Abundant Adrenal-specific Transcription of the Human P450c21A “Pseudogene”*

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Human adrenal steroid 21-hydroxylase (P450c21) is encoded by the CYP21A1 (21B) gene located in the class III region of the HLA locus. A tandemly duplicated gene designated CYP21A1P (21A), which lies 30 kilobases upstream, contains several point mutations and an 8-base pair deletion so that it cannot encode P450c21 protein; as a result, it is generally considered to be a pseudogene. We previously showed that two additional genes, XA and XB, lie on the opposite strand of DNA overlapping the 3’-ends of the 21A and 21B genes. We have now identified a third pair of duplicated overlapping genes in this locus, termed YA and YB, whose transcriptional orientation is the same as 21A and 21B and opposite to XA and XB. YA transcripts use the 21A promoter, have 5’-ends that are similar to 21B mRNA, and have ~10–20% of the abundance of 21B transcripts, but have unique 3’-ends. The YA gene encodes a 7.5-kilobase RNA that overlaps XA completely and a 3.0-kilobase RNA that excludes most of XA. The YB gene appears to be similar in size and organization to YA. The YA and YB genes extend beyond the limit of the duplication in this locus, hence, their cDNAs are distinguishable by differences in their 3’-sequences. YA and YB transcripts are expressed only in the fetal and adult adrenal glands, but their cDNAs do not contain a long open reading frame. Although the function of these genes is not yet clear, the complex genetic organization of three overlapping genes (21/X/Y) appears to be unique among higher eukaryotes. As YA transcription is initiated by the 21A 5’-flanking DNA and includes 21A sequences, the designation of 21A as a “pseudogene” merits reconsideration.

Steroid 21-hydroxylase (P450c21) is an adrenal-specific microsomal cytochrome P450 required for the synthesis of both glucocorticoids and mineralocorticoids (1). Genetic lesions in the human P450c21 gene locus cause congenital virilizing adrenal hyperplasia (2, 3), a common disorder affecting ~1 in 12,000 persons (4); thus, this gene locus has been the subject of intensive study. The human genome contains two genes formally termed CYP21AlP and CYP21A1 (5) and generally referred to as 21A and 21B. These genes are located in the class III region of the HLA locus on chromosome 6p21.1 and are duplicated in tandem with the C4A and C4B genes, which encode the two forms of the fourth component of serum complement. These four genes are arranged 5’-C4A/21A/C4B/21B-3’ and have the same transcriptional orientation (Fig. 1, upper panel). The 21B gene encodes the P450c21 protein. The 21A gene has 98% nucleotide sequence identity to 21B (6–8), reflecting concerted evolution (9–11) at this locus, but 21A has various mutations, producing frameshifts and three premature stop codons (6–8); hence, it cannot encode a P450 protein. Gene conversion events in the duplicated C4 (12–15) and 21 genes are common, resulting in ~85% of the mutations causing congenital virilizing adrenal hyperplasia (2, 3, 16–21) and suggesting an extremely high degree of recombinational activity in this locus.

We recently identified a pair of genes termed XA and XB encoded on the DNA strand opposite from the 21A and 21B genes and overlapping the last exon of each (Fig. 1) (22–24). The XB gene encodes a protein with remarkable homology to the extracellular matrix protein tenasin (23–25). The XA gene gives rise to a stable adrenal-specific transcript of unknown function; it is >99% identical to XB, but is truncated at its 5’-end and contains a deletion closing the reading frame (23).

We now report the adrenal-specific expression of another pair of duplicated genes, operationally termed genes YA and YB, with the same transcriptional orientation as 21A and 21B and overlapping the XA and XB genes, respectively. The YA gene utilizes the 21A promoter and extends to a point just upstream from the C4B gene. The YA gene encodes 7.5- and 3.0-kb transcripts that differ by the inclusion of a 4.5-kb intron, which spans all but 80 bases of the XA gene. The YB gene appears to be similar to the YA gene, although its 3’-end is distinct, and its exonic regions have extensive overlap with exons of the XB gene. The complex organization of three overlapping genes within this locus appears to be unique in the human genome. The function of the X and Y genes is not clear. The transcription of the YA locus may facilitate single-stranded DNA breaks that could be involved in gene conversion events. Furthermore, RNA/RNA hybrids may form between 21B and X mRNAs and between X and Y RNAs; such hybrids might influence splicing, transport, or translation of the protein-coding mRNAs within this locus.

