Coulter, Fullerton, CA) and a CEQ 2000 DNA Analysis System (Beckman Coulter). Fourteen deletion constructs were generated by nested PCR of the primary clone using the following sense primers: 5'-TCTCTAGAGGTTAATCTAAATCTAAGAATTCA-3' (2C9 -380 and 2C19 -380), 5'-ATTTCTAGAGCATCAGATTATTTACTTCA-3' (2C9 -340), 5'-ATTACGCGTGCATCAGATTGTTTACTTCA-3' (2C19 -340), 5'-TCTAGAGTGCTCTCAATTATGATGGTG-3' (2C9 -320), 5'-TCTAGACAGTGCTCTCAATTATGAC-3' (2C19 -320),

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5'-TTTTCTAGAAATACCTAGGCTCCAACCAAGT-3' (2C9 -255), 5'-TCTAGAATTACCAATACCTAGGCTTCAA-3' (2C19 -255), 5'-ATACGCGTAAGGAGAACAAGACCAAAGGAC-3' (2C9 -195 and 2C19 -195), 5'-TTTCTAGATATCAGTGGGTCAAAGTCCT-3' (2C9 -160 and 2C19 -160) and 5'-ATCTAGATTTCAGAAGGAGCATATAGT-3' (2C9 -140 and 2C19 -140). The antisense primer used was the same as that used in genome cloning. The obtained 5'-deletion fragments except for 2C9 -195, 2C19 -340 and 2C19 -195 were transferred into the pGL3-Basic vector as described above. 2C9 -195, 2C19 -340 and 2C19 -195 were inserted into the pGL3-Basic vector by *Mlu*I and *BamH*I digestion. All constructs are named as shown in parentheses.

The cDNA clone of mouse HNF4α2 was isolated from mouse liver cDNA by PCR amplification, and was subcloned into pTARGET mammalian expression vector (Promega) by *EcoR*I digestion, resulting in pHNF4α2 as described in elsewhere (Furihata et al., in press).

Site-directed mutagenesis

Site-directed mutagenesis was carried out as described elsewhere (Furihata et al., 2004). To introduce mutations into the reporter plasmids, complementary primers harboring a few mutations were designed for each target site as follows: 5'-GGAGAACAAGACCT
__GGACATTTTATTTTTATCTGTATCAGTGGG-3' and 5'-CCCACTGATACAGATAAAAATAAAATGTCCA___GGTCTTGTTCTCC-3' for the *CYP2C9*

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DR1-Amt; 5'-CTGTATCAGTGGGTCT __GTCCTTTCAGAAGGAGCATATAGTGG-3' and 5'-CCACTATATGCTCCTTCTGAAAGGACA GACCCACTGATACAG-3' for the *CYP2C9* DR1-Bmt; 5'-CGAAGGAGAACAAGACCT __GGACATTTTATTTTTATCTCTATCAGTGG-3' and 5'-CCACTGATAGAGATAAAAATAAAATGTCCA__GGTCTTGTTCTCCTTCG-3' for the *CYP2C19* DR1-Amt; 5'-CTCTATCAGTGGGTCT __GTCCTTTCAGAAGGAGCATATAGTGGG-3' and 5'-CCCACTATATGCTCCTTCTGAAAGGACA___GACCCACTGATAGAG-3' for the *CYP2C19* DR1-Bmt. The mutagenic sites are underlined, and spaces indicate deletions of 2-base pair nucleotides.

Cell culture, transient transfection and dual luciferase assay

FLC7 cells (Kawada et al., 1998), a human hepatocellular carcinoma cell line, were kindly provided by Dr. S. Nagamori (Kyorin University, Tokyo, Japan). FLC7 cells were maintained at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium/F-12 (DMEM/F12, Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum, and 50 units/ml penicillin and 50 μg/ml streptomycin.

FLC7 cells were plated at a density of 1.8 x 10^5 cells/well in 24-well plates one day before transfection. The reporter plasmids (200 ng/well) were cotransfected with pHNF4α (100 ng/well) and phRL-TK Vector (Promega, 4 ng/well) into FLC7 cells by *Trans*IT®-LT1 (Mirus, Madison, WI).

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Twenty-four hours after transfection, luciferase reporter activities were measured as previously described (Kobayashi et al., 2004). The *Renilla* luciferase activity derived from the control plasmid phRL-TK was used to normalize the results of the firefly luciferase activity of reporter plasmids. Experiments were performed in triplicate, and each value is the mean ± S.D. from three or four separate assays.

Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed by using a ChIP-IT kit (Active Motif, Carlsbad, CA) according to the manufacturer's protocol. Human liver (from a 56-year-old Caucasian male) was supplied by the National Disease Research Interchange (Philadelphia, PA) through HAB Research Organization (Tokyo, Japan), and this study was approved by the Ethics Committee of Chiba University (Chiba, Japan). The human liver tissue (2.4 g) was isolated and chopped on ice and then cross-linked by 1% formaldehyde for 12 min. Cross-linking was stopped by the addition of glycine solution. The chromatin was sheared by using an Ultrasonic disruptor UD-201 (TOMY, Tokyo, Japan) at 25% power with 14 pulses. Nine μg of the sheared chromatin was immunoprecipitated with either control mouse IgG or anti-HNF4α IgG (2ZH1415H). After incubating for 4 h at 4 °C with gentle rotation, salmon sperm DNA/protein G agarose was added to the mixture and it was further incubated for 1.5 h under the same conditions. The DNA fragment was purified and used as a template for PCR. The DNA sequences around DR1 elements of the *CYP2C9* and the *CYP2C19* genes were amplified by using the sense primers

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5'-CAACCAAGTACAGTGAAACTG-3' (for *CYP2C9*) and 5'-CAGAATGTACAGAGTGGGCAC-3' (for *CYP2C19*) and the anti-sense primers 5'-TAACACTCCATGCTAATTCGG-3' (for CYP2C9) and 5'-AACACTCCATGCTAATTAAGT-3' (for *CYP2C19*). The specificity of the CYP2C9 and CYP2C19 primers was verified by the lack of amplification from sheared genomic DNA than the intended target. PCR conditions were as follows: 94°C for 2 min, followed by 94°C for 30 sec, 47°C (for *CYP2C9*) or 50°C (for *CYP2C19*) for 30 sec, and 72°C for 30 sec, 40 cycles. The amplicons were visualized by ethdiumbromide staining, and the sequence of each amplicon was confirmed by direct DNA sequence.

Determination of mRNA levels

To measure the CYP2C9 and CYP2C19 mRNA levels, cDNA prepared from total RNA of the same human liver used for ChIP assays was subjected to quantitative real-time PCR with an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). The mRNA levels of CYP2C9 and CYP2C19 were determined by using Gene Expression Assays (Applied Biosystems), gene expression products for CYP2C9 and CYP2C19, respectively. The mRNA levels were normalized against glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNA determined by Pre-Developed TaqMan Assay Reagents for GAPDH (Applied Biosystems).

Statistical analyses

Data are presented as means ± S.D. The *p* values for each experimental comparison were

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determined using Student's *t* test.

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Results

Identification of two DR1 elements in the *CYP2C19* gene

A comparison of the 5'-flanking regions of the *CYP2C9* and *CYP2C19* genes is shown in Fig. 1. The 5'-flanking region from -2 kbp to the translation start site of the *CYP2C9* gene was 88.8% identical to that of the *CYP2C19* gene. We searched for the DR1 element in the *CYP2C19* promoter by using a searching program for nuclear receptor binding sites (http://www.nubiscan.unibas.ch/, Podvinec et al., 2002) and found two putative DR1 elements (score $\geq$ 0.75). No other DR1 element was identified with this score in this region. The upstream and downstream elements identified in the *CYP2C19* promoter had the same sequences as those of two DR1 elements of the *CYP2C9* promoter to which it has been reported that HNF4α can bind (Chen et al., 2005; Ibeanu and Goldstein, 1995). The upstream elements and the downstream elements in both genes are hereafter referred to as the DR1-A element and the DR1-B element, respectively.

