Assignment of the Functional Gene for Human Adrenodoxin to Chromosome 11q13→qter and of Adrenodoxin Pseudogenes to Chromosome 20cen→q13.1

Yves Morel,* James Picado-Leonard,* Du-An Wu,† Chi-Yao Chang,† T. K. Mohandas,‡ Bon-chu Chung,† and Walter L. Miller*

*Department of Pediatrics and the Metabolic Research Unit, University of California, San Francisco; †Institute of Molecular Biology, Academia Sinica, Nankang, Taipei; and ‡Department of Pediatrics and Division of Medical Genetics, Harbor/UCLA Medical Center, Torrance, CA

Summary

Adrenodoxin is a small iron/sulfur protein serving as an electron-transport intermediate for all mitochondrial forms of cytochrome P450. Southern blots of normal genomic DNA cleaved with six restriction endonucleases probed with full-length human adrenodoxin cDNA revealed complex patterns indicating the presence of multiple adrenodoxin genes. Southern blots of DNA from a panel of mouse/human somatic cell hybrids identified cross-hybridizing adrenodoxin DNA in two loci, chromosome 11q13→qter and chromosome 20cen→q13.1. Examination of adrenodoxin clones from a genomic DNA library in phage lambda revealed some clones bearing gene fragments interrupted by introns and other clones bearing processed pseudogenes. By probing the mouse/human hybrids with unique intronic DNA and by correlating restriction maps of the phage clones with that of uncloned genomic DNA, we show that the authentic transcribed adrenodoxin gene lies on chromosome 11, while pseudogenes lie on chromosome 20.

Introduction

The conversion of cholesterol to pregnenolone is the first and rate-limiting step in the synthesis of all steroid hormones. This complex reaction involves three steps, 20-hydroxylation, 22-hydroxylation, and cleavage of the cholesterol side-chain, all mediated on the single active site of a specific mitochondrial form of cytochrome P450 termed P450scc (for review see Miller and Levine 1987). Each of these three steps requires a separate pair of electrons, donated by NADPH and transferred to P450scc via two electron-transport intermediates: a flavoprotein termed adrenodoxin reductase followed by an iron-sulfur protein termed adrenodoxin. These two proteins also

serve to transport electrons from NADPH to P450c11, which mediates steroid 11-hydroxylase, 18-hydroxylase, and 18-methyl oxidase (aldehyde synthetase) activities (Yanagibashi et al. 1986). The microsomal steroidogenic P450 enzymes, P450c17 (17-hydroxylase/17,20 lyase) (Chung et al. 1987; Picado-Leonard and Miller 1987), P450c21 (21-hydroxylase) (Chung et al. 1986a; Higashi et al. 1986), and P450arom (aromatase) (Chen et al. 1988) employ a different electron-transport system, lacking an iron-sulfur protein.

P450scc is found in all human steroidogenic tissues: adrenal (DiBlasio et al. 1987), testis (Voutilainen and Miller 1988), ovary (Voutilainen et al. 1986), and placenta (Chung et al. 1986b; Voutilainen et al. 1986), and is encoded by a single gene lying on chromosome 15 (Chung et al. 1986b). The other steroidogenic enzymes are also expressed in a tissue-specific manner but are chromosomally unlinked: P450c17 lies on chromosome 10 (Matteson et al. 1986), P450c21 is on chromosome 6

(Higashi et al. 1987), and P450c11 is on chromosome 8 (Chua et al. 1987). By contrast, adrenodoxin is found in nonsteroidogenic as well as in steroidogenic tissues (Voutilainen et al. 1988). We have recently cloned several full-length cDNAs for human adrenodoxin (Picado-Leonard et al. 1988). Sequencing of these cDNAs and probing of RNA transfer blots indicates that there are several human adrenodoxin mRNAs, varying in size from 1.0 to 3.8 kb. All available cDNA sequencing and restriction-mapping data, however, indicate that these mRNAs arise from a single functional gene encoding a pre-mRNA with multiple poly(A) addition sites (Picado-Leonard et al. 1988).

