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Regulation of human *CYP11B1* and *CYP11B2* promoters by transposable elements and conserved *cis* elements

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ABSTRACT

CYP11B1 and *CYP11B2* responsible for the final steps of cortisol and aldosterone synthesis, respectively, are believed to be duplicate genes with distinctive promoters. Our sequence analysis uncovers that these two genes share great homology in the proximal upstream regions, but insertion of Alu and L1 elements drives promoters divergent. Each *CYP11B* promoter contains two Alu elements embedded in a truncated L1 element, breaking L1 into three disconnected fragments. Alu functions as an enhancer in both genes regardless of orientation and copy number. Insertion of Alu upstream of a SV40 promoter also elevates promoter activity. However, the effect of Alu on *CYP11B1* is blocked by a second L1 element (CYP11B1-L1.2) inserted between the first one and the conserved proximal upstream region. Although CYP11B1-L1.2 is 5′-truncated and lacks a functional ORF, replacing it with a fluorescent gene demonstrates that the element can be transcribed from the *CYP11B1* core promoter in an opposite direction and a smaller magnitude compared to *CYP11B1*. Deletion of CYP11B1-L1.2 greatly increases *CYP11B1* promoter activity and restores the enhancing effect of Alu. The Ad5 and SF-1 binding elements conserved in the proximal core promoter play a role in basal expression of both genes. Mutation of the Ad5 site reduces promoter activity to the minimal level. ERRα is the transcription factor interacting with Ad5 during basal expression. The core promoters of both genes are also conserved in mouse and rat despite the fact that the sites corresponding to cre, Ad5, and SF-1 in rodent *Cyp11b1* promoters deviate from consensus.

1. Introduction

Adrenal corticosteroid production is essential for life. In humans, cortisol and aldosterone are the major glucocorticoid and mineralocorticoid produced by the adrenal cortex, respectively. Cortisol regulates a wide range of physiological processes such as energy metabolism, stress response and immunosuppression, while aldosterone mainly controls electrolyte balance, water homeostasis, and thus blood pressure. Cortisol and aldosterone synthesis are catalyzed basically by the same group of mitochondrial and microsomal enzymes except (1) cortisol synthesis requires an extra 17α-hydroxylation reaction catalyzed by CYP17, and (2) the final synthetic steps are catalyzed by two similar but different CYP11B enzymes. CYP11B1 containing only 11β-hydroxylase activity is responsible for 11β-hydroxylation of 11-deoxycortisol to cortisol. CYP11B2 also named aldosterone synthetase catalyzes 11β-hydroxylation, 18-hydroxylation, and 18-oxidation of 11-deoxycorticosterone to yield aldosterone [1].

Human *CYP11B1* and *CYP11B2* genes are closely related. They are located in tandem on chromosome 8. These two genes share 95% identity in the coding regions and 90% identity in introns, but they have very dissimilar 5′ upstream sequences. The promoter dissimilarity suggests that *CYP11B1* and *CYP11B2* are under distinct transcriptional regulation [2]. In accordance with this suggestion, CYP11B1 and CYP11B2 display different zonal expression patterns. CYP11B1 is present primarily in the zona fasciculata/reticularis of the human adrenal cortex, whereas CYP11B2 expression is limited to the zona glomerulosa [3]. Expression levels of *CYP11B1* are relatively high compared to *CYP11B2*. People carrying a chimeric gene in which the upstream region and 5′ coding sequence of *CYP11B1* is fused to the 3′ coding sequence of *CYP11B2* suffer from hyperaldosteronism because of excessive expression of a CYP11B2-like chimeric enzyme [4,5]. The difference in expression level contributes to a higher daily secretion of cortisol (10–20 mg/d) over aldosterone (100–150 μg/d) [6].

Using a computational alignment program MISA, we located two clusters of common sequence segments in *CYP11B1* and *CYP11B2* promoter regions. One cluster is situated in the proximal upstream region, while the other is present in two copies in the distal upstream region. Homology search identified the latter as Alu, a transposable element widely spread in the human genome.

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Fig. 1. Alignment of human *CYP11B1* and *CYP11B2* proximal upstream sequences. Nucleotides are numbered relative to the transcription start site. Identical residues are highlighted with gray shade, whereas gaps are indicated with dashes. TATA box, cre, Ad5, and SF-1 binding site are marked on the top of sequences. The T residue of the *CYP11B2* SF-1 binding site has been reported to be C in some people.

The Alu repeats are surrounded by sequences of another family of transposable elements, L1. The insertion of Alu and L1 elements leads to divergence in the upstream regions. The impacts of these conserved sequence elements and transposable elements on basal *CYP11B1* and *CYP11B2* gene expression were determined in this study.

2. Materials and methods

2.1. Computational sequence analysis

Homology between sequences was searched and aligned using MISA (Multiple Indexing Sequence Alignment) developed by Dr. TW Pai [7,8] and BLAST provided by the National Center for Biotechnology Information, USA (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Transposable elements were located using CENSOR provided by Genetic Information Research Institute, USA (http://www.girinst.org/censor/index.php).

2.2. Construction of reporter plasmids

Human *CYP11B1* and *CYP11B2* promoters were assembled from DNA fragments PCR amplified from genomic clones RP11-706C16 and RP11-304E16 (Children's Hospital Oakland-BACPAC Resources Center, Oakland, CA, USA), respectively, and cloned into a promoterless pGL2 vector (Promega, Madison, WI, USA). Promoter sequences were validated by sequencing. Hybrid reporter plasmids pCYP11B1(Alu.2)/11B2(−1334) and pCYP11B1(Alu.2)/11B2(−905) were created by insertion of the CYP11B1-Alu.2 element (−4511 to −4057) in front of the 1334-bp and 905-bp *CYP11B2* promoters, respectively. CYP11B1-Alu.2 was also cloned upstream of the SV40 promoter of pGL2-control (Promega) in both orientations to form pCYP11B1(Alu.2) and pCYP11B1(Alu.2r). Green fluorescent protein (GFP) displaced the luciferase reporter of pCYP11B1(−4511dL1) to form pCYP11B1(−4511dL1)-GFP, and displaced the L1.2 region of pCYP11B1(−4511) to generate pCYP11B1(L1→GFP). In addition, the 1354-bp *CYP11B1* promoter was fused to GFP in the anti-sense orientation to produce pCYP11B1(−1354AS)-GFP.

2.3. Site-directed mutagenesis and deletion

Mutations and internal deletions were generated by two-step PCR. In the first-step PCR reaction, two complementary oligonucleotides containing the desired mutation or deletion junction were separately paired with an upstream primer and a downstream primer to amplify the sequences upstream and downstream of the mutation or deletion site. The PCR products were then 1:1 mixed. 1% of the mixture was used as templates, and the upstream and downstream primers were again used as primers for the second-step PCR reaction. After sequencing verification, the product with the desired mutation or deletion was used to replace the wild type sequence via DNA recombination.

2.4. Transfection analysis

Promoter activity was assessed by lipofectamine transfection (Invitrogen, Carlsbad, CA, USA) of individual promoter-luciferase plasmids with pSV40-βgal at a 15:1–25:1 ratio into human adrenocortical H295R cells. To learn the effect of ERRα, ERRα expression plasmid (Open Biosystems, Huntsville, AL, USA) and vector were separately cotransfected with indicated reporter plasmids. The transactivation effects of glucocorticoid receptor and estrogen receptor on CYP11B1-Alu.2 were assayed by transfection of the expression plasmid pRShGR (ATCC, Manassas, VA, USA) and pSG5-ER [9], respectively, into H295R cells along with the reporter plasmid pCYP11B2(Alu.2) or its vector pGL2-control. After transfection, cells were treated with indicated concentrations of cortisol (Sigma-Aldrich, St. Louis, MO, USA) or 17β-estradiol (Sigma-Aldrich) for 24 h. Promoter activity was determined by βgal–normalized luciferase activity as described previously [10]. The transcriptional status of CYP11B1-L1.2 was checked by lipofectamine transfection of indicated GFP reporter plasmids into H295R cells. Green fluorescence was detected by fluorescent microscopy.

2.5. Identification of Ad5-binding protein

The Ad5-binding protein was isolated by avidin–biotin complex DNA (ABCD) binding assay. The nuclear extract used in this binding assay was prepared from H295R cells using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Rockford, IL, USA), while the Ad5 oligonucleotide probe (sense: 5′-gcctccagcccTGACCTCTGCCCTCggtct-3′ and antisense: 5′-agaccGAGGGCAGAGGTCAgggctggaggc-3′) was labeled using the Biotin 3′ End DNA Labeling Kit (Thermo Fisher Scientific). One mg of H295R nuclear extract was incubated with one pmole of Ad5 probe in the binding buffer (12 mM HEPES, pH 7.9, 60 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM dithiothreitol, 12% glycerol, and 50 ng/μL dI–dC) at room temperature (RT) for 20 min. The DNA-protein complex was captured by incubation with 100 μL of buffer-washed streptavidin magnetic particles (Roche Applied Science, Indianapolis, IN, USA) at 4 °C for 3.5 h with gentle rotation. The beads were then washed five times in TE buffer (10 mM Tris–HCl, pH 8.0, and 1 mM EDTA) before boiling in SDS PAGE sample buffer.

After separation by 10% SDS–PAGE and staining with Coomassie blue, protein bands were in-gel digested with sequencing grade, modified trypsin (Promega) and purified through a C18 Zip-Tip