Binding of HNF4α to the DR1-A and DR1-B elements of the *CYP2C9* and *CYP2C19* promoters *in vitro*

EMSAs were performed to examine whether HNF4α could bind to the DR1-A and DR1-B elements in the *CYP2C9* and *CYP2C19* promoters. We used a nuclear extract prepared from HepG2 cells because it has been reported that this cell line endogenously expressed HNF4α (Ihara et al., 2005; Furihata et al., in press). As shown in Fig. 2A, specific protein-DNA complexes were formed when the

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radiolabeled probe containing either the DR1-A element (DR1-A WT) or the DR1-B element (DR1-B WT) was incubated with HepG2 nuclear extracts (lanes 3 and 9, respectively). These complexes migrated at the same position as that of the one formed with the radiolabeled probe of HNF4α consensus (HNF4 WT, lane 1). The formation of the complexes was eliminated by the addition of self competitors (DR1-A WT, lane 4; DR1-B WT, lane 10) or unlabeled HNF4 WT (lanes 6 and 12). However, complex formation was not inhibited in the presence of mutated competitors (DR1-A MT, lane 5; DR1-B MT, lane 11; HNF4 MT, lanes 7 and 13).

To determine the proteins forming these complexes, supershift assays were performed using IgGs against HNF4α. The addition of anti-HNF4α IgGs to the mixture resulted in generation of a supershifted band when either DR1-A WT or DR1-B WT was used as a probe (Fig. 2B, lanes 3 and 7, respectively). Control mouse IgGs, used as a negative control, did not affect the formation of any complexes (lanes 4 and 8). These results indicate that HNF4α can bind to both the DR1-A and DR1-B elements of the *CYP2C9* and *CYP2C19* promoters.

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Effects of HNF4α on the transcriptional activity of the *CYP2C9* and *CYP2C19* promoters in FLC7 cells

Co-transfection analyses were performed by using human hepatocarcinoma FLC7 cells to examine whether HNF4α played different roles in the transactivation of the *CYP2C9* and *CYP2C19* promoters. We have determined that this cell line does not express endogenous HNF4α (Furihata et al., in press). Several constructs containing various lengths of the *CYP2C9* promoter region and the HNF4α

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expression vector were co-transfected into FLC7 cells (Fig. 3, left panel). The levels of the transcriptional activities of the five constructs containing two DR1 elements (2C9 -2k, 2C9 -380, 2C9 -340, 2C9 -320 and 2C9 -255) in the presence of HNF4α were increased to 4.9-, 2.4-, 4.2-, 4.0- and 3.5-fold, respectively, compared to those in the absence of HNF4α. However, the transcriptional activity of 2C9 -195 was not increased by HNF4α despite the fact that this construct contained two DR1 elements. Deletion of the DR1-A element (2C9 -160) or both elements (2C9 -140) from the promoter region abolished its response for transactivation by HNF4α. The same experiments were also performed using eight different deletion constructs of the *CYP2C19* promoter (Fig. 3. right panel). In contrast to the results obtained from the CYP2C9 constructs, the levels of the transcriptional activities of CYP2C19 constructs were not increased in the presence of HNF4α. Deletion of the DR1-B elements of CYP2C9 (2C9 -140) and CYP2C19 (2C19 -140) from the promoter regions abolished the transcriptional activities in the presence and absence of HNF4α.

Mutation analyses were performed to examine whether HNF4α required two DR1 elements for its transactivation ability (Fig. 4). As for the CYP2C9 constructs, HNF4α could stimulate the level of the promoter activity of the wild-type construct (2C9 -2k) to approximately 4-fold, but the introduction of mutation of each DR1 element resulted in complete loss of transactivation of the mutated *CYP2C9* promoter (2C9 DR1-Amt, 2C9 DR1-Bmt) by HNF4α (Fig. 4. left panel). On the other hand, the levels of the transcriptional activities of constructs of the *CYP2C19* promoter (2C19 -2k, 2C19 DR1-Amt and 2C19 DR1-Bmt) were not increased by HNF4α (Fig. 4. right panel). These results indicate that HNF4α

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can increase the level of transcriptional activity of the *CYP2C9* promoter but not that of the *CYP2C19* promoter and that this activation occurred only when two DR1 elements of the *CYP2C9* promoter were simultaneously functional. Introduction of mutation of DR1-B elements decreased transcriptional activities of the *CYP2C9* and *CYP2C19* promoters in the presence and absence of HNF4α.

Binding of HNF4α to the *CYP2C9* promoter but not to the *CYP2C19* promoter *in vivo*

ChIP assays were performed using human liver to examine whether HNF4α could bind to the *CYP2C9* and *CYP2C19* gene promoters *in vivo* (Fig. 5). Following DNA extraction of the immunoprecipitated chromatin, PCR was performed to detect the occupancy of DR1 elements of the *CYP2C9* and *CYP2C19* genes by HNF4α. As for *CYP2C9*, the DR1 elements were much more abundant in DNA extracted from chromatin immunoprecipitated with anti-HNF4α IgG than in that with control mouse IgG (Fig. 5, upper panel). On the other hand, no DNA fragment around the DR1 elements of the *CYP2C19* gene was detected in both extracted DNA samples (Fig. 5, lower panel). We also determined the expression levels of CYP2C9 and CYP2C19 mRNAs in the same liver used for ChIP assays by using quantitative real-time PCR. The expression level of CYP2C9 mRNA was 82.5-times higher than that of CYP2C19 mRNA.

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Discussion

The present study showed that two DR1 elements were located in the *CYP2C9* promoter (Fig. 1) and that the transcriptional activities of the *CYP2C9* promoter were increased by exogenous HNF4α (Fig. 3). The introduction of mutation to each DR1 element resulted in complete loss of transactivation (Fig. 4). These results are consistent with the results presented in a recent report (Chen et al., 2005). We also performed the same experiments for the *CYP2C19* promoter. In contrast to the case of the *CYP2C9* promoter, transactivation by HNF4α was not observed in the *CYP2C19* promoter in spite of the existence of two DR1 elements (Fig. 3). In addition, HNF4α could bind to the DR1 elements located in the *CYP2C9* promoter but not to those in the *CYP2C19* promoter *in vivo* (Fig. 5). These results suggest that HNF4α participated in the transactivation of at least -2 kbp of the *CYP2C9* promoter but not that of the *CYP2C19* promoter.

Significant decreases in the levels of CYP3A4, CYP3A5, CYP2A6, CYP2B6, CYP2C9, and CYP2D6 mRNAs have been observed in HNF4α-deficient human hepatocytes (Jover et al., 2001), and several studies have demonstrated that transcription of *CYP3A4*, *CYP2A6* and *CYP2D6* genes are regulated by HNF4α via DR1 elements located in their promoters (Tirona et al., 2003; Pitarque et al., 2005; Cairns et al., 1996). Our results and the results of a recent study by Chen et al. (2005) showed that HNF4α was involved in the expression of the *CYP2C9* gene. Therefore, these findings suggest that HNF4α plays important roles in regulation of the expression of these *CYP* genes in the human liver. On

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the other hand, it has been reported that HNF4α is not involved in transactivation of the *CYP2C18* promoter, although a DR1 element is located in this promoter (Ibeanu and Goldstein, 1995). Considering the fact that the expression level of CYP2C18 mRNA in the human liver is very low compared with the expression levels of other genes of the *CYP2C* subfamily (Goldstein et al., 1994), it is possible that the lack of a functional DR1 element in the *CYP2C18* promoter contributed to this low level of expression of CYP2C18 mRNA in the human liver. Accordingly, the same idea would also explain why the expression level of CYP2C19 is lower than that of CYP2C9. That is, the existence of "functional" DR1 elements in the regulatory region of the *CYP2C9* gene would be crucial factors for its higher level of expression than that of the *CYP2C19* gene in the human liver.