MATERIALS AND METHODS

cDNA Library Construction and Screening—Total adrenal RNA was extracted in 4 m guanidinium thiocyanate followed by ultracentrifugation over a CsCl cushion as described (26). Pellets were resuspended in 10 mM Tris-HCl (pH 7.6), 1 mM EDTA, 0.1% SDS;

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1 The abbreviations used are: kb, kilobase(s); PIPES, 1,4-piperazineethanesulfonic acid; RT/PCR, reverse-transcribed polymerase chain reaction.
The limits of the duplication of this locus are shown by dashed lines. The positions of genomic probes G1-G3 are shown (see text). The 10-kb regions designated A and B are expanded below. Lower panels. For a YB cDNA clone, shown below the line. Exons of the YA and YB cDNAs are shown below the line. Sequences to the left of the duplication limit are homologous in the A and B regions. Jagged ends of Y clones represent partial-length cDNAs; clones YA-17 and OB-9 are polyadenylated.

**RESULTS**

Cloning of YA cDNAs—Northern blots of fetal adrenal RNA probed under high stringency with the YA region between 21A and C4B revealed transcripts larger than those of the YA RNA (23). Similar transcripts are also seen on long exposures of Northern blots of human fetal adrenal RNA probed with 21B cDNA. From 39 positive clones, we purified three longer than 2.5 kb (the size of XA transcripts). Clone YA-3 was 2.6 kb long and had a 5′-sequence identical to that of 21A, beginning at base -80 (numbered according to Ref. 6). This cDNA was spliced in a fashion analogous to 21B, except that the first two introns were included, and a splice junction was present 24 bp downstream from the TGA codon in exon 10 of 21A (bases 2704-2706 in Ref. 6). The ninth exon of YA-3 overlaps the cap site of the YA RNA (23). The final two exons of YA-3 lie between XA and C4B. Each splice junction conformed to the GT/AG rule.
Clone YA-3 was partial-length because it did not contain a poly(A) tail, although there is a canonical polyadenylation signal in the genomic sequence 300 bp downstream from the end of this clone (bases -682 to -677 in Ref. 23 and Fig. 2). To delineate the 3′-end of the YA cDNA, the 39 original positive clones were rescreened with a 1-kb PCR-amplified genomic probe (see G2 in Fig. 1). This probe extended from the limit of the duplication to the polyadenylation signal and identified four additional clones. One of these, clone YA-17, was polyadenylated, containing a poly(A) tail 12 bases downstream from the predicted AAATAAA polyadenylation signal. As shown in Fig. 1A, the last two introns were excised from YA-17 as in YA-3; however, the 5′-most exon of this clone continued 240 bp upstream without splicing. The intron/exon organization of the other three YA clones was the same as that of YA-17, extending the sequence of this exon 1.2 kb upstream from the alternate splice junction present in YA-3 (Fig. 1A). The sequences of these cDNAs correspond to the sequences of the corresponding genomic DNA (6-8, 23).

The YA-3 cDNA does not contain a long open reading frame: the P450c21 reading frame terminates 35 bp into intron 2 of 21A and should amplify any shorter spliced cDNAs preferentially. To determine if there was a short YB RNA analogous to short YA and to determine the relative abundances of the various Y transcripts, we performed RNase protection assays. We used a 611-bp SacI fragment of clone YA-3 encompassing 199 bp of the last exon of 21A (having sequence identical to that of 21B), 333 bp of sequence common to YA and YB, and 79 bp specific to YA (Fig. 3). This probe can distinguish both the long and short forms of both YA and YB. The long forms of YA and YB are ~10–20% of the abundance of P450c21B, while the short forms are very much less abundant. The reason for the apparent difference in abundance of the long and short forms of YA seen in Figs. 2 and 3 is unclear; there may be differences among various adrenal glands, and the long form is more subject to degradation or may not have transferred completely, leading to disproportionately lower representation on the Northern blot (Fig. 2).

