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Immunology

Mapping of steroid 21-hydroxylase genes adjacent to complement component *C4* genes in *HLA*, the major histocompatibility complex in man

(congenital adrenal hyperplasia/cytochrome P-450)

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ABSTRACT **The genes for four components (C) of complement in the human major histocompatibility complex (*HLA*) have been aligned previously in a series of overlapping cosmid cloned inserts. Those inserts, which contained the two *C4* genes *C4A* and *C4B*, hybridized with human adrenal mRNA, indicating that they contain a gene expressed in the adrenal. The mRNA fraction of 2.4 kilobases (kb) hybridizes with genomic DNA of 4.5 kb, which is duplicated and lies about 1.5 kb 3′ of both the *C4A* and the *C4B* complement genes. Sequencing of a 430-base section and comparison with the published cDNA sequence of bovine cytochrome P-450 21-hydroxylase, peptide sequences of porcine 21-hydroxylase, and a cDNA sequence of a rat liver cytochrome P-450 identified the gene as coding for human steroid 21-hydroxylase [steroid,hydrogen-donor:oxygen oxidoreductase (21-hydroxylating), EC 1.14.99.10]. Mapping of the gene was helped by use of a synthetic oligonucleotide based on the bovine cDNA sequence.**

The genes coding for the complement components C2, factor B, and two classes of C4 (C4A and C4B) lie between *HLA-B* and *HLA-D* in the major histocompatibility complex (*HLA*) in man (1). These genes have been mapped relative to each other, showing that those for C2 and factor B lie less than 1 kilobase (kb) apart and are separated from the *C4A* gene by about 30 kb (2). The *C4B* gene is about 10 kb from the *C4A* gene and, on a chromosome from one individual, a second *C4B* gene was found 10 kb further away (3).

The most common form of congenital adrenal hyperplasia is due to a defect of steroidogenesis in the adrenal in which the enzyme steroid 21-hydroxylase [steroid,hydrogen-donor:oxygen oxidoreductase (21-hydroxylating), EC 1.14.99.10] is wholly or partly missing or inactive (4). This inherited defect is closely linked to the *HLA* complex (5). The 21-hydroxylase enzyme is a cytochrome P-450, and a cDNA clone coding for it has been obtained from bovine adrenal mRNA (6). The derived amino acid sequence showed strong homology with cystine-containing peptide sequences of porcine 21-hydroxylase (7), and there was limited homology between the cDNA sequence of the bovine 21-hydroxylase and of a rat liver cytochrome P-450 (8).

To extend the characterization of the section of the *HLA* complex containing the complement genes, we tested the ability of cosmids (*cos*-containing plasmids) and their subcloned fragments to hybridize with human adrenal mRNA. This identified the position of the 21-hydroxylase genes in *HLA*, and use of a synthetic oligonucleotide based on the known cDNA and amino acid sequences helped to define the position of the genes.

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MATERIALS AND METHODS

Preparation and Analysis of Genomic DNA. Genomic DNA was prepared from whole blood by the procedure of Bell *et al.* (9). For restriction enzyme analysis of DNA, 8–10 μg of genomic DNA was digested with one or two restriction endonucleases (5 units/μg of genomic DNA) according to the manufacturer's instructions (New England Biolabs). DNA fragments were separated by electrophoresis in 0.7% agarose gels and then treated as described by Wahl *et al.* (10) before transfer to nitrocellulose by the procedure of Southern (11). After baking for 2 hr at 80°C, the filters were prewashed, prehybridized, and hybridized as described for RNA blots. Hybridized filters were washed as described for RNA filters and then autoradiographed at −70°C.