The reason for the different effects of HNF4α on transactivation of the *CYP2C9* and *CYP2C19* genes is currently unknown. However, the results obtained from our study provided some clues for understanding this difference. HNF4α could not transactivate the *CYP2C9* promoter in the absence of the region from -255 bp to -195 bp (-255 bp / -195 bp), although two DR1 elements were still present in the promoter (Fig. 3), suggesting that the region -255 bp / -195 bp of the *CYP2C9* promoter is necessary for HNF4α to up-regulate the transcription of the *CYP2C9* gene. One of possible explanation of these results is that other HNF4α binding sites exist in the region -255 bp / -195 bp of the *CYP2C9* gene and they can help the action of HNF4α that is recruited to the downstream elements. However, no DR1 elements were found in this region of the *CYP2C9* promoter by a searching program for nuclear receptor binding sites (http://www.nubiscan.unibas.ch/, Podvinec et al., 2002), and HNF4α could not bind to this

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region in EMSA by using an oligonucleotide probe ranging from -255 bp to -195 bp (data not shown). Therefore, effects of the region -255 bp / -195 bp on transcription of the *CYP2C9* gene are unlikely to be mediated by the direct binding of HNF4α to this region. Another possibility is that a certain factor, which assists of HNF4α-mediated transactivation of the *CYP2C9* promoter, specifically binds to the region -255 bp / -195 bp of the *CYP2C9* gene but not to the *CYP2C19* gene. Actually, there are 8-bp differences between the region -255 bp / -195 bp of the *CYP2C9* promoter and the region -257 bp / -197 bp of the *CYP2C19* promoter. The factor that binds to the region -255 bp / -195 bp of the *CYP2C9* promoter may stabilize the binding of HNF4α to the DR1 element of the *CYP2C9* promoter, or it may recruit cofactors that are required for function of HNF4α. However, no complexes were formed in EMSAs using HepG2 nuclear extracts and an oligonucleotide probe ranging from -255 bp to -195 bp (data not shown). A searching program for transcriptional factors could not identify any factors that fulfill these requirements. Thus, further detailed study is needed to elucidate the role of the region -255 bp / -195 bp of the *CYP2C9* promoter in HNF4α function.

Deletion and mutation of DR1-B elements decreased transcriptional activities of the *CYP2C9* and *CYP2C19* promoters in the presence and absence of HNF4α (Fig. 3 and 4). A putative binding site of CCAAT enhancer-binding protein was found in the down stream of DR1-B elements with partly overlapped. Therefore, the binding of CCAAT enhancer-binding protein to the *CYP2C9* and *CYP2C19* promoters may be inhibited by deletion and mutation of DR1-B elements, resulting in the decrease of basal activities of *CYP2C9* and *CYP2C19* promoters.

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In conclusion, we demonstrated that HNF4α is one of the important factors regulating of promoter activity of the *CYP2C9* gene but not that of the *CYP2C19* gene in the human liver. The direct binding of HNF4α to two DR1 elements of the *CYP2C9* promoter is essential for HNF4α-mediated transactivation of the *CYP2C9* promoter. In addition, this transactivation requires certain factors that facilitate the function of HNF4α via the region from -255 bp / -195 bp of the *CYP2C9* promoter. The results of the present study suggest that HNF4α is one of the determinants for the difference between expression levels of CYP2C9 and CYP2C19 in the human liver.

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Relationship between *CYP2C9* and *2C19* genotypes and tolbutamide methyl hydroxylation and *S*-mephenytoin 4'-hydroxylation activities in livers of Japanese and Caucasian populations. *Pharmacogenetics* **7**:103-113.

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Footnotes

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Address reprint requests to: Dr. Kaoru Kobayashi, Laboratory of Pharmacology and Toxicology, Graduate School of Pharmaceutical Sciences, Chiba University.

Inohana 1-8-1, Chuo-ku, Chiba 260-8675, Japan. E-mail: kaoruk@p.chiba-u.ac.jp. Tel / Fax: +81-43-226-2895

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Legends of figures

Fig. 1. Nucleotide sequences of the promoter regions of the *CYP2C9* and *CYP2C19* genes.

Nucleotides are arbitrarily numbered in negative numbers from the ATG coding for the initiation codon (+1). Differences in nucleotide sequence are highlighted in bold letters, and putative HNF4α binding sites (DR1-A and DR1-B) are indicated by arrows.

Fig. 2. Binding of HNF4α to the DR1-A and DR1-B elements of the *CYP2C9* and *CYP2C19* promoters *in vitro*.

(A) EMSAs were performed using nuclear extracts prepared from HepG2 cells with the following probes: HNF4 WT in lane 1, DR1-A WT in lanes 2-7 and DR1-B WT in lanes 8-13. Oligonucleotide competitors were added with 50-fold excess amounts of the following probes: DR1-A WT in lane 4, DR1-A MT in lane 5, DR1-B WT in lane 10, DR1-B MT in lane 11, HNF4 WT in lanes 6 and 12, and HNF4 MT in lanes 7 and 13. Symbols (+) and (-) indicate the presence and absence of the nuclear extracts or competitors, respectively.

(B) Supershift assays were performed using antibodies specific for HNF4α. Anti-HNF4α IgG (2 μg) was added to the reaction mixtures in lanes 3 and 7. Control IgG (2 μg) was used as a negative control

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(lanes 4 and 8). Symbols (+) and (-) indicate the presence and absence of the nuclear extracts or IgGs, respectively. The arrow indicates supershifted bands.

Fig. 3. Effects of HNF4α on transcriptional activity of the *CYP2C9* and *CYP2C19* promoters in FLC7 cells.

Deletion constructs (200 ng) of the *CYP2C9* or *CYP2C19* promoter were co-transfected with 100 ng of HNF4α expression vector (pHNF4α, open bars) or 100 ng of an empty vector (pT, closed bars). Two HNF4α binding sites are shown in circles. Each value is the mean ± S.D. of relative activity (Firefly/*Renilla*) for four separate experiments, each performed in triplicate. $^{*}p<0.05$ and $^{**}p<0.01$ compared with the empty vector. Luc, luciferase.

Fig. 4. Mutation analysis for two HNF4α binding sites of the *CYP2C9* and *CYP2C19* promoters in FLC7 cells.

Reporter constructs (200 ng) of the *CYP2C9* or *CYP2C19* promoter were co-transfected with 100 ng of HNF4α expression vector (pHNF4α, open bars) or 100 ng of an empty vector (pT, closed bars). Two HNF4α binding sites are shown in circles, and mutations are indicated by crosses. Each value is the mean ± S.D. of relative activity (Firefly/*Renilla*) for three separate experiments, each performed in

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triplicate. **p*<0.05 and ***p*<0.01 compared with the empty vector. Luc, luciferase.

Fig. 5. Binding of HNF4α to the DR1-A and DR1-B elements of the *CYP2C9* and *CYP2C19* promoters *in vivo*.