We now report the probing of DNA from a panel of mouse/human somatic cell hybrids, showing that adrenodoxin genes or genelike sequences lie on human chromosomes 11 and 20. The hybridizing DNA is found on three major HindIII fragments of 12.5, 10, and 8.3 kb and on a faintly hybridizing band of 9 kb. The 12.5- and 8.3-kb fragments are assigned to chromosome 11, while the 10-kb fragment is on chromosome 20. PstI digestion produced bands of 16 and 3.2 kb lying on chromosome 11 and a band of 3.8 kb lying on chromosome 20. Analysis of the mouse/human somatic cell hybrids with a cloned portion of an intron from the functional gene showed that this gene is on chromosome 11. Analysis of the PstI digestion patterns from the cloned gene and from a pair of cloned processed pseudogenes confirmed that the functional gene(s) is on chromosome 11. The presence of highly repetitive DNA flanking the pseudogene clones precluded preparing unique probes from these genes. However, it is most likely that chromosome 20 contains the processed pseudogenes.

Material and Methods

Preparation and Blotting of DNA

DNA was isolated from human lymphocytes according to a method described by Y.M. and coworkers (Y. Morel, M. David, M. G. Forest, H. Betuel, G. Hauptman, J. Andre, J. Bertrand, and W. L. Miller, unpublished data) and from a panel of mouse/human somatic cell hybrids (Mohandas et al. 1986) according to a method described by Yen et al. (1984). DNA was similarly isolated from a panel of cell lines (see fig. 5) bearing deletions of portions of chromosome 11 or chromosome 20 (Mohandas et al. 1980,

1984). At least 30 G-banded metaphases from each of the mouse/human somatic cell lines were analyzed karyotypically at the time the cells were harvested for DNA extraction. DNA samples were digested with PstI (Amersham, IBI, or Boehringer-Mannheim) or HindIII (IBI) and displayed by electrophoresis through 0.8% or 1.0% agarose gel before transfer to nylon membranes (Amersham). Hybridization was done in 50% formamide, 5 × SSC, 1% NaDodSO$_4$, 1 × Denhardt's solution, 100 μg herring-sperm DNA/ml, 100 μg torula yeast RNA/ml, 50 mM Tris HCl (pH 7.5) at 42 C for 24 h. Washing was done twice in 2 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate) at room temperature to remove excess probe, then three times for 30 min in 0.1 × SSC, 1% NaDodSO$_4$ at 50 C.

Probes

DNA was isolated from the cloning vectors by endonuclease digestion and agarose-gel electrophoresis and labeled to 10^9 cpm/μg with ^{32}P[dCTP] (Amersham) by oligonucleotide-primed labeling (Feinberg and Vogelstein 1983). The 1,250-bp full-length human adrenodoxin cDNA clone λhadx-7 has been described elsewhere (Picado-Leonard et al. 1988). The isolation of specific probes for the various human adrenodoxin genes and pseudogenes from a human genomic DNA library (Lawn et al. 1978) is described in Results.