Preparation and Analysis of Cloned DNA. Cosmid and plasmid DNA were extracted from bacterial colonies by using the alkaline $NaDodSO_4$ method of Birnboim and Doly (12) and were characterized by restriction endonuclease analysis as described above for genomic DNA. DNA fragments required for further restriction analysis or use as probes were subcloned by blunt-end ligation with the cloning vector pAT153/Pvu II/8 (13), which had been digested previously with *Pvu* II and treated with alkaline phosphatase (14). Colonies containing recombinant plasmids were identified by colony hybridization (14). DNA fragments further characterized by nucleotide sequencing were end-labeled with [α-^{32}P]dATP by using the Klenow fragment of DNA polymerase I and were purified by polyacrylamide gel electrophoresis. The nucleotide sequence of purified and single-end-labeled fragments was determined on both strands by the procedure of Maxam and Gilbert (15).

RNA Blots. RNA was extracted from human postmortem adrenal tissue, collected fresh, and stored in liquid nitrogen, by the 4 M guanidine thiocyanate procedure (16).

For RNA blots, ≈10 μg of RNA was fractionated by electrophoresis in 1% agarose gels containing 2.2 M formaldehyde in 1× running buffer [0.2 M 3-(*N*-morpholino)propanesulfonic acid, pH 7.0/50 mM sodium acetate/5 mM EDTA] after first being denatured for 15 min at 55°C in 2.2 M formaldehyde/50% formamide (vol/vol) (14). Prior to overnight transfer of RNA to nitrocellulose, the agarose gel was treated stepwise at room temperature in deionized water for 5 min, 50 mM NaOH/10 mM NaCl for 45 min at room temperature, 0.1 M Tris·HCl (pH 7.5) for 45 min, and 3 M NaCl/0.3 M Na citrate for 1 hr (14). After the nitrocellulose filter was baked at 80°C for 3 hr, it was prewashed in 1 M NaCl/50 mM Tris·HCl, pH 7.5/5 mM EGTA/0.1% $NaDodSO_4$ and then prehybridized at 42°C in 50% formamide solution (14) for at least 2 hr before hybridization at 42°C for

Abbreviations: HLA, human leukocyte antigen; C2, C4, and factor B, components of complement; bp, base pair; kb, kilobase pair.

