Multiple transcripts of the *CYP21* gene are generated by the mutation of the splicing donor site in intron 2 from GT to AT in 21-hydroxylase deficiency

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Abstract

Maturation of primary RNA transcripts of eukaryotic genes often involves the removal of introns and joining of exons. The fidelity of RNA splicing is dependent on the identity of the nucleotide (nt) sequences at exon/intron boundaries. Most importantly, the highly conserved intronic 5'GT and 3'AG sequences are essential for correct splicing. Substitution of GT by any other nt leads to incomplete mRNA and a disruption of protein structure. We describe here the results of our transfection experiments in COS-1 cells with a *CYP21* genomic construct that contained an IVS 2+1G→A mutation. Analysis of the

transcripts by RT-PCR revealed that two different transcripts were generated by this mutant genome. In all the splicing products, we found that the entire exon 2 was deleted. Surprisingly, 30% of the transcripts from this mutant *CYP21* genome were accompanied by an inclusion of 3' intron 2 sequences due to the use of a different splice acceptor site. This is the first report of the molecular characterization of a splice donor site mutation in *CYP21* via transcription in COS-1 cells.

Journal of Endocrinology (2001) **171**, 397–402

Introduction

Congenital adrenal hyperplasia (CAH, OMIM 201910) is one of the most common inborn errors of metabolism. More than 90% of CAH cases are caused by mutations of the gene which encodes steroid 21-hydroxylase (P450c21, *CYP21*) on human chromosome 6p21.3. Deficiency of steroid 21-hydroxylase prevents the conversion of 17-hydroxyprogesterone to 11-deoxycorticosterone, leading to the abolition of the synthesis of cortisol/aldosterone and the overproduction of androgen.

To date, 56 mutations in the *CYP21* gene have been reported (Krawczak & Cooper 2000, Lee 2001). Fifteen of these mutations are 'gene conversion mutations' due to intergenic recombination of DNA sequences between *CYP21* and the *CYP21P* pseudogene (Lee 2001). The other 41 mutations described are not present in the pseudogene, and are likely to be spontaneous mutations. Dysfunctional protein with mutated functional domains due to different missense mutations has been reported. Mutations occur at the membrane association region (Picado-Leonard & Miller 1988, Hu *et al.* 1996, Lajic *et al.* 1999), the substrate-binding site (Picado-Leonard & Miller 1988), and the heme-binding site (Chiou *et al.* 1990, Nikoshkov *et al.* 1998). Obviously, mutations in these regions have a deleterious effect on enzyme activity.

Gene deletions or insertions of *CYP21* (Lee 2001), including complete gene deletion or the common 707 del8 mutation, always lead to a complete loss of enzyme activity. Finally, a common 655A/C→G mutation within intron 2 has been reported in CAH patients (Higashi *et al.* 1988). S1 mapping and COS-7 cells transfection experiments have revealed that this mutation causes the introduction of an upstream acceptor site resulting in the synthesis of a truncated or an abnormal protein (Higashi *et al.* 1988). In addition, transversion mutations at the splicing junction in intron 1 and intron 7 of the *CYP21* gene have been reported in CAH patients (Wedell & Luthman 1993, Lajic & Wedell 1996). The AG to GG mutation at the splicing acceptor site in intron 1 (Lajic & Wedell 1996) and the GT to CT mutation at the donor site in intron 7 (Wedell & Luthman 1993) both disrupt the canonical consensus sequences which are crucial for splicing processing. However, the effect on the splicing process caused by these mutations in the *CYP21* gene has not been further studied.

In this study, we provide evidence to explain the consequence of the IVS 2+1G→A mutation in the *CYP21* gene found in two CAH patients. Amplification of the mutant *CYP21* gene directly from the CAH patient in combination with transfection of COS-1 cells has enabled us to analyze the genetic defects in the splicing process *in vitro*.