Results

Restriction Pattern in Normal Individuals

Genomic DNA prepared from several unrelated persons was digested with PstI, XbaI, KpnI, EcoRI, HindIII, and TaqI and analyzed by Southern blotting with the full-length human adrenodoxin cDNA from λhadx-7; typical data are shown in figure 1. A complex pattern of bands was seen with each enzyme used, suggesting the presence of multiple adrenodoxin genes or pseudogenes. We chose to analyze the panel of mouse/human somatic cell hybrids with PstI and HindIII. The reproducibility of the fragment sizes obtained with these enzymes is seen in DNA from four unrelated individuals, as shown in figure 2. PstI yields strongly hybridizing bands of 16 and 3.8 kb and weakly hybridizing bands of 3.2 and 2.4 kb. HindIII yields strongly hybridizing bands of 12.5, 10, and 8.3 kb and weakly hybridizing bands of 9 and 2 kb.
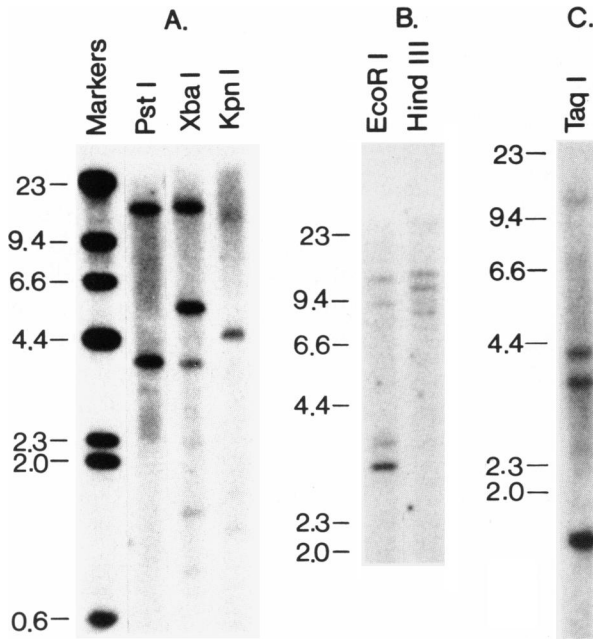
Figure 1 Southern blots of human genomic DNA digested with various restriction endonucleases and probed with hadx-7 cDNA. The lanes are designated by the restriction endonuclease used. The figure is a composite from three gels, each run with its own set of molecular-weight standards: Panels A and B show DNA from one person; panel C shows DNA from another person. However, all individuals examined showed the same TaqI pattern.
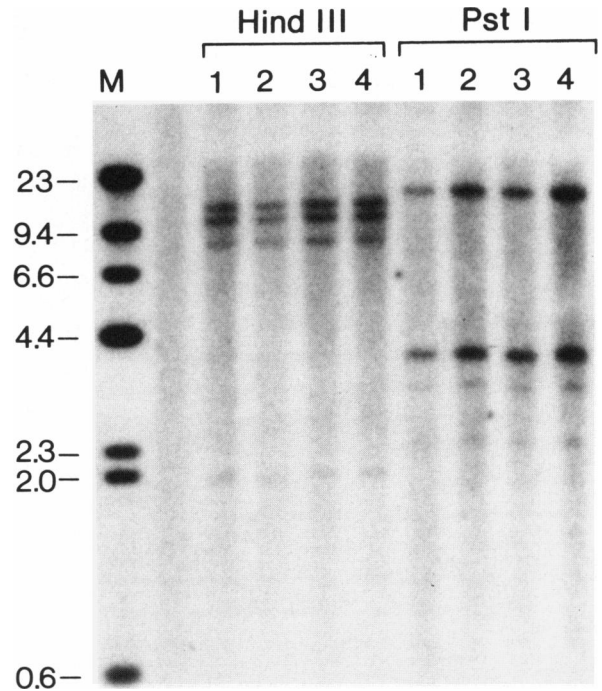
Figure 2 Southern blot of genomic DNA from four individuals cleaved with PstI or HindIII and probed with λhadx-7 cDNA. The lanes are designated with numbers referring to the four individuals. Lane M, molecular-weight standards (HindIII-cleaved bacteriophage lambda DNA).