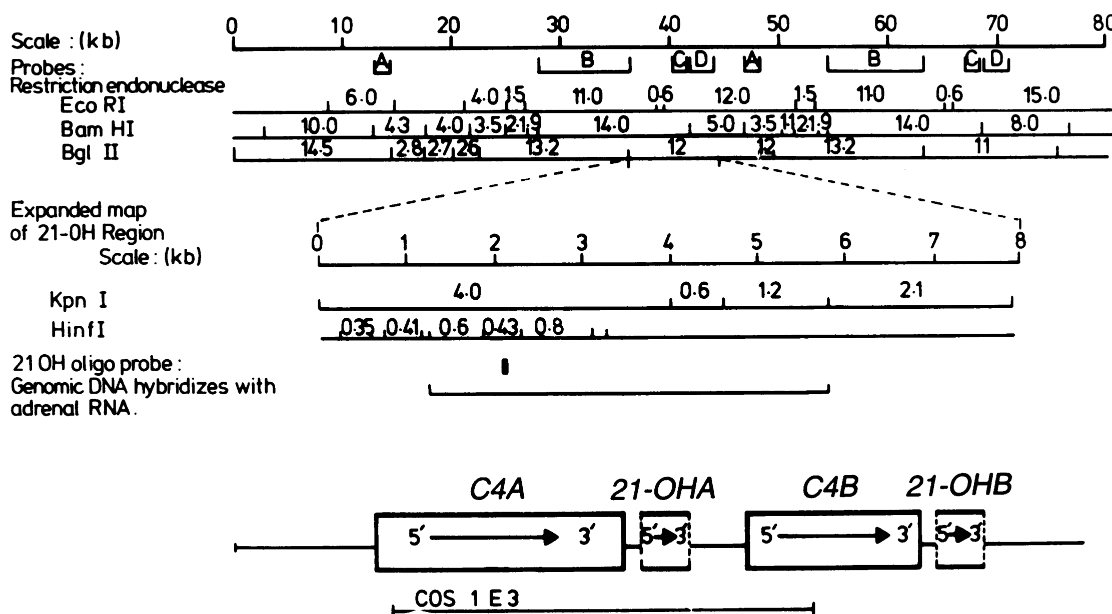

FIG. 1. Molecular map of *HLA* region containing genes for C4A, 21-hydroxylase A (*21-OHA*), C4B, and 21-hydroxylase B (*21-OHB*). The map represents an extension of published maps (2, 3) of approximately 15 kb in the 3′ flanking region of the *C4B* gene. This region was mapped by Southern analysis of uncloned genomic DNA probed with C4 cDNA or 1.2- (probe C) and 2.1- (probe D) kb *Kpn* I genomic fragments from cosmid 1E3. The approximate limits of the 21-hydroxylase gene, underlined in the expanded map, were determined by probing blots of human adrenal RNA with labeled *Hin*fI fragments prepared from a subclone of the 4-kb *Kpn* I fragment of cosmid 1E3 and with probes C and D. The *Hin*fI sites were mapped only in the 4.0-kb *Kpn* I fragment. The 25-base synthetic oligonucleotide prepared against the known bovine 21-hydroxylase cDNA sequence was shown to hybridize to the 430-bp *Hin*fI fragment that was isolated, and the nucleic acid sequence was determined (see Fig. 4). Cosmid 1E3 has been described (2, 3). Probes: A, 0.5-kb *Bam*HI/*Kpn* I fragment from the full-length C4A cDNA insert of pAT-A (21); B, 2.4-kb *Bam*HI fragment from the 3′ end of C4B cDNA insert of pAT-F (21); C, 1.8-kb *Kpn* I genomic fragment from cosmid 1E3, which includes both the 1.2- and 0.6-kb *Kpn* I fragments; D, 2.1-kb *Kpn* I genomic fragment from cosmid 1E3.

16–18 hr with nick-translated (Amersham) (17), heat-denatured DNA probes with a specific activity of 10^8 cpm·μg^{-1} (0.5 $\mu Ci \cdot ml^{-1}$) (1 Ci = 37 GBq). After hybridization, filters were washed at room temperature four times for 15 min each in 0.3 M NaCl/0.03 M Na citrate, followed by four washes at 68°C for 15 min each in 0.15 M NaCl/0.015 M Na citrate, and then were air-dried before autoradiography at −70°C.

When the 25-nucleotide-long synthetic oligonucleotide (Celltech, London) was used as a probe, the filters were prehybridized (for at least 2 hr at 42°C in the same solution as that described above except that the 50% formamide step was omitted) before hybridization for 16 hr at 46°C with 4 × 10^6 cpm·$pmol^{-1}$ (2.5 $\mu Ci \cdot ml^{-1}$) of polynucleotide kinase (Boehringer Mannheim) labeled 5′-[^{32}P]oligonucleotide probe (18). After hybridization, filters were washed in 1 M NaCl/0.1 M Na citrate/0.1% $NaDodSO_4$ at room temperature four times for 15 min each and then twice in the same solution at 46°C for 15 min each.

RESULTS

Molecular Map of Human *C4* Region. Fig. 1 illustrates a molecular map of the *C4* region of *HLA* on chromosome 6 in man, based on the ordering of overlapping cloned genomic fragments (20) and Southern analysis of uncloned genomic DNA (2, 3). The map was extended about 15 kb in the 3′ flanking region of *C4B* by single and double digests of uncloned genomic DNA with several restriction enzymes and was probed with *Kpn* I fragments of 1.2 kb (probe C) and 2.1 kb (probe D) from cosmid 1E3.

In an attempt to identify additional coding sequences within this region, fragments of cloned cosmid DNA were excised from agarose gels and labeled by nick-translation for use as probes on human adrenal and liver RNA fractionated on formaldehyde denaturing gels and transferred to nitrocellulose.