Clone YA-3 included the first two introns of 21A. To determine if the inclusion of these introns is a general feature of YA RNAs, we performed RNase protection assays with a 396-bp EcoRI fragment of YA-3 that includes exons 3 and 4 and 205 bases of intron 2. This probe spans the 8-bp deletion in exon 3 of 21A so that spliced and unspliced forms of P450c21B and YA RNAs can be differentiated. Fig. 4A shows an abundant 156-bp protected fragment corresponding to properly spliced P450c21B and YA RNAs can be differentiated. Fig. 4A shows an abundant 156-bp protected fragment corresponding to properly spliced P450c21B and YA RNAs can be differentiated. Fig. 4A shows an abundant 156-bp protected fragment corresponding to properly spliced P450c21B and YA RNAs can be differentiated. Fig. 4A shows an abundant 156-bp protected fragment corresponding to properly spliced P450c21B and YA RNAs can be differentiated. Fig. 4A shows an abundant 156-bp protected fragment corresponding to properly spliced P450c21B and YA RNAs can be differentiated. Fig. 4A shows an abundant 156-bp protected fragment corresponding to properly spliced P450c21B and YA RNAs can be differentiated. Fig. 4A shows an abundant 156-bp protected fragment corresponding to properly spliced P450c21B and YA RNAs can be differentiated. Fig. 4A shows an abundant 156-bp protected fragment corresponding to properly spliced P450c21B and YA RNAs can be differentiated. Fig. 4A shows an abundant 156-bp protected fragment corresponding to properly spliced P450c21B and YA RNAs can be differentiated. Fig. 4A shows an abundant 156-bp protected fragment corresponding to properly spliced P450c21B and YA RNAs can be differentiated. Fig. 4A shows an abundant 156-bp protected fragment corresponding to properly spliced P450c21B and YA RNAs can be differentiated. Fig. 4A shows an abundant 156-bp protected fragment corresponding to properly spliced P450c21B and YA RNAs can be differentiated. Fig. 4A shows an abundant 156-bp protected fragment corresponding to properly spliced P450c21B and YA RNAs can be differentiated. Fig. 4A shows an abundant 156-bp protected fragment corresponding to properly spliced P450c21B and YA RNAs can be differentiated. Fig. 4A shows an abundant 156-bp protected fragment corresponding to properly spliced P450c21B and YA RNas can be differentiated. Fig. 4A shows an abundant 156-bp protected fragment corresponding to properly spliced P450c21B and YA RNas can be differentiated. Fig. 4A shows an abundant 156-bp protected fragment corresponding to properly spliced P450c21B and YA RNas can be differentiated. Fig. 4A shows an abundant 156-bp protected fragment corresponding to properly spliced P450c21B and YA RNas can be differentiated. Fig. 4A shows an abundant 156-bp protected fragment corresponding to properly spliced P450c21B and YA RNas can be differentiated. Fig. 4A shows an abundant 156-bp protected fragment corresponding to properly spliced P450c21B and YA RNas can be differentiated.

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poly(A') RNA (20 ng) gave products of 550 and 450 bp, but only the larger band (corresponding to RNA retaining both introns) hybridized to a 21A genomic probe (Fig. 4C). Thus, clone YA-3 represents the majority of YA RNA, but the splicing of introns 1 and 2 of 21A is somewhat variable.

Adrenal-specific Expression of Y Genes—Because the YA and YB RNAs appear to arise from the 21A and 21B promoters, we expected that, like P450c21 mRNA, they would be expressed only in the adrenal cortex. To test this, we performed RNase protection assays with a 357-bp FokI fragment encompassing the 3'-end of YA-3 and crossing the duplication boundary. This probe distinguishes YA RNA (357-bp protected band) from YB RNA (97-bp protected band). Both fetal and adult adrenal RNAs protected readily detectable bands of 97 and 357 bp, but RNAs prepared from other tissues did not (Fig. 5). Longer exposures detected very low expression of YA in brain and liver (data not shown). The origins of the ubiquitous minor band at 210 bp and of the abundant adult-specific 220-bp band are unknown.

FIG. 3. RNase protection assay of Y transcripts. A 694-nucleotide cRNA probe was gel-purified and hybridized to 20 µg of total fetal adrenal RNA or tRNA. Only fetal adrenal RNA protected probe fragments. As diagrammed to the right, short YA mRNAs protected all 611 bases of the probe corresponding to the SacI insert. Short YB transcripts protected a probe fragment truncated at the limit of the duplication. Because the probe was derived from a short YA clone, long Y RNAs protected probe fragments truncated at the splice duplication boundary. This probe distinguishes YA RNA from P450c21B transcripts plus the 5'-portions of the probe hybridizing to long YA and YB RNAs. Markers indicated to the left are HpaII-cut pBluescript.

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flanking sequences of 21A and 21B are 98.6% identical, including nearly complete identity in regions that appear to be important for 21B gene transcription (31). Despite this great similarity in the 21A and 21B promoters, there are substantial differences in Y and P450c21 RNA abundances, suggesting that post-transcriptional mechanisms may influence the relative abundances of YA, YB, and P450c21 RNAs. This very high degree of nucleotide sequence identity between the duplicated A and B loci appears to represent an example of concerted evolution driven by frequent genetic exchange (9-11). However, if the YA gene product has a function, there might be additional selective pressure favoring such concerted evolution.