Figure 1 Analysis of secondary PCR products of *CYP21* gene subclones with linker primers and RT-PCR products of expression plasmid expressed in COS-1 cells. (a) Analysis of 1.0% agarose gel. Lane 1; 2.8 kb PCR product of the *CYP21* gene with the IVS 2+1G→A mutation amplified with primers BamH-21/XhoI-21. Lane 2; 8.2 kb pCDNA3 plasmid with inserted fragment digested with restriction enzyme BamHI. 'mk' was Lambda DNA-HindIII-digested marker. (b) Analysis on 1.5% agarose gel. Lanes 1 and 2; RT-PCR products from pCDNA3 expressed in COS-1 cells amplified with primers E1/E3, with normal *CYP21* and mutant *CYP21* genes respectively. Lanes 3 and 4; PCR products amplified with primers cdn62-5'/C4 from the RT-PCR product of mutant *CYP21* genes subcloned into the pGEM-T vector. Lane 5; normal *CYP21* gene subclone. 'mk' was a 100 bp marker. (c) Sequence analysis of the mutant *CYP21* gene subcloned in pCDNA3 expression plasmid. The arrow points to the position of the base change.

Materials and Methods

Subjects

The two CAH patients found to carry the IVS 2+1G→A mutation on one of the chromosomes were described previously (Lee *et al.* 1998).

Mutated *CYP21* gene amplification and construction of expression plasmid

Genomic DNA from one of the CAH patients carrying the IVS 2+1G→A mutation was directly amplified by differential amplification with allele-specific primers (BF1 and 21BR, Table 1) as previously described (Lee *et al.* 1996, Lee 2001). The amplified *CYP21* gene was used for secondary amplification with primers containing BamHI and XhoI linkers (BamH-21 and XhoI-21, Table 1) to

generate a 2.8 kb fragment covering the *CYP21* gene sequence from -76 to 2734 (Higashi *et al.* 1986). After complete digestion of the 2.8 kb PCR fragments with BamHI and XhoI, the digested fragment was separated on 1.5% agarose gel and purified. The purified DNA (Fig. 1a, lane 1) was cloned into the 5.4 kb pCDNA3 mammalian expression vector (Invitrogen Corporation, Carlsbad, CA, USA). After restriction map analysis (data not shown) and DNA sequencing (Fig. 1c), a clone containing the mutant *CYP21* gene (Fig. 1a, lane 2) was obtained. A vector containing the normal *CYP21* gene was also constructed. All the constructs were sequenced to confirm their identity.

Transfection of COS-1 cells with recombinant pCDNA3 plasmid containing either normal or mutated *CYP21* gene

The CsCl gradient-banded pCDNA3 constructs with mutated *CYP21* or the normal *CYP21* gene were individually

Table 1 Primers used for CYP21 gene amplification, expression plasmid construction, RT-PCR amplification and DNA sequencing

	Sequence (5' → 3')	Function	Location (nt)*
Amplification of CYP21 genes			
BF1	CCCAGGTGGGGGCGGACACTA	Forward	– 314 to – 294
21BR	AATTAAGCCTCAATCCTCTGCAGCG	Reverse	3177 to 3152
Amplification with BamHI/XhoI linkers for expression vector			
BamH-21**	ACAGGATCCGTCACACAGCAGGAGGGATGGC	Forward	– 74 to 53
XhoI-21	TGACTCGAGGTACCCGGCTGGCATCGGTCCTGCC	Reverse	2722 to 2742
RT-PCR amplification			
E1	TTCTTGCACTTGCTGCA	Forward	106 to 122
E3	TCCATGGAGTCACGGAT	Reverse	786 to 770
Cdn62–5'	CAGAAATTCGGGCCCATCTACAGGgTgC	Forward	157 to 184
C4	AGGGCTGAGCGGGTGAGCTTC	Reverse	759 to 739
DNA sequencing			
T7	TAATACGACTCACTATAGGGCGA	Forward	—

*nt sequence was based on Higashi *et al.* (1986).

**Underlined bases present a restriction site introduced for expression vector cloning. Lowercase represents a modified base.

transfected into COS-1 cells using Lipofectamine Plus Reagent (Life Technologies, Grand Island, NY, USA). After 3 days incubation in Dulbecco's modified Eagle's medium containing 15% fetal bovine serum, the COS-1 cells were harvested and total cellular RNA was purified using Trizol reagent (Life Technologies). The purity and the amount of RNA prepared were determined by the reading at OD_{268/280} in a GeneQuant II RNA/DNA calculator (Amersham Pharmacia Biotech Inc., Piscataway, NY, USA). Total RNA from transfected

COS-1 cells was subjected to RT-PCR amplification using primers E1/E3 (Table 1).