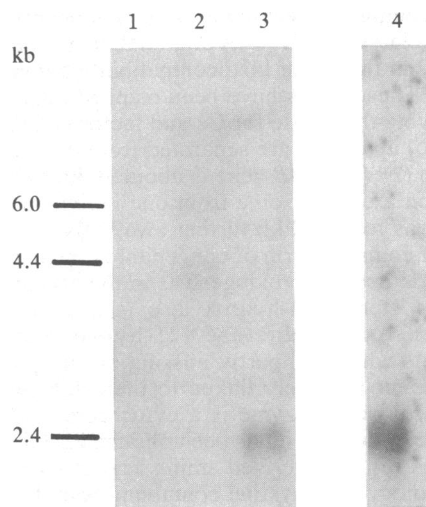

FIG. 2. Blot of human liver and adrenal RNA showing that a genomic fragment from a *C4*-containing cosmid and a bovine 21-hydroxylase-specific synthetic oligonucleotide hybridize to a common fraction of adrenal RNA. Approximately 10-μg samples of RNA were denatured and fractionated in 1% agarose gel containing 2.2 M formaldehyde and then transferred to nitrocellulose for hybridization as described. Lanes: 1, 18S fraction of human liver RNA; 2, 28S fraction of human liver RNA; 3 and 4, total human adrenal RNA. Probe C hybridized to a 2.4-kb fraction and faintly to a 4.4-kb fraction of human adrenal RNA (lane 3). However, only faint bands at 2.4 (lane 1), 4.4, and 6.0 (lane 2) kb were seen when probe C was hybridized to the size-enriched fractions of human liver RNA. The 21-hydroxylase-specific synthetic oligonucleotide hybridized to the common 2.4-kb fraction of adrenal RNA but not to the 4.4-kb fraction (lane 4). Fraction sizes were determined from size markers included in the denaturing agarose gel.

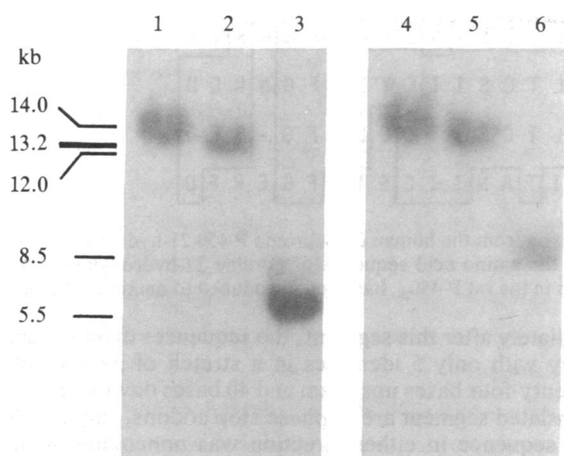

FIG. 3. Southern analysis of cosmid 1E3 hybridized with a bovine 21-hydroxylase-specific synthetic oligonucleotide shows that the gene for 21-hydroxylase is adjacent to *C4A*. Approximately 1-μg samples of cosmid 1E3 were digested to completion with the indicated restriction endonuclease, separated in 0.7% agarose gel, and treated for Southern blotting as described. The restriction endonucleases used were as follows: *Bam*HI (lanes 1 and 4), *Bgl* II (lanes 2 and 5), and *Bam*HI/*Bgl* II (lanes 3 and 6). Both the 3′ C4 cDNA probe and the 21-hydroxylase oligonucleotide hybridized to the 14-kb *Bam*HI fragment (lanes 1 and 4), showing that the fragment overlaps both genes. However, after subsequent digestion with *Bgl* II, probe B hybridized to the 8.5-kb fragment (lane 6), and the oligonucleotide probe hybridized to the 5.5-kb fragment, localizing the 21-hydroxylase coding sequence 3′ to *C4A*. The 13.2- and 12-kb *Bgl* II fragments hybridizing with probe B (lane 5) and 21-hydroxylase oligonucleotide (lane 2), respectively, were not separated clearly. Fragment sizes were estimated by including standards in the gel.