Assignment of Adrenodoxin Sequences to Two Chromosomes

DNA from a panel of 17 mouse/human somatic cell hybrids was digested with PstI and analyzed by Southern blotting and hybridization to λhadx-7 cDNA (figs. 3A, 4). The two prominent bands of 16 and 3.8 kb seen in human genomic DNA were readily identified, but the fainter 3.2- and 2.4-kb bands could not be detected readily in the hybrid cell lines, presumably owing to dilution with the large excess of mouse DNA. Hybridization of the λhadx-7 cDNA to mouse genomic DNA under the conditions used to probe the hybrids failed to detect endogenous mouse adrenodoxin DNA fragments. Therefore, all the hy-
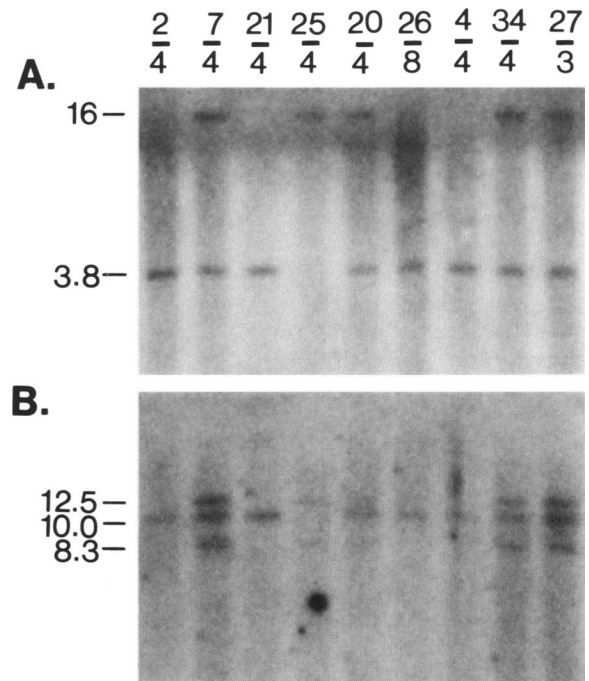
Figure 3 Southern blots of DNA from the mouse/human somatic cell hybrids. A, digestion with PstI probed with λhadx-7 cDNA; B, digestion with HindIII. The numbers above each lane identify the individual cell lines. All mouse/human cell lines in figs. 3 and 4, except line 116/4, are derived from the same human parent.
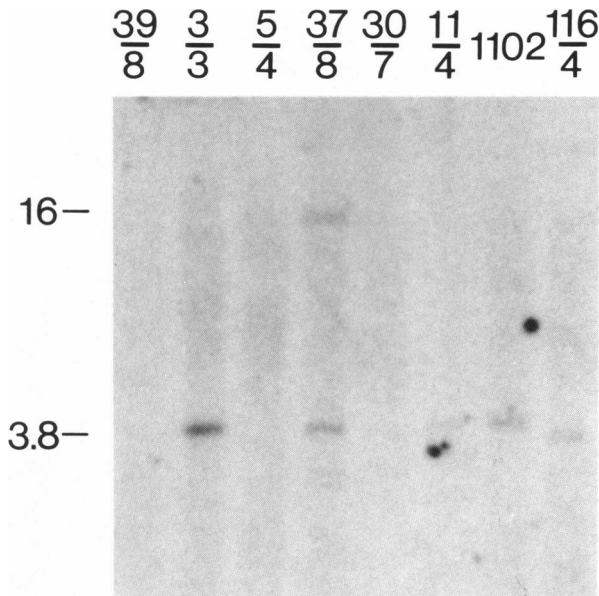
$$\frac{39}{8} \quad \frac{3}{3} \quad \frac{5}{4} \quad \frac{37}{8} \quad \frac{30}{7} \quad \frac{11}{4} \quad 1102 \quad \frac{116}{4}$$

16—

3.8—

Figure 4 Southern blots of DNA from additional somatic cell hybrid lines, not shown in fig. 3. All lanes show mouse/human cell lines, except for lane 11/4, which is a hybrid of Chinese-hamster and human cells, and lane 1102, which is a nonhybrid Chinese-hamster control.

bridizing DNA in the mouse/human hybrid cell lines is due to the human DNA. This is confirmed by finding the same bands in total human genomic DNA (figs. 1, 2) and in the mouse/human hybrids. Cell line 11/4 is a Chinese-hamster/human cell line (Mohandas et al. 1979); control line 1102 containing PstI-cut Chinese-hamster DNA alone shows a faint band at 3.9 kb, one indistinguishable from the human band at 3.8 kb (fig. 4). Therefore, the identity of the 3.8-kb Pst band in line 11/4 cannot be determined unambiguously. Some cell lines contained both the 16- and the 3.8-kb fragments, but others contained only one or the other of these. By comparison of this pattern with the karyotypic composition of each cell line, we assign the 16-kb PstI fragment to chromosome 11 and the 3.8-kb PstI fragment to chromosome 20 (table 1).

The hybridization and washing conditions used were highly stringent; hence, the adrenodoxin gene sequences lying on chromosomes 11 and 20 must be highly homologous. To confirm that adrenodoxin genomic sequences exist on two chromosomes, DNA from 9 of the 17 cell lines was digested with HindIII and probed with λhadx-7 cDNA. The 10-kb HindIII band cosegregates with the 3.8-kb PstI band (chromosome 20) while the 12.5- and 8.3-kb HindIII

bands cosegregate with the 16-kb PstI band (chromosome 11) (fig. 3B). Thus, the assignments to chromosomes 11 and 20 are confirmed independently with two restriction digests.

Chromosomal Subassignments

To localize these adrenodoxin genes further, DNA was prepared from a series of mouse/human hybrids bearing deletions of portions of chromosome 11 (Mohandas et al. 1980) or of chromosome 20 (Mohandas et al. 1984). This DNA was digested with PstI and probed with λhadx-7 cDNA (fig. 5). The 3.2-kb PstI fragment seen in total human DNA (figs. 1, 2) could not be detected in the hybrids in figure 3 but was easily detected in figure 4, permitting assignment of this band to chromosome 11. The 3.2- and 16-kb PstI bands are found in all cell lines containing chromosome 11, including line CF37 (fig. 1, lane 9). Karyotypic analysis shows that CF37 bears only a portion of chromosome 11, q13→qter; hence, this adrenodoxin genomic DNA is assigned to 11q13→qter.

