The $-^{104}$G nucleotide of the human CYP21 gene is important for CYP21 transcription activity and protein interaction

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ABSTRACT

CYP21 gene encodes the steroid 21-hydroxylase (P450c21) that is involved in steroidogenesis in the adrenal cortex. Mutations occurring on CYP21 which convert it to the neighboring pseudogene, CYP21P, are found in patients with congenital adrenal hyperplasia (CAH), an autosomal recessive disease. We previously reported that the CYP21P pseudogene had lower transcription activity when compared with the active CYP21 gene. The sequences determining the basal transcription activity of the human CYP21 gene are located within the 166 bp region upstream from the transcriptional start site. Within this region, only 4 nucleotides are different between the active CYP21 and the CYP21P pseudogene; they are located at the –117, –104, –101 and –94 positions from the start site of the gene. Here, we report that the CYP21 gene-specific G nucleotide sequence at the –104 position is crucial for interaction with nuclear proteins from the adrenal gland. These results therefore suggest that the single G sequence of the human CYP21 gene is crucial for the expression of its basal transcription activity, and this may be influenced by the interaction with specific nuclear proteins from the adrenal gland.

INTRODUCTION

P450c21 (steroid 21-hydroxylase), a microsomal cytochrome P450 enzyme, mediates the biosynthesis of mineralocorticoids and glucocorticoids (1). P450c21 is synthesized in the adrenal cortex where it receives electrons from NADPH via a membrane-bound reductase. It converts progesterone and 17-hydroxysterone to deoxycorticosterone and 11-deoxycortisol, respectively. These products are then converted to cortisol, corticosterone and aldosterone. Enzymatic deficiency of P450c21 is strongly associated with congenital adrenal hyperplasia (CAH), a common inherited disease of adrenal steroidogenesis in humans with an incidence of 1 in 15,000 (2,3). CAH disease can be classified into classical and non-classical types, according to phenotypic differences (4). The classic type of CAH is further divided to salt-wasting and simple virilizing forms based on the patient’s ability to preserve salt.

The gene encoding the P450c21 (CYP21) is located on human chromosome 6p21.1, within the class III region of the major histocompatibility complex (HLA) locus (5). CYP21 gene contains 10 exons extending 3.4 kb (6,7). Two CYP21 genes, the active CYP21 gene and the pseudogene, CYP21P, are arranged in tandem next to the serum complement C4 genes (C4A and C4B) in the array of 5′-C4A-CYP21P-C4B-CYP21-3′, with the same transcription direction (6,8,9). Recently, more pairs of duplicated genes, such as XA, XB, XB-S; YA, YB and ZA, ZB genes have been found within this locus (10–12). Among these duplicated genes, XA, XB-S, ZA and ZB genes, as well as CYP21 and CYP21P genes, are specifically expressed in the adrenal gland. Because they are a pair of duplicated genes, there is >98% sequence similarity between CYP21 and CYP21P genes, including the 5′-flanking region, the exon and the intron sequences (6–8). This high similarity in DNA sequence causes frequent genetic recombination. Therefore, mutations characterized in CAH patients are either point mutations, small deletions, or gene conversion from the active CYP21 gene to the neighboring non-functional CYP21P gene sequence, resulting in defective enzymatic activity (6–8,13–15).

The 1.6 kb 5′-flanking sequence of CYP21P gene was shown to have lower basal transcription activity than that of CYP21 gene in both mouse Y1 and human NCI-H295 cell lines (16). This result was consistent with the observation that there is less YA transcript in adrenal gland than that of CYP21 transcript, since YA and CYP21P genes use the same DNA region as the promoter element for expression. The differential basal transcription activity between CYP21 and CYP21P genes was determined by the DNA region covering 166 bp from the transcriptional start site of the gene (16). In this study, we performed transient transfection experiments and electrophoresis mobility shift assay (EMSA) to further dissect the gene-specific sequences within that 166 bp region of the human CYP21 gene for its importance in transcriptional activity.