Chromatin immunoprecipitation (ChIP) assays were performed using the sheared genomic DNA extracted from human liver (9 μg), control mouse IgG (3 μg) and anti-HNF4α IgG (3 μg). M, DNA size marker; Input, control sheared genomic DNA; HNF4α, sheared genomic DNA immunoprecipitated with anti-HNF4α IgG; Control, sheared genomic DNA immunoprecipitated with control mouse IgG; N.C., non-template control.

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Fig. 1.

```
2C9    TGTTAAATCATATATTCCCAACTGGTTATTAATCTAAGAATTCAGAATTTTGAGTAATTGCTTTTGCATCAGATTATTTA:-321
2C19   ************A**************************************A********T**************G****:-326

2C9    CTTCAGTGCTCTCAATTATGATGGTGCATTAGAACCATCTGGGTTAACATTTGTTT...TTTATTACCAATACCTAGGCT:-244
2C19   *********************C********G******CT************T***GTT*********************:-246

                                                                     DR1-A
2C9    CCAACCAAGTACAGTGAAACTGGAATGTACAGAGTGGACAATGGAACGAAGGAGAACAAGACCAAAGGACATTTTATTTT:-164
2C19   T*****T******A******CA***************G**C***G*********************************:-166

                DR1-B
2C9    TATCTGTATCAGTGGGTCAAAGTCCTTTCAGAAGGAGCATATAGTGGACCTAGGTGATTGGTCAATTTATCCATCAAAGA: -84
2C19   *****C**************************************G*************C**C***************: -86

2C9    GGCACACAC..CGAATTAGCATGGAGTGTTATAAAAGGCTTGGAGTGCAAGCTCATGGTTGTCTTAACAAGAAGAGAAGG:  -6
2C19   *********ACTT***********************A******************C****************G*******:  -6
                                     TATA

          +1
2C9    CTTCAATG:   3
2C19   ********:   3
```

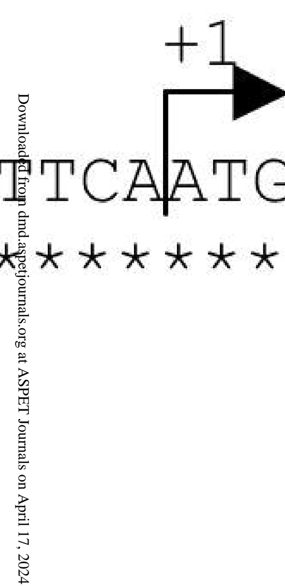

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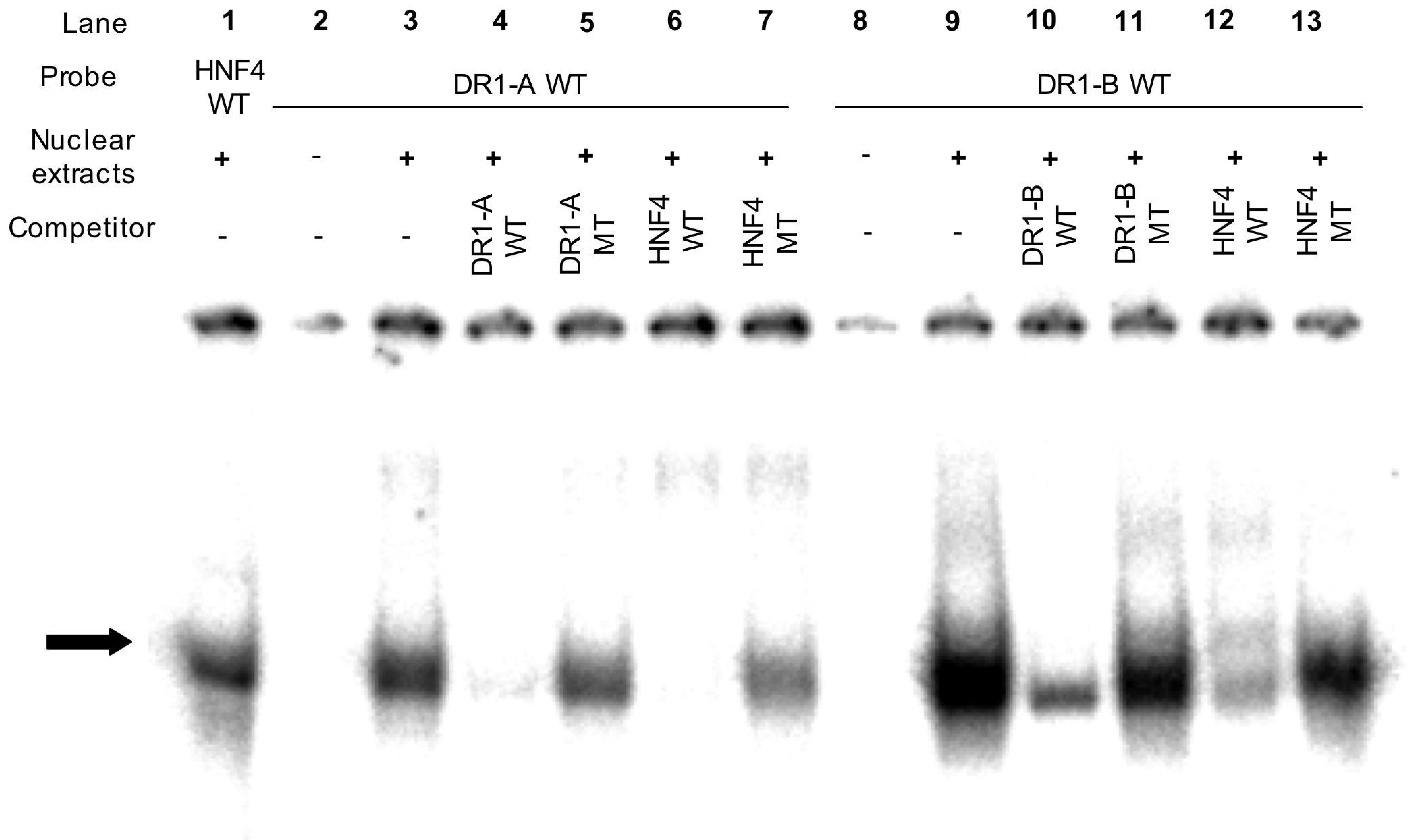
Fig. 2.

A.



B.

Lane 1 2 3 4 5 6 7 8

Probe: DR1-A WT (lanes 1–4); DR1-B WT (lanes 5–8)