Similarly, cell lines bearing the long arms of chromosome 20 retain the 3.8-kb PstI fragment, but those lacking the long arm of chromosome 20 do not (cell lines CF31-24 [fig. 1, lane 4] and CF31-1 [fig. 1, lane 5]). Cell lines CF89-3a (fig. 1, lane 7) and CF80-8 (fig. 1, lane 8) bear a portion of chromosome 20, extending from q13.1 to qter. These cell lines lack the 3.8-kb PstI band. Therefore, this adrenodoxin genomic DNA is assigned to 20cen→q13.1.

Assignment of Adrenodoxin Genes and Pseudogenes

The HindIII bands assigned to chromosome 11 totaled ~21 kb, while the bands assigned to chromosome 20 totaled only 10 kb; but the 9-kb and 2-kb bands were too faint to be seen on the gels of the hybrid cell DNAs. Similarly, the PstI bands assigned to chromosome 11 totaled >19 kb, those to chromosome 20 only 3.8 kb, and the 2.4-kb band was too faint to be seen. Thus, the adrenodoxin sequences on chromosome 11 appear to be spread over a much larger distance than do those on chromosome 20. As there are no HindIII sites in the cDNA (Picado-Leonard et al. 1988) and as HindIII appears to cut the gene on chromosome 11 but not the gene(s) on chromosome 20, we hypothesized that the "authentic" gene lies on chromosome 11 and that the hybridizing DNA on chromosome 20 represents a pseudogene (Morei et al. 1987).

To investigate this hypothesis further, we probed a

Table I

Correlation of Human Chromosomes in Hybrid Cell Lines and Hybridization Patterns

	CELL LINE																	DISCORDANT No. of PstI Bands	
	2	7	21	25	20	26	4	34	27	39	3	5	37	30	116	11	35		
	4	4	4	4	4	8	4	5	3	8	3	4	8	7	4	4	4	16 kb	3.8 kb
Chromosome:																			
1	+	−	−	−	−	+	+	−	−	−	+	−	−	−	+	−	−	11	6
2	+	−	−	−	+	−	+	−	+	−	+	+	−	−	+	−	−	9	6
3	−	+	−	−	+	+	+	−	−	+	+	+	−	+	+	−	+	12	9
4	+	+	−	+	+	+	+	+	−	−	+	−	+	+	+	−	+	8	5
5	(+)	−	−	+	(+)	+	−	−	+	−	−	+	+	(+)	+	−	−	7	8
6	+	+	−	+	−	+	+	+	+	−	+	(+)	+	+	+	−	+	9	6
7	+	(−)	+	−	+	+	+	+	−	+	+	−	+	−	−	−	+	10	5
8	+	(+)	−	+	+	+	+	+	+	+	+	+	(+)	−	+	−	+	8	5
9	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	7	12
10	−	−	+	+	−	(+)	+	+	−	−	+	−	−	+	−	−	−	9	8
11	(−)	+	−	+	+	−	−	+	+	−	−	−	+	−	−	−	−	0	7
12	+	(+)	+	+	+	+	−	−	+	−	−	−	+	(+)	+	−	+	7	6
13	−	−	−	−	+	+	+	−	−	−	+	(+)	−	+	−	−	−	10	9
14	+	+	+	−	+	+	+	+	−	−	+	+	+	+	−	−	+	10	5
15	+	−	−	−	(−)	+	+	−	+	+	+	+	−	−	+	−	+	13	8
16	+	−	−	−	+	(+)	−	−	−	−	−	−	−	−	+	−	−	8	7
17	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	9	6
18	+	−	−	−	+	+	+	−	+	−	+	−	−	+	+	−	+	11	6
19	+	−	+	(+)	−	+	+	(+)	−	−	+	+	−	−	+	−	−	11	6
20	+	+	+	−	+	(+)	+	+	+	−	+	−	+	(−)	+	−	−	7	0
21	−	−	+	+	+	+	−	−	+	−	−	+	+	+	+	−	−	7	8
22	+	−	−	−	+	+	−	(+)	−	−	−	+	+	+	+	−	−	8	7
X	−	−	−	−	+	−	−	−	−	−	−	+	−	−	+	+	−	8	11
Y	−	−	(+)	−	−	−	−	−	−	−	−	+	−	−	−	−	−	9	10
PstI bands:																			
16 kb	−	+	−	+	+	−	−	+	+	−	−	−	+	−	−	−	−		
38 kb	+	+	+	−	+	+	+	+	+	−	+	−	+	−	+	−	−		