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MATERIALS AND METHODS

Cell cultures and transcriptional activity analysis

Mouse adrenocortical tumor cells, Y1 cells (17), were grown at 37°C in a 5% CO2 incubator in F10 medium (GIBCO, Grand Island, NY, USA) supplemented with 0.2% sodium bicarbonate, 10% horse serum, 2.5% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 µg/ml). CsCl-gradient double banded plasmid DNA was transiently transfected into Y1 cells using the calcium phosphate precipitation method (18). Plasmid RSV-CAT was used as the internal control to normalize transfection efficiency. Transcriptional activity was analyzed by primer extension of total cellular RNA from transfected cells using a CAT29 primer (5′-TTTAGCTTCTTTAGCTGAGAAAAACATC-3′) as described by Chang et al. (19). Band intensities were quantified using the Image-Pro Plus version 1.0 program (Media Cybernetics, Inc., MD, USA).

Preparation of nuclear extract

Cultured cells grown to 70% confluence were scraped into 1 ml of extraction buffer (10 mM Tris, pH 7.4, 10 mM NaCl, 3 mM MgCl2, 0.5% NP-40, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 2 µg/ml leupeptin). The crude nuclear extracts were prepared following the procedure described by Dignam et al. (20). The protein concentration in the final nuclear extract preparation was determined using a protein assay kit (BioRad Laboratories, Hercules, CA, USA).

EMSA

Oligonucleotides and their complementary strands were end labeled, annealed and used as probes to bind nuclear extracts from mouse Y1 cells as previously described by Guo et al. (21). The protein–DNA binding reaction was conducted in a 20 µl reaction mixture with 2 µg poly dI–dC, 20 µg nuclear protein extract, and 32P-labeled oligonucleotide probe in 15 mM HEPES, pH 7.9, 0.1 mM EDTA, 0.5 mM DTT and 5 mM MgCl2. Competition assay was conducted by preincubation of the nuclear extract with unlabeled DNA for 15 min before the addition of the labeled probe.

Southwestern analysis

Nuclear extract (40 µg) from mouse Y1, human NCI-H295, HeLa cells or bovine adrenal cortex, was boiled at 100°C for 5 min and separated on an 8% SDS–polyacrylamide gel. The proteins were transferred electrophoretically onto nitrocellulose membrane and renatured to the native conformation by soaking in the renaturing buffer (10 mM HEPES, pH 7.5, 100 mM KCl, 0.1 mM EDTA, 10 mM MgCl2, 0.1 mM ZnSO4, 1 mM DTT, 10% glycerol and 5% non-fat milk) to remove SDS (22). The binding of labeled DNA to the nitrocellulose blots was done in the above buffer with the addition of 13.33 µg/ml of poly dI–dC at room temperature for 1 h. The filters were then washed in washing buffer (16% glycerol, 20 M HEPES, pH 7.9, 100 mM KCl, 6.25 mM MgCl2, 0.5 mM EDTA, 0.5 mM DTT and 0.25% non-fat milk) and exposed to Kodak BIOMAX film.

Sequences of oligonucleotides (36mer)

Oligonucleotides used for gel retardation and mutagenesis assays were designed according to the genomic sequences of human CYP21 and CYP21P genes (11). All these oligonucleotides were synthesized and bought from Life Technologies (Grand Island, NY, USA). The following oligonucleotides are those with the sense-strand sequence.

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21B: -123GGGGGACCAGGTGACCCCATCCGAGTGGG-88,
21A: -123GGGCTGGGTCGCCGGAGGTTCAACGGTTGGG-88,
Mu1: -123GGGGGGGACCAGGTGACCCCATCCGAGTGGG-88,
Mu2: -123GGGGGGGACCAGGTGACCCCATCCGAGTGGG-88,
Mu3: -123GGGGGGGACCAGGTGACCCCATCCGAGTGGG-88,
Mu4: -123GGGGGGGACCAGGTGACCCCATCCGAGTGGG-88,
Mu2:3: -123GGGGGGGACCAGGTGACCCCATCCGAGTGGG-88
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Reporter plasmid construction and site-directed mutagenesis