Nuclear extract: - + + + - + + +

Antibodies: - - HNF4α control - - HNF4α control

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Supershift

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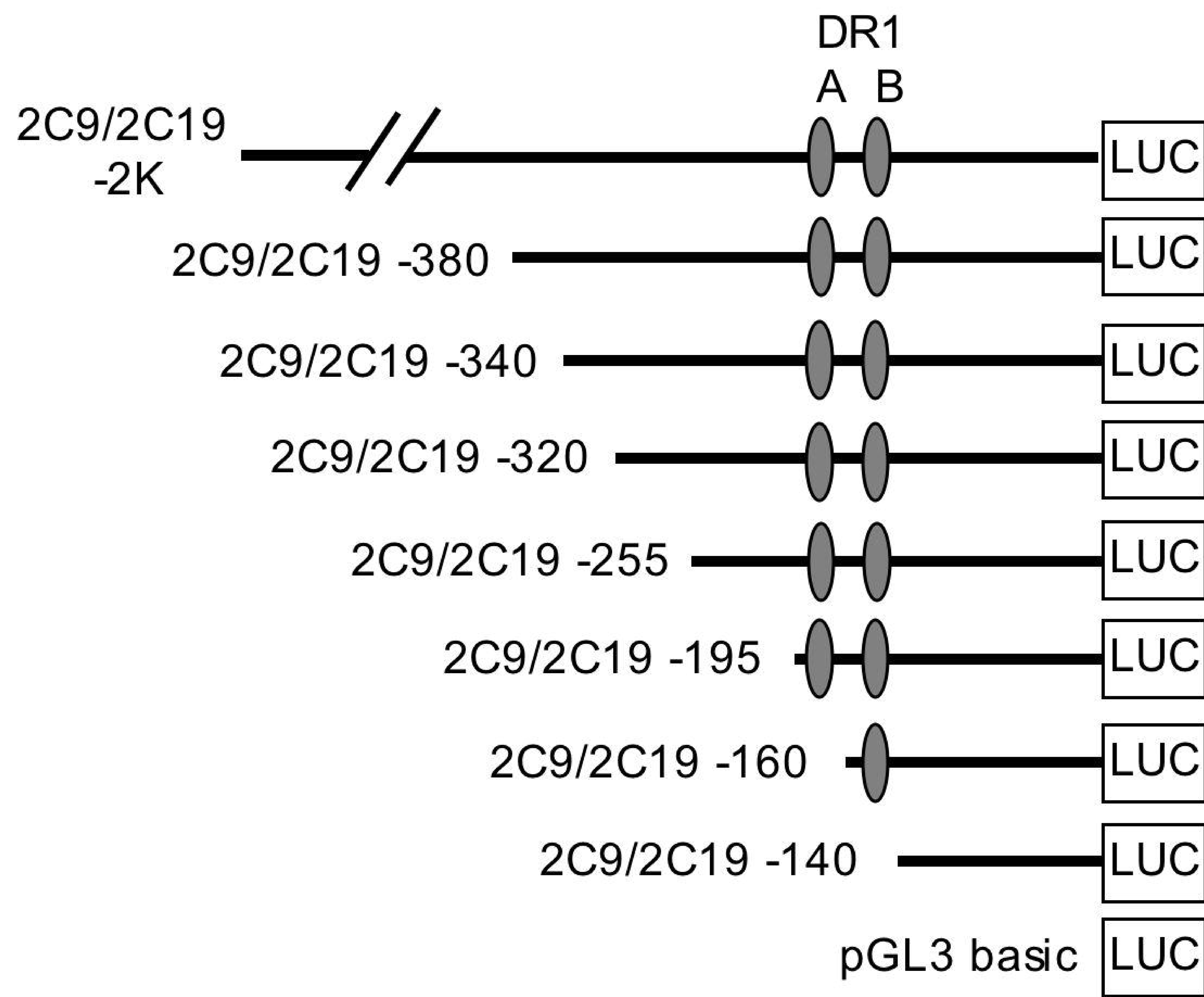
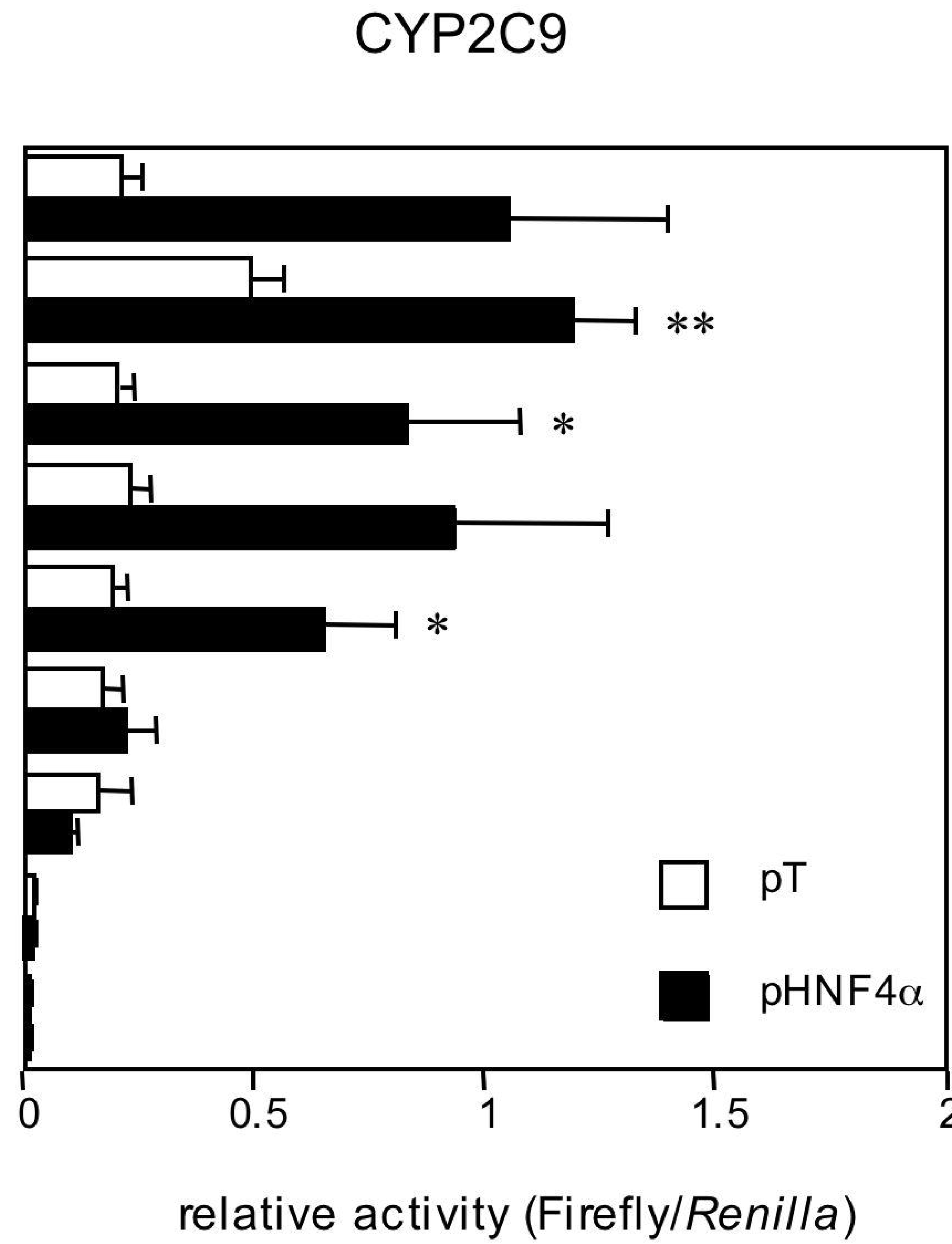
Fig. 3.