Analysis of RT-PCR product

After RT-PCR amplification, the PCR products were further subcloned into the pGEM-T vector system (Promega Corporation, Madison, WI, USA) following the manufacturer's instructions. After transformation, the colonies were screened by PCR amplification with the

Figure 2 Sequence analysis of the subcloned RT-PCR products using the T7 primer; (a) 134 bp of mutated RT-PCR subclone amplified with primers T7/C4, (b) 153 bp of mutated RT-PCR subclone amplified with primers T7/C4.

primers cdn62–5′/C4 (Table 1). The PCR product was electrophoresed in 1.5% agarose gel to check the DNA product.

DNA sequencing

Basically, the PCR product amplified from the clones identified was directly used as a template for DNA sequencing. The reaction mixture of the ABI PRISM Dye Termination Cycle Sequencing Kit (Perkin-Elmer, Foster City, CA, USA) was applied to the DNA cycling reaction. The protocol was used as described previously (Lee *et al.* 1998).

Results

Expression of mutated gene in COS-1 cells

To analyze the effect of splicing site mutation on RNA processing, the transcription of these constructed plasmids was analyzed in COS-1 cells 72 h after transient transfection. Total RNA from transfected cells was subject to RT-PCR amplification using primers E1/E3 (Table 1). One single 296 bp band (Fig. 1b, lane 1) was present in RT-PCR products of RNA from cells transfected with plasmid containing the normal *CYP21* gene, indicating the correct RNA processing. A small amount of 683 bp band of DNA contaminant was also observed whose size corresponded to the predicted PCR product from the genome template. However, there were two RT-PCR products of RNA from cells transfected with mutant *CYP21* plasmid (Fig. 1b, lane 2) and both were smaller than the one that amplified from the normal *CYP21* RNA (Fig. 1b, lane 1).

Sequencing analysis of the alternation of RNA splicing in mutant CYP21 gene

To analyze the transcript of RNA splicing of the mutant *CYP21* gene, the RT-PCR product was further sub-cloned into the pGEM-T vector. The colony obtained was subject to PCR amplification using primers cdn62–5′/C4 (Table 1) and sequencing analysis. Two different inserts of 134 and 153 bp sizes were found (Fig. 1b, lanes 3 and 4). These two fragments were smaller than the transcript product of the normal *CYP21* gene (224 bp, Fig. 1b, lane 5). From sequencing analysis, it appeared that the 134 bp fragment lacked the entire introns 1, 2 and exon 2 (Fig. 2a), whereas the 153 bp fragment had a deletion of intron 1, entire exon 2 and part of intron 2. In other words, the 153 bp fragment had sequences connecting exon 1 directly to the last 19 nucleotides (nts) of intron 2 (Fig. 2b). There was no other transcript product found in RT-PCR products of RNA from the mutant *CYP21* gene.

Table 2 Analysis of mRNA transcript by RT-PCR amplification expressed in COS-1 cells

	Splicing processing	Frequency (%)*	Size (bp)**
Mutant type			
Wild type	Normal splicing	—	224
Type I	Exon 2 skipped	70.7 (70/99)	134
Type II	A 19 bp insertion of the last intron 2 between exon 1 and exon 3	29.2 (29/99)	153

*Screened from 99 colonies of pGEM-T plasmid.
**PCR product amplified with primers cdn62–5′/C4.

Observation of the frequency of splicing in mutant CYP21 gene in vitro

To measure the frequency of preferential sites used for RNA splicing in the mutant *CYP21* gene, 99 transformed colonies were sequenced to identify the splicing sites. Results showed that there were 71 clones containing the 134 bp fragment lacking the entire exon 2 (70.7%) and 29 clones (29.2%) that had the 153 bp product produced from the splicing event that connected exon 1 to the last 19 nts of intron 2 (Table 2).