The similarity between the 21A and 21B promoter sequences made it difficult to map the precise cap sites of the YA and YB RNAs as the 5'-ends of YA, YB, and P450c21B RNAs are identical. Both the human (6, 7) and bovine (32) 21B genes have three cap sites; the site 10–11 bp from the ATG translational start codon initiates most P450c21B mRNA transcription, but we cannot tell if all three cap sites are used to initiate Y transcription or whether the minor cap sites might be the primary ones used for Y transcripts.

The YA RNAs include the first two introns of 21A. A portion of the second intron of 21B is included in a common mutant 21B allele containing a gene microconversion of nucleotide 656 from C to G as is found in the normal 21A gene (33). Nuclease protection experiments using a 21B intron 2 probe showed that this single base change prevented normal splicing of P450c21B mRNA in COS-7 cells (33). This probe also protected other bands when hybridized to normal adrenal RNA (33); these probably represent YA RNA. Our RNase protection and RT/PCR analysis of normal human adrenal RNA verified retention of intron 2 in YA transcripts (Fig. 4). Sequences corresponding to the first intron of 21B were also lost unspliced in YA (Fig. 4, B and C), even though this sequence is identical in the 21A and 21B genes. This intron might be spliced in a very small population of mRNAs undetectable by our RT/PCR assay.

The function of Y RNAs is not known. About 14% of wild-type human chromosomes 6 carry a deletion within the C4/21/X locus extending from the 5'-flanking DNA of C4A through the 21A and XA genes; similarly, ~15% of alleles causing severe 21-hydroxylase deficiency carry a deletion extending from the middle of 21A to the exactly corresponding point in the 21B gene, again deleting the XA gene (2, 3, 19). Persons homozygous for the former deletion are normal, suggesting that no functional genes reside in this DNA. However, an intact Y gene is preserved in both deletions: the YB gene in the former case and a hybrid YA/YB gene in the latter case. Among >800 alleles analyzed (2, 3), no deletion has been described that disables a Y gene without also disabling its corresponding X gene (for example, a short deletion at the 5'-end of 21A or 21B). These data suggest that preservation of overlapping pairs of X and Y genes may be important.

It is not presently known if the Y RNAs encode proteins. The longest YA open reading frame is initiated by a good ATG consensus sequence and encodes a 290-amino acid protein similar to residues 113–306 of P450c21. However, to use this ATG codon, the translational machinery would have to skip several other good ATG codons, including the “authentic” P450c21 translational start site. Even if the Y RNAs do not encode protein, they may still serve a function. The long forms of YA and YB are potentially able to form double-stranded hybrids with XA or XB RNA, which can also hybridize to P450c21 mRNA. The short form of YA overlaps the first 84 bases and the last 5 bases of XA. Because the first 500 bases of XA are not included in XB transcripts (22, 23), the short form of YA is complementary to XB only over its last few bases so that it is unlikely that XB/short YA hybrids are formed in vivo. Irrespective of function, the transcription of the 21A/YA locus may have important consequences. Transcription transiently unravels DNA, increasing its susceptibility to single-stranded breaks. Such breaks may be involved in the gene conversion events causing congenital virilizing adrenal hyperplasia.

There are several examples of overlapping genes in higher eukaryotes. In most cases, the functional consequences of such overlaps are unclear; however, three recent examples suggest possible functions. First, the overlapping bovine fibroblast growth factor transcript in Xenopus oocytes forms double-stranded hybrids with bovine fibroblast growth factor transcripts, allowing extensive post-transcriptional editing of the bovine fibroblast growth factor mRNA by an RNA unwinding activity (34). Second, an antisense transcript from the p53 gene is induced during maturation of cultured murine erythroleukemia cells and interferes with normal processing
and transport of p53 mRNA from the nucleus (35). Third, stability of the prespore gene EB4-PSV transcript of Dictyostelium is regulated by an antisense transcript; EB4 is constitutively expressed, but its mRNA only accumulates when the antisense transcript is absent (36). The sequences of our several YA, YB, XA, and XB cDNAs match those of the corresponding genomic DNA exactly, providing no evidence of RNA editing. It is intriguing to speculate that Y transcripts might hybridize to X mRNAs and prevent the formation of X/P450c21 hybrids that could impair P450c21 mRNA processing, translocation, or translation; thus, Y transcripts might “protect” P450c21 mRNA from untoward interactions with X transcripts. Resolution of the potential function of the Y transcripts must await identification of a human adrenal cell line that produces these mRNAs.

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