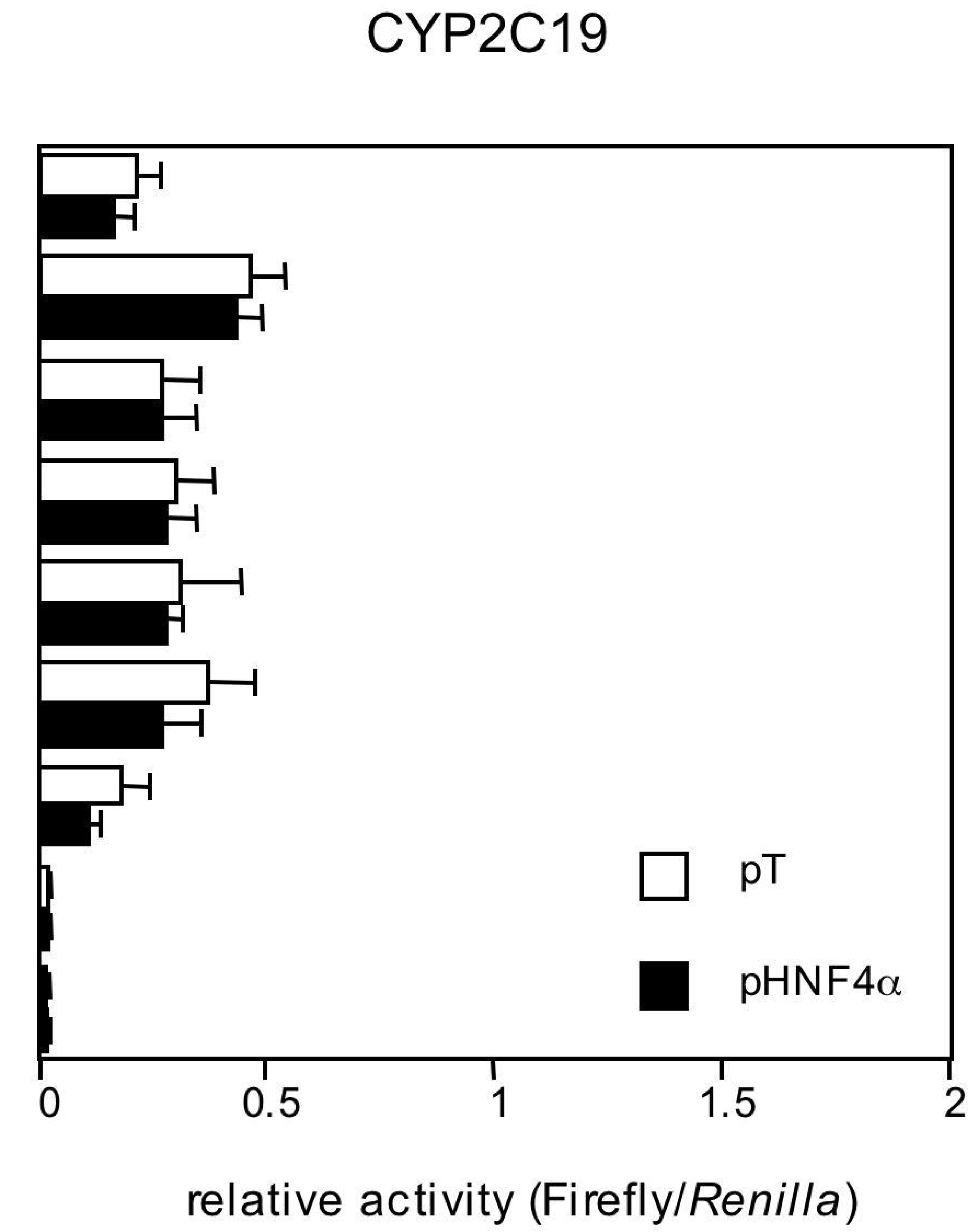


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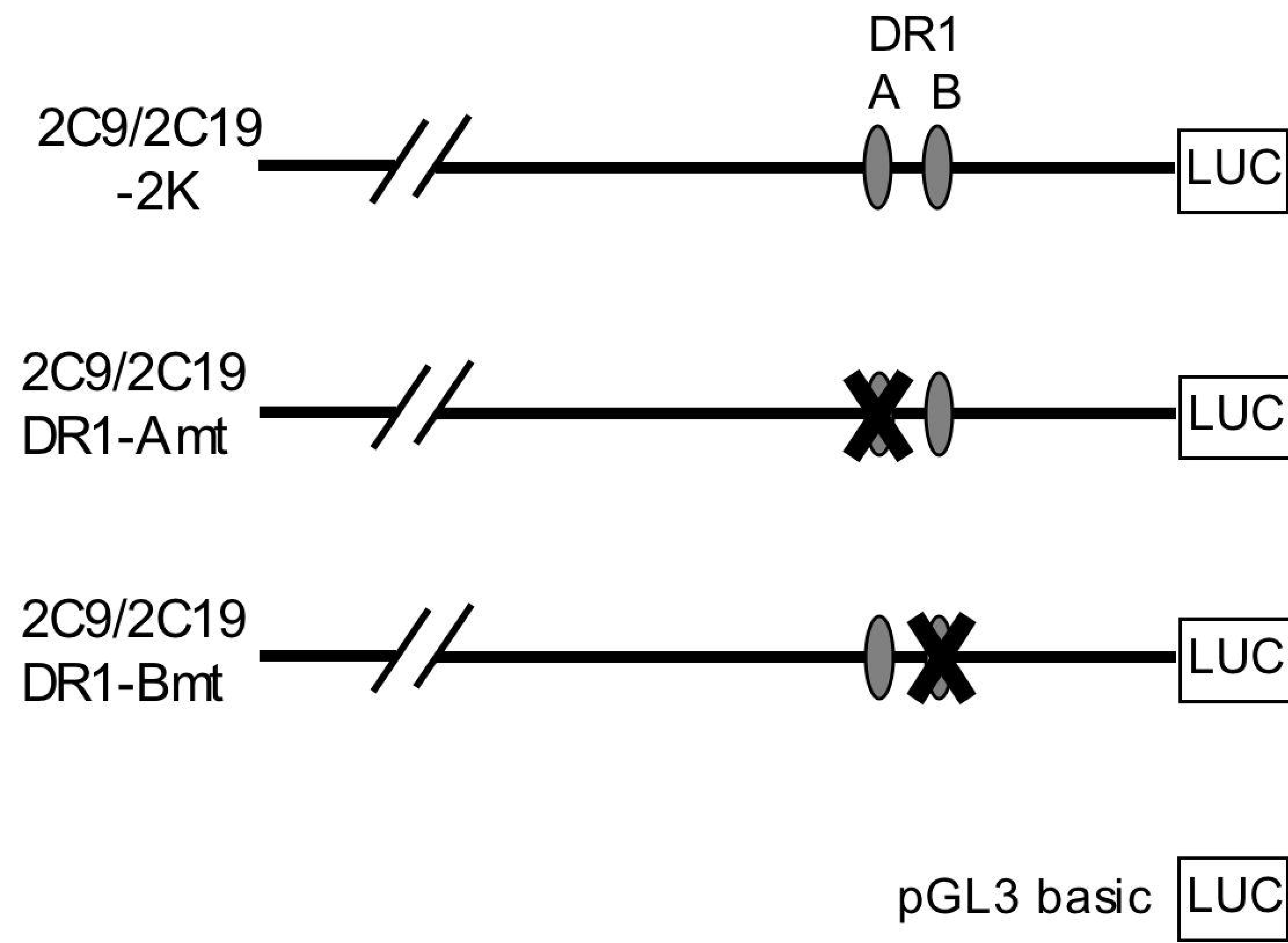
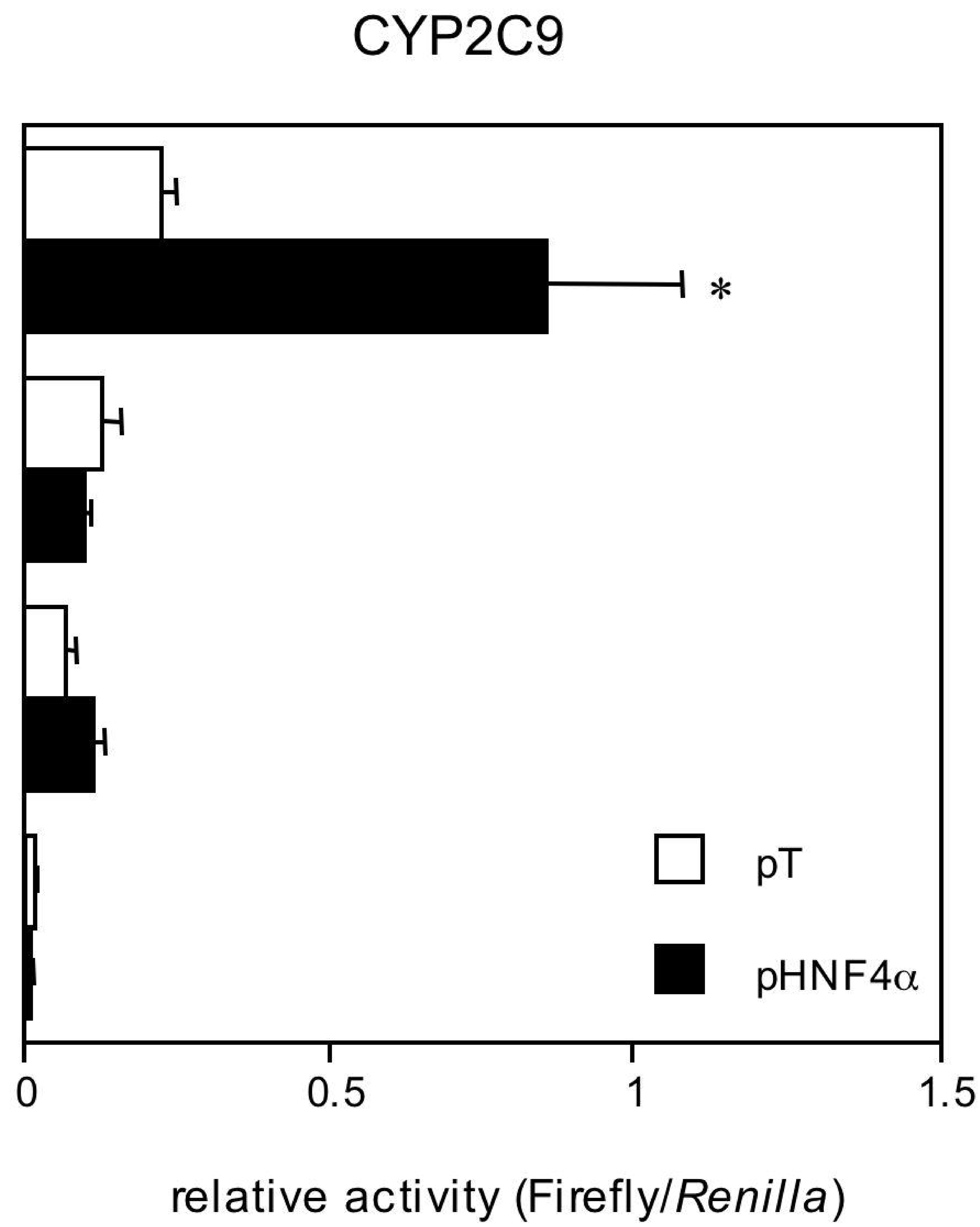
Fig. 4.