Discussion

Using differential PCR (Lee *et al.* 1996, Lee 2001) combined with single-strand conformation polymorphism analysis (Lee *et al.* 1998), we characterized the transcription pattern of the splicing donor site mutated *CYP21* gene with GT changed to AT in intron 2 (Fig. 3a). This *CYP21* mutation was found in one of the chromosomes of two CAH patients we have screened before (Lee *et al.* 1998). Both patients showed salt-wasting and exhibited a picture of clitoromegaly after birth (Lee *et al.* 1998). We had speculated that this splicing site mutation might either lead to skipping of exon 2, or result in the use of cryptic splice donor sites creating a premature TAA stop codon located at nts 46–48 of the intron 2 based on sequence prediction (Lee *et al.* 1998). In the case of an alternative usage of a cryptic donor site this would result in the formation of a truncated, unstable protein with no enzymatic activity. However, no sequences resembling the consensus splice donor site are present within intron 2. To find out the real effect of this mutation, transfection studies of mutant *CYP21* gene into COS-1 cells were carried out, followed by RT-PCR analysis of the resulting mRNA (Fig. 1). To our surprise, our experiments revealed the formation of two types of transcripts from this mutant *CYP21* gene. Type I transcript lacked the entire exon 2 as expected. However, the second transcript (type II) also

Figure 3 Structure and transcription of the normal and mutated IVS 2 splice donor sites. (a) Sequence changes in the mutated *CYP21* gene. The structure of the normal gene is shown with exons represented by solid boxes and introns by the solid line. The nt difference found in the mutant allele is indicated. (b) The cytoplasmic RNA produced in COS-1 cells by normal and mutant genes. Normal exons are shown as solid blocks and the RNA sequences removed by splicing are indicated by a dashed line. nts of the last 19 bp of intron 2, present in mutated type II transcript, are shown as an open box.

lost exon 2 but contained an insertion of the last 19 nt of intron 2 between exons 1 and 3 (Figs 2 and 3).

Our data show that the IVS 2+1G→A mutation completely inhibits the use of this splice donor site. This result emphasizes the requirement of the invariability of GT dinucleotides at the 5' splicing sites for correct RNA processing. The effect of such a mutation may differ from gene to gene. In vertebrates, exon skipping at the 5' splicing site was found in at least 18 naturally occurring mutations (Talerico & Berget 1990). However, transcripts resulting from exon skipping are only a minor splice variant in β^0 -thalassemia in humans, in which the main product results from the use of a downstream splice donor within the following intron (Chen *et al.* 1989). Furthermore, in the human chorionic somatomammotropin-like gene (Wieringa *et al.* 1983) and the rabbit β -globin gene (Treisman *et al.* 1982) resulting from splice donor mutations, exon skipping does not occur at all. In both cases, a cryptic splice site within the preceding exon was used.

We report in this study that the type I transcript lacking exon 2 is the predominant splicing product, proving that exon skipping is the most important result of the IVS 2+1G→A mutation. However, the less common type II

transcript still constituted approximately one-third of the mRNAs produced (Table 2), making it more than a rare splice variant. In this transcript, skipping of exon 2 is found together with an insertion of the last 19 nts of intron 2, due to the use of a cryptic splice acceptor site downstream from exon 3. This result indicates that mutation of a splice donor site may not only influence the use of the donor site itself, but also influences the use of the downstream splice acceptor site. Although, the molecular basis of this preference is still unclear, our study once more emphasizes that it is impossible to predict the effects of splice mutations on mRNA formation, and that they can only be revealed by *in vitro* or *in vivo* studies.

Four splicing site mutations in the *CYP21* gene have been reported so far (Higashi *et al.* 1988, Wedell & Luthman 1993, Lajic & Wedell 1996, Lee *et al.* 1998). The effect for only one of these mutations (656 A/C→G) was proven from mRNA studies (Higashi *et al.* 1988). By way of differential amplification of the *CYP21* gene, we have established a reliable method to analyze the effect of mutations occurring at either splicing donor or acceptor sites of the *CYP21* gene.

Acknowledgements

We wish to thank Dr Erik A Sisterman for critical reading of this manuscript. This work was supported by grants NSC 89-2314-B039-008 from the National Scientific Council of the Republic of China.

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Received 24 July 2001

Accepted 9 August 2001