NOTE.—Correlations of the human chromosomes present in each hybrid cell line with the hybridization patterns seen in the Southern blots (figs. 3, 4 and other data). The names of the cell lines are shown across the top, and the human chromosomes are indicated in the left-hand column. + = Presence of a human chromosome in >30% of analyzed cells; (+) = 10%–20%; (−) = 5%–9%; − = not detected. The two columns on the right designate the number of cell lines discordant for each chromosome. Note that the 16-kb PstI band shows no discordances for chromosome 11 and that the 3.8-kb PstI band shows no discordances for chromosome 20. The blots of DNA from cell line 35/4 were run separately and are not shown; cell line 35/4 has the same human parent as all other cell lines except 116/4.

human genomic DNA library with λhadx-7 and isolated four unique nonoverlapping phage clones. One of the clones, λhadxG-1, contains ~14 kb of DNA, including much highly repetitive DNA. Genomic fragments corresponding to various portions of the cDNA were subcloned and sequenced, showing 100% identity with the cDNA sequence and standard "canonical" intron/exon splice junctions (C.-Y. Chang and B. Chung, unpublished data). Thus, this phage appears to bear part of an authentic gene encoding adrenodoxin mRNA. HindIII mapping of this phage shows an 8.2-kb fragment containing a small 130-bp exon; this probably corresponds to the 8.3-kb fragment seen in total genomic DNA (figs. 1, 2). The other exon in this phage clone lies on a HindIII fragment interrupted by an EcoRI cloning site; hence, its size cannot be correlated with the genomic DNA fragments.

Fragments of genomic phage λhadxG-1 that did not hybridize to λhadx-7 but that lie between other hybridizing regions were identified as introns. Various intronic subclones were used to probe human genomic DNA, and one, a 500-bp AluI fragment from a 1.0-kb SmaI/EcoRI clone, hybridized in a pattern indicating that it lacked repetitive sequences. When hybridized to HindIII-cut DNA, this probe,
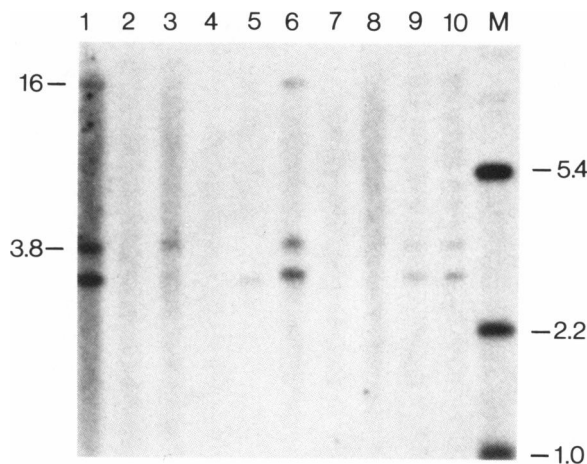
Figure 5   Southern blots of *Pst*I-cut DNA from cell lines bearing partial deletions of chromosome 11 and chromosome 20. Lanes 1 and 10, control human genomic DNA; lanes 2–9, DNA from mouse/human hybrids bearing the following chromosomes or chromosomal fragments:

Lane	2	3	4	5	6	7	8	9
ch 11	−	−	−	+	+	−	−	q13→ter
ch 20	−	+	p	p	q	q13.1→qter	q13.1→qter	+
Cell line	$\frac{39}{8}$	$\frac{2}{4}$	31-24	31-1	21-2-11	89-9a	80-8	37-6/12

termed 0.5 Alu, identified a 4.4-kb fragment. Since no 4.4-kb *Hin*dIII fragment was seen when cDNA was used as probe, the 0.5 Alu probe was identifying a 4.4-kb *Hin*dIII fragment lying entirely within a large intron. When 0.5 Alu was used to study DNA from the mouse/human somatic cell hybrids, the 4.4-kb *Hin*dIII band was assigned to chromosome 11 (not shown). Thus, chromosome 11 bears a large adrenodoxin gene transcribed into adrenodoxin mRNA. The assignment of the gene bearing this intron to chromosome 11 is confirmed by hybridization of 0.5 Alu to the 16-kb *Pst*I band found in human genomic DNA (reprobing of fig. 2, not shown) and in the mouse/human hybrid DNAs (reprobing of fig. 3*A*, not shown). Another phage clone, λhadxG-4, contains the same array of introns and exons found in λhadxG-1. The sequences of exons corresponding to adrenodoxin protein are identical in these two genomic clones, but their 3′-untranslated regions differ somewhat. This may be due to allelic variation or could represent two tandemly arranged adrenodoxin genes on chromosome 11.

Two other nonoverlapping phage clones, λhadxG-2 and λhadxG-3, contained processed pseudogenes. These phage have significantly different restriction maps, and the pseudogenes bore numerous sequence differences, with each other and with the cDNA.

Thus, these two phage clones represent two different pseudogenes and not alleles. Phage λhadxG-2 contains a 9-kb *Hin*dIII fragment and a 2.4-kb *Pst*I fragment, apparently corresponding to the 9-kb *Hin*dIII and 2.4-kb *Pst*I bands seen in the Southern blots in figures 1 and 2. The chromosomal assignment of these pseudogenes could not be determined directly, as the DNA flanking both pseudogenes was repetitive and hence could not be used as a probe. However, since the mouse/human somatic cell hybrid data clearly indicate that there are sequences hybridizing to adrenodoxin cDNA on chromosome 20, we believe that it is most likely that these correspond to the pseudogenes in λhadxG-2 and λhadxG-3.

Discussion

Hybridization of human adrenodoxin cDNA to Northern blots of RNA prepared from a variety of human tissues shows that adrenodoxin mRNA or a very similar mRNA is widely distributed (Voutilainen et al. 1988). Thus, adrenodoxin mRNA is found in tissues lacking mRNA for P450scc or other steroidogenic enzymes. This finding suggests that adrenodoxin serves as an electron-transport intermediate for other, nonsteroidogenic mitochondrial cytochromes P450 (hence, the historical name "ad-

renodoxin" is unnecessarily parochial). The wide distribution of this adrenodoxin-like mRNA could be due to transcription of other gene(s) yielding adrenodoxin-like mRNAs having the same sizes and cross-hybridizing under highly stringent conditions. Finding a single adrenodoxin gene (as there is a single P450scc gene) would rule out the latter possibility. However, the discovery of multiple adrenodoxin genes or genelike sequences is compatible with either possibility. Extensive search of three human genomic DNA libraries indicates the presence of one or two human adrenodoxin genes having introns (C.-Y. Chang and B. Chung, unpublished data) and the presence of two processed genes lacking introns. Since these processed genes contain many nucleotide differences from the three cDNAs sequenced (Picado-Leonard et al. 1988) and from the "authentic" gene on chromosome 11, it appears likely that the processed, intronless genes are nonfunctional pseudogenes. Duplicated genes bearing considerable homology have been found on different chromosomes. These include human genes for opal suppressor phosphoserine tRNA (McBride et al. 1987), ornithine aminotransferase (Ramesh et al. 1987), and trifunctional folate-dependent enzyme (Rozen et al. 1987).

It is possible that chromosome 11 might bear two or more repeated adrenodoxin genes, presumably maintaining very close nucleotide sequence homology through "concerted evolution" (Hood et al. 1975; Liebhaber et al. 1981; Miller and Eberhardt 1983). The PstI and HindIII hybridization patterns show that adrenodoxin gene components are spread over ≥20 kb of chromosome 11, certainly enough DNA to accommodate more than one adrenodoxin gene. Examination of genomic clones with the human adrenodoxin cDNA probe indicates that it is most likely that chromosome 11 contains a single gene whose exons are widely spread by very large introns. Thus, all present data indicate that a unique functional adrenodoxin gene on chromosome 11q13→qter is widely expressed in human tissues, while the pseudogene(s) on chromosome 20cen→q13.1 are unexpressed.

Acknowledgments

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