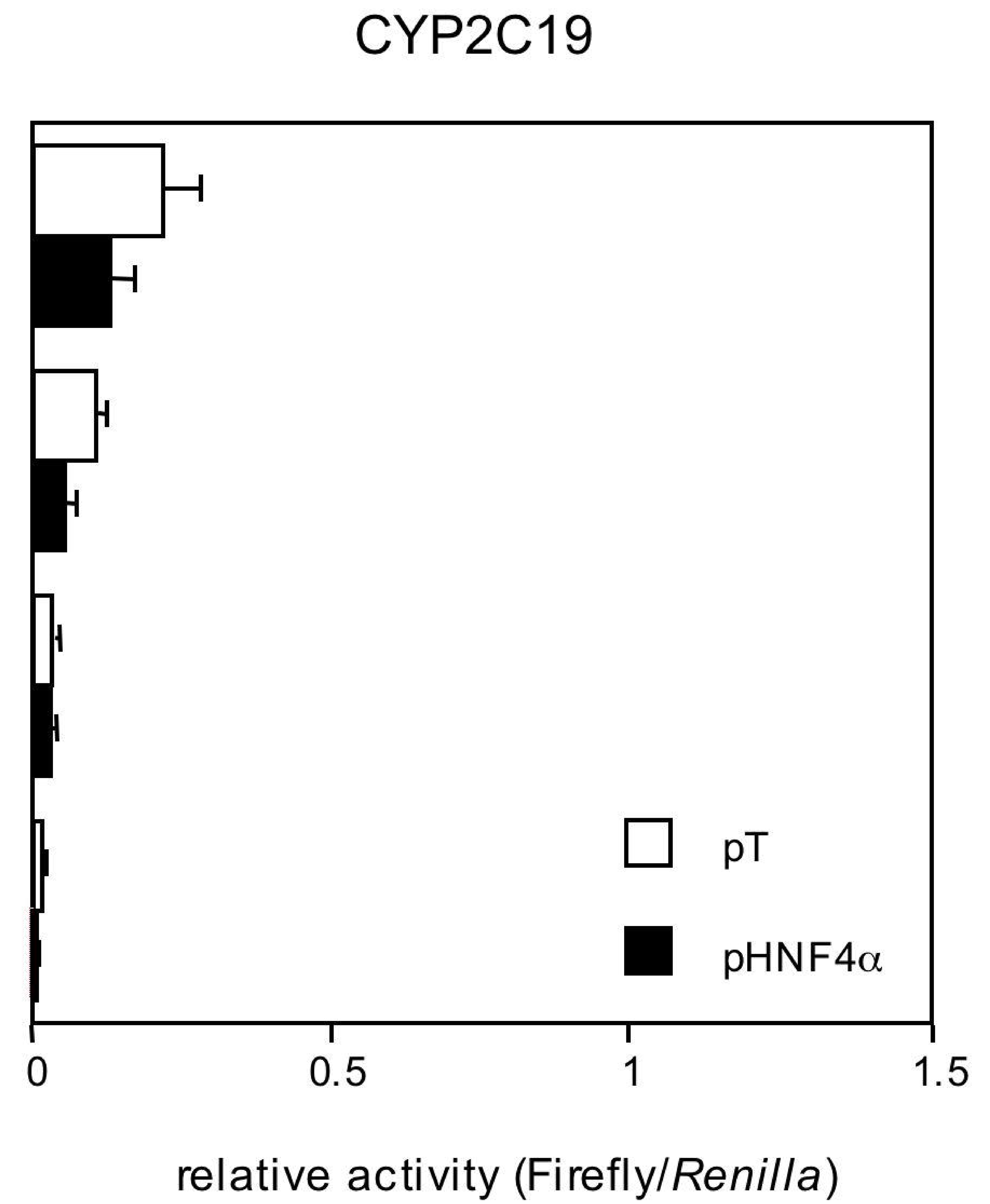


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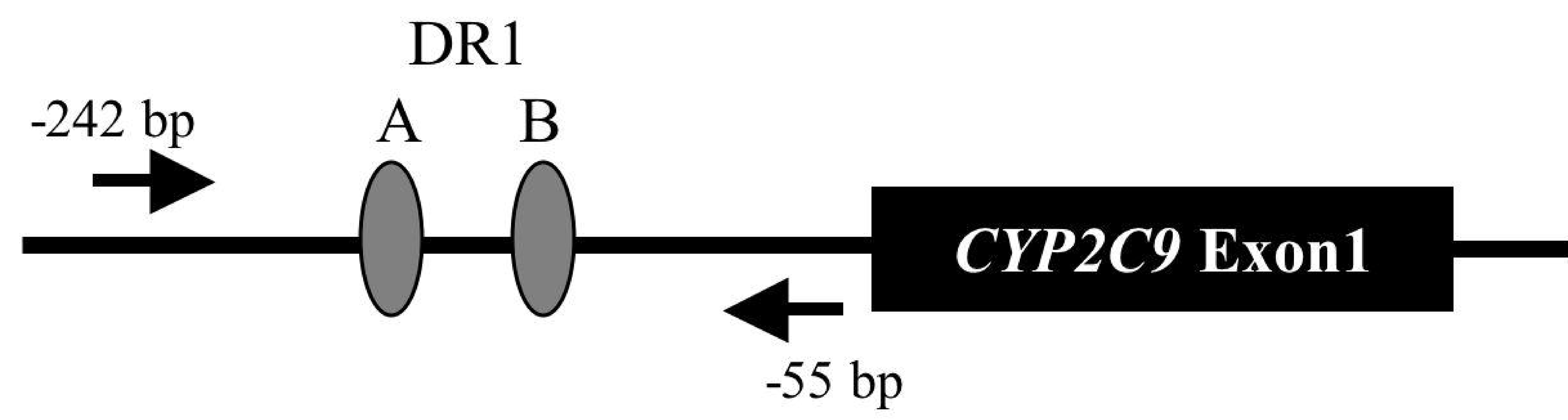
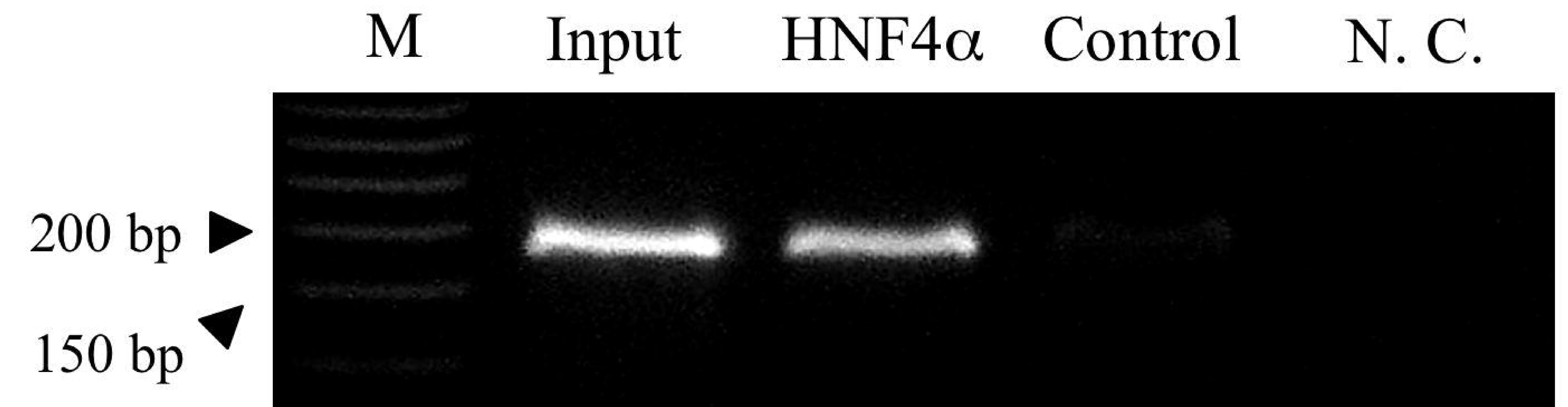