Adrenal RNA was chosen because it represents the site of synthesis of 21-hydroxylase, a cytochrome P-450, which had been mapped by inherited deficiency near *C4* in the *HLA* class III region (5). The 1.8-kb *Kpn* I fragment from cosmid 1E3 was found to hybridize strongly to a fraction of adrenal RNA of ≈2.4 kb and faintly to a similar-size fraction of 18S human liver RNA (Fig. 2). A second fraction of ≈4.4 kb was seen to hybridize weakly and with similar intensity in both liver and adrenal RNA. The 1.8-kb *Kpn* I fragment contains both the 1.2- and 0.6-kb *Kpn* I fragments (Fig. 1).

In order to resolve the approximate limits of the gene coding for the 2.4-kb fraction of adrenal RNA, the 4-kb *Kpn* I genomic fragment was subcloned and further fragmented by digestion with *Hin*fI. After the *Hin*fI sites were ordered by restriction mapping, the 350-, 410-, 600-, and 800-bp fragments were end-labeled with [α-^{32}P]dATP by filling in with the Klenow fragment of DNA polymerase I and were used as probes on human adrenal RNA as described above. Whereas the 350-bp band did not appear to hybridize, the 410-bp fragment hybridized to the 4.4-kb fraction, and the 600- and 800-bp fragments hybridized strongly to the 2.4-kb fraction and only faintly to the 4.4-kb fraction (data not shown). This result, combined with the observation that the 1.8-kb fragment but not 2.1-kb *Kpn* I fragments hybridized to the 2.4-kb fraction of adrenal RNA localized the gene within a 4.5-kb region 3′ to *C4A*.

The identity of the gene was further defined by preparation of a synthetic oligonucleotide complementary to a 25-nucleotide region of the published bovine 21-hydroxylase cDNA sequence showing homology with the rat liver cytochrome P-450_{pb} cDNA nucleotide sequence (ref. 6; rat liver cytochrome P-450_{pb} is induced by phenobarbital and specific for steroid 21-hydroxylation). By using relatively mild hybridization conditions, the oligonucleotide specific for bovine 21-hydroxylase was shown to hybridize to the 2.4-kb fraction of human adrenal RNA (Fig. 2). The 4.4-kb band did not hybridize, suggesting that it did not contain 21-hydroxylase coding sequence.

Hybridization of the oligonucleotide to a Southern blot of the cosmid 1E3 double-digested with *Bam*HI and *Bgl* II showed that the oligonucleotide sequence mapped to the