The −166/+1 DNA fragment of human CYP21 or CYP21P genes was amplified using polymerase chain reaction (PCR) from genomic clones containing either CYP21 or CYP21P genes (11). The DNA fragments obtained were blunt-end ligated to a pCAT-Enhancer reporter vector (Promega, Madison, WI, USA) and transformed into TG-1 cells. The resulting plasmid was named p2ChemE#66, p21-SE455 or pMu2-1E99. The −166/+1 region of the CYP21 gene from p2ChemE#66 was then transferred to the M13mp18 vector using PstI and XbaI. The recombinant M13 single-stranded DNA substituted with uracil by growth in Escherichia coli CJ236 (dat-1 ung-1) was used as a template for synthesis of a mutant strand by priming with synthetic oligonucleotide. Mutants were then selected by growing in the wild-type strain. The −166/+1 CYP21 DNA fragment from the obtained mutant M13 recombinant was then transferred back to the pCAT-Enhancer vector. The cloned inserts in all the plasmid constructs used for transfection experiments were verified by DNA sequencing using the dyeoxy method with Sequenase (US Biochemical Corp., Cleveland, OH, USA).

RESULTS

The protein binding ability of human CYP21 gene nucleotides at the −117, −104, −101 and −94 positions

We have previously shown that sequences within the 166 bp region from the transcription start site of the human CYP21 gene determine its basal transcription activity (16). Sequences within that region are shown in Figure 1. Four nucleotide sequences at −117, −104, −101 and −94 positions are different between CYP21 and CYP21P genes (11). All these oligonucleotides were designed according to the genomic sequences of human CYP21 and CYP21P genes (11). Sequences within the −117, −104, −101 and −94 positions are different between CYP21 and CYP21P genes within the 166 bp region. Gel mobility shift assay was performed to analyze the role of those four gene-specific sequences of the CYP21 sequence for interaction with nuclear proteins from mouse Y1 cells (sequences of the oligonucleotides used in the assay are shown in Materials and Methods). When the 32P-labeled oligonucleotide probe (21B) (−123/−88) was used to interact with nuclear proteins from Y1 adrenal cells, multiple DNA–protein complexes were observed (Fig. 2A). Excess unlabeled 21B DNA competed well with these DNA–protein interactions, indicating a specific interaction between DNA and proteins. Similar complexes of less intensity were seen when the 32P-labeled oligonucleotide 21A probe containing the same region of the CYP21P sequence was used to interact with the nuclear extract (Fig. 2B). In addition, the 21B oligonucleotide
competed for protein binding much more efficiently than 21A itself (Fig. 2B).

To clarify the difference in binding ability of each oligonucleotide, we quantified the observed bands on X-ray film. The molar excess of competitor DNA added was plotted against the percentage of competition. In order to achieve 50% competition for 21B binding with nuclear proteins, approximately five times more 21A than 21B DNA was required as the competitor (Fig. 2C). These results further confirm our previous report that the CYP21P sequence binds to nuclear proteins with lower affinity than that of CYP21 when the –117/–94 region of DNA is used as a probe (16).

Since there are only 4 nucleotide (nt) differences within the –123/–88 region between the CYP21 and CYP21P genes, the importance of individual gene-specific nucleotides at the –117, –104, –101 and –94 positions of the CYP21 gene interaction with nuclear proteins was further analyzed. Synthetic oligonucleotides (Mu1, Mu2, Mu3 and Mu4) were used to compete with probe 21B for nuclear protein binding (Fig. 3A). Mu1, Mu3 and Mu4 all competed with the 21B probe interaction with nuclear proteins well, and a 50-fold excess of the competitor DNA abolished most of the DNA–protein interactions. However, the Mu2 competitor DNA in which the –104 nt changed from G to A (i.e., from CYP21 to CYP21P sequences) had lower competitive ability. Even with a 100-fold excess of the Mu2 competitor DNA, the 21B probe still bound with nuclear proteins.

The competition curves of Mu3 and Mu4 were close to that of the 21B oligonucleotide (Fig. 3B). Similar amounts of Mu3, Mu4 and 21B oligonucleotides were required to obtain a 50% competition effect on the 21B probe interaction with nuclear proteins, whereas the competition curves of both Mu1 and Mu2 shifted to a much lower position than that of the 21B competition curve (Fig. 3C). These results indicate that Mu3 and Mu4 oligonucleotides have lower protein binding affinity than the 21B oligonucleotide.

Figure 1. Partial nucleotide sequences at the 5′flanking region of the human CYP21 gene. The nucleotide sequence of CYP21 is shown with the transcriptional start site as +1. The TATA core sequence is depicted with bold letters. The CYP21P sequence is shown below only when it is different from CYP21. Putative transcription factor binding sites (16) are marked by a square box. Sequences of the oligonucleotides used in this study are underlined.

Figure 2. Nuclear protein bindings of the –123/–88 region of CYP21 and CYP21P. The nucleotide sequences of 21B and 21A are shown at the top of the figure. (A) EMSA of the interaction of the 21B probe with nuclear proteins from adrenocortical Y1 cells. The competitor DNA and its molar excess amounts (10, 50 and 100×) used in the reaction are indicated on top of each lane. Shifted DNA–protein complexes are indicated on the side of the autoradiograph. (B) EMSA of the interaction of 21A probe with nuclear proteins from adreno cortical Y1 cells. (C) Quantitative evaluation of competition against the 21B probe binding with Y1 nuclear proteins. The competition effect (arbitrary value of 0 in the reaction without specific competitor DNA) is plotted against the molar excess of competitor DNA. The value for each point is the average from two different experiments. The dashed line indicates the level of 50% competition.

The influence of nucleotides at the –117 and –101 positions on protein binding to the –104 position

We further analyzed the influence of nearby nucleotide changes at positions –117 and –101 in the presence of a G→A transition at position –104 on protein binding. Oligonucleotides containing substitutions at nucleotide positions –117 and –104 (Mu1-2) or –104 and –101 (Mu2-3) were used to compete the 21B binding with nuclear proteins. Our results showed that both Mu1-2 and Mu2-3 oligonucleotides had much lower protein binding ability when compared with 21B (Fig. 5A), since the DNA–protein complexes were still apparent when 50- or even 100-fold excess amounts of competitor DNA were used. The competition curves...
of Mu1-2 and Mu2-3 were similar to that of 21A which differs from 21B by 4 nt (Fig. 5B). The amount of competitor DNA needed to reduce binding by 50% was the same for Mu1-2, Mu2-3 and 21A oligonucleotides, indicating that Mu1-2 and Mu2-3 oligonucleotides bind to nuclear proteins with an affinity similar to 21A.

The above results indicate that the –104 sequence of the CYP21 gene is important for interaction with nuclear proteins. We therefore analyzed the transcription activity of plasmid containing the –166/+1 region of either the native CYP21 (p2'ChemE #66), CYP21P (p21-5E #55) sequences, or the CYP21 sequences with a single nucleotide at the –104 position changed to the CYP21P sequence (pMu2-1E#9) (Fig. 6A). All the plasmids were transiently transfected into mouse Y1 cells and the transcription activity was analyzed by primer extension. The data were normalized with the transcript from the internal control RSV-CAT plasmid in which the reporter CAT expression was controlled by the promoter of Rous sarcoma virus. Our results showed that the –166/+1 region of the CYP21 gene has ~8-fold (average from three separate experiments) higher transcription activity than the same region containing the CYP21P sequences (Fig. 6B). When a single nucleotide at position –104 changed to the CYP21P sequence (pMu2-1E#9), the transcription activity decreased to 20% of normal (p2'ChemE #66) (Fig. 6B).

**DISCUSSION**

In this study, we have provided evidence showing that the $^{104}G$ nucleotide of the human CYP21 gene is critical for the expression of its basal transcription activity. When the $^{104}G$ nucleotide was replaced by the CYP21P sequence, the transcription activity decreased by 80% (Fig. 6). This single nucleotide at position –104 is also important for physical interaction of CYP21 with nuclear protein(s) from adrenal cells (Figs 3 and 5).