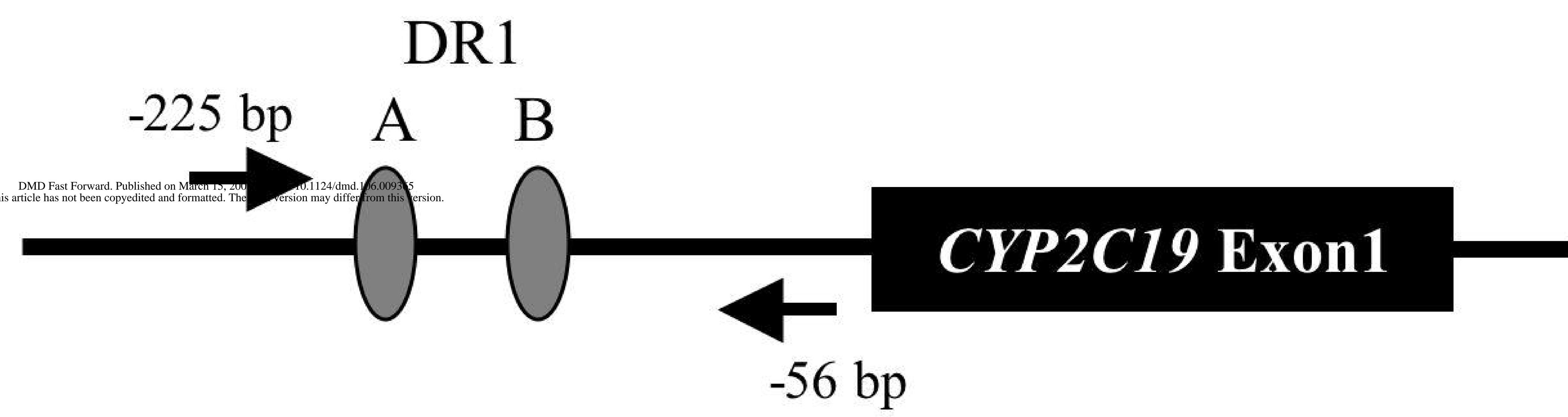
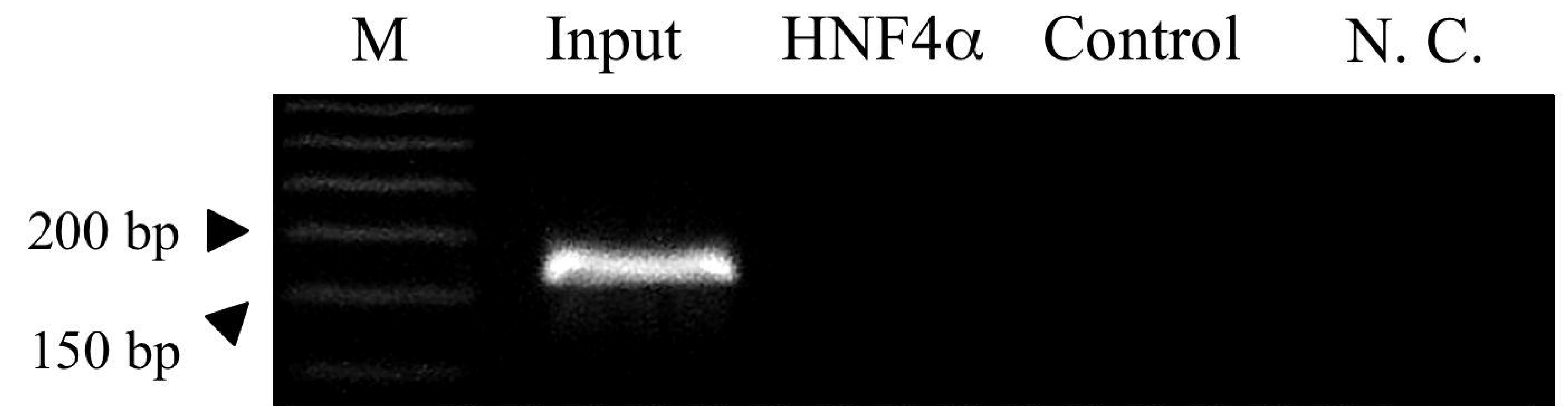
Fig. 5.









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