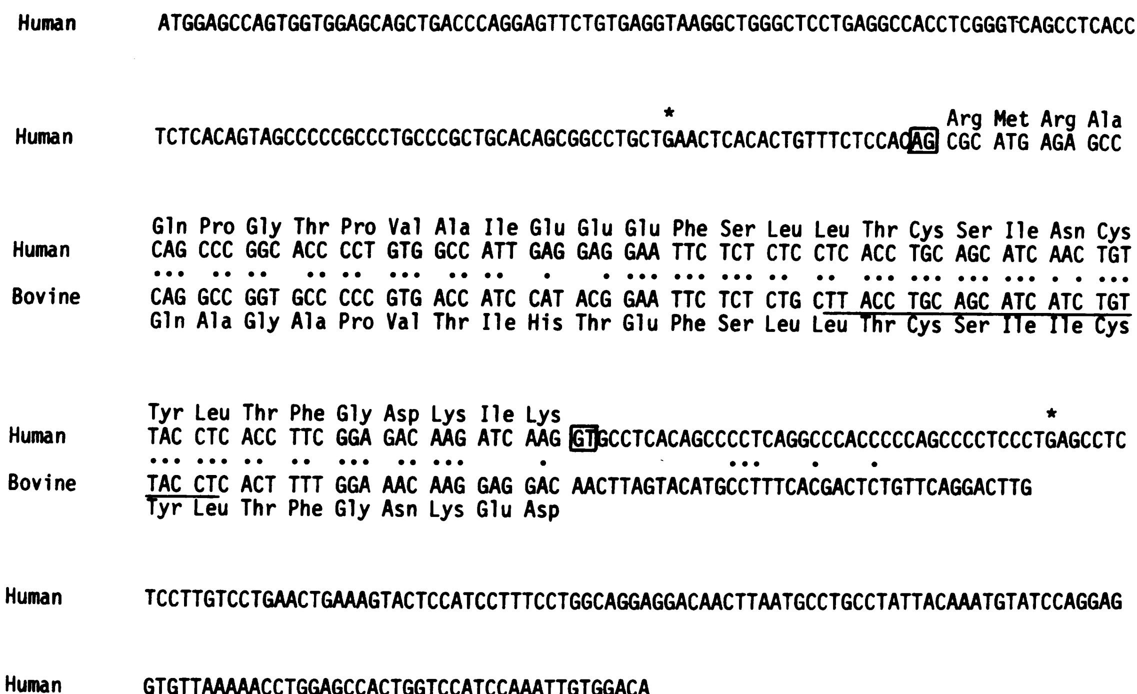

FIG. 4. Base sequence of the *Hin*fI fragment (Fig. 1) showing the postulated exon of the human 21-hydroxylase gene with the derived amino acid sequence. The spliced sites are boxed. *, In-phase stop codons. The relevant cDNA sequence of the bovine enzyme and its derived amino acid sequence are shown below the human sequence. Dots above nucleotides indicate identical bases in the two DNA sequences. The sequence of the synthetic oligonucleotide used to map the human gene is underlined.

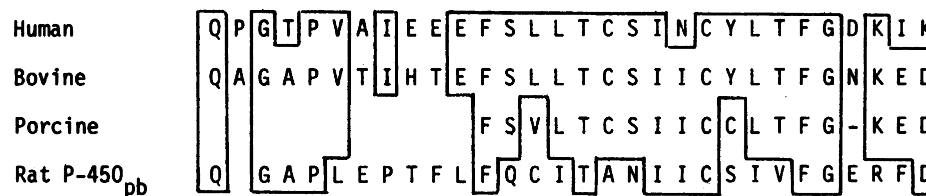

FIG. 5. Comparison of the derived amino acid sequence of the postulated exon from the human cytochrome P-450 21-hydroxylase with the derived sequences of bovine P-450 21-hydroxylase and rat liver P-450_{pb} and the amino acid sequence of porcine 21-hydroxylase. Identical residues are boxed. —, Unidentified residue (in the porcine sequence). The gap in the rat P-450_{pb} has been introduced to maximize homology.

same 4.5-kb region as did probe C. As shown in Fig. 3, the 14-kb *Bam*HI fragment hybridized with both probes B and C; however, after double digestion with *Bam*HI and *Bgl* II, probe B hybridized to the 8.5-kb fragment in the *C4A* gene, whereas probe C hybridized to the 5.5-kb fragment containing the 21-hydroxylase gene. This showed that the 21-hydroxylase-specific oligonucleotide hybridized 3′ of the *C4A* gene on cosmid 1E3.

Comparison of the Human and Bovine 21-Hydroxylase Nucleotide Sequence. The precise location of the oligonucleotide sequence within the 3′ flanking region of the *C4A* gene was determined by hybridizing it to a Southern blot of the subcloned 4-kb *Kpn* I genomic fragment digested with *Hin*fI as described above. The 430-bp *Hin*fI fragment was found to hybridize to the synthetic oligonucleotide and was isolated for nucleotide sequence determination (Fig. 4). The sequence was translated and compared to the sequence of bovine 21-hydroxylase (6). A segment of 90 bases was shown to have 77% homology with the bovine cDNA sequence. Immediately after this segment, the sequences diverge substantially with only 5 identities in a stretch of 39 nucleotides. Twenty-four bases upstream and 40 bases downstream of the translated segment are in-phase stop codons, suggesting that the sequence in either direction was noncoding or intron. The tentative location of the intron/exon boundaries was determined by comparison of the sequence with the consensus sequences of splice junctions shown by Mount (19). The dinucleotides A-G and G-T at the 3′ and 5′ ends of introns, respectively, are boxed in Fig. 4. Thus, the postulated exon of the human 21-hydroxylase gene codes for 34 amino acids. No other sequences homologous to the bovine sequence were found in the 430-bp *Hin*fI fragment.