In transient transfection assay, the plasmid containing the –166/+1 region of CYP21 had much higher transcription activity than CYP21P. This is consistent with our previous report (16), except that the transcription activities of CYP21 and CYP21P showed a more prominent difference in the present study. In the present study, we used the native TATA sequence (TATAA) of the human CYP21 gene in the constructs used for transient transfection assay, whereas the TATA sequence of the rat $\beta$-globin gene
Figure 5. The influence of double nucleotide substitutions from CYP21 to CYP21P specific sequences on the nuclear protein binding with the 21B probe. The nucleotide sequences of Mu1-2 and Mu2-3 are shown at the top of the figure. (A) EMSA of the interaction of the 21B probe with nuclear proteins from Y1 cells competed by Mu1-2 and Mu2-3 DNAs. The competitor DNA and its molar excess amounts (10, 50 and 100×) used in the reaction are indicated on top of each lane. Shifted DNA–protein complexes are indicated on the side of the autoradiograph. (B) Quantitative evaluation of Mu1-2 and Mu2-3 competitions.

(CATAAAA) was used in the previous studies to analyze the regulation of CYP21 expression (16,23,24); this may account for the discrepancy. The TATA core sequence may affect the activity of regulatory elements (25–29). To our knowledge, this is the first report using the native TATA promoter sequence of the human CYP21, in combination with regulatory elements to study transcription activity, and the results therefore more appropriately reflect the natural transcription activity of the human CYP21 gene. In addition, within this C4/CYP21 gene locus, another YA transcript is known to be driven by the CYP21P promoter (11). The 8-fold difference in the transcription activity between CYP21 and CYP21P genes shown in this study is comparable with the 10–20% abundance of the YA transcript when compared with CYP21 mRNA (11).

A single nucleotide alteration at the –104 position from the CYP21 to the CYP21P sequence reduced most of the transcription activity of the CYP21. Therefore, a mutation occurring at this promoter/regulatory region of the active CYP21 to the non-functional CYP21P sequence, subsequently lowering the expression of functional c21-hydroxylase, may be another genetic defect to be considered in the CAH patients.

Multiple DNA–protein complexes formed when the –123/–88 region of the human CYP21 gene interacted with the nuclear proteins from Y1 adrenal cells (Fig. 2). Our competition experiments and Southwestern assay results confirm that the CYP21P sequence binds with the same nuclear proteins, but less efficiently compared with the CYP21 sequence (Figs 2 and 4). Moreover, the sequences at –104 and/or –117 of the CYP21 gene are crucial to its interaction with nuclear proteins (Figs 3 and 5). As shown in Figure 3, the single nucleotide sequence replaced by the CYP21P sequence at the –104 position decreased the affinity to bind nuclear protein(s). Therefore, we feel that interaction with nuclear protein(s) around the –104G nucleotide of the CYP21 influences the expression of the transcription activity of the human CYP21 gene.

The CYP21 specific sequences around the –117 position constitute a perfect consensus binding site (GGGC(GG)G) for the transcription factor Sp1 (30). A single nucleotide change at the –117 position from C to T (Mu1) disrupted the Sp1 consensus binding sequence and lowered the protein binding ability (Fig. 3). However, a change of this single nucleotide at the –117 position (Mu1) did not interrupt the nuclear protein interaction to the extent of that caused by the single nucleotide change at position –104 (Mu2) (Fig. 3). Simultaneously changing the –117 and –104 nucleotides to the CYP21P sequences further reduced the protein binding ability to the level of that of the native CYP21P pseudogene.
(Fig. 5). It is likely that other nuclear protein(s) interacting at or near the −104 position may affect Sp1 binding at the −117 position, or vice versa. However, there is no known consensus transcriptional factor binding sequence around the −104 position of the CYP21. So far, in searching for sequence similarities, sequences around the −104 position (−112GTGGGAGGG−103) of the CYP21 gene have only one position different from the binding sequences (GGGGGAGGG) of a human histone transcription factor (H4TF1) (31). Further investigation is required to elucidate the protein factors involved in binding to the −104 position of the human CYP21 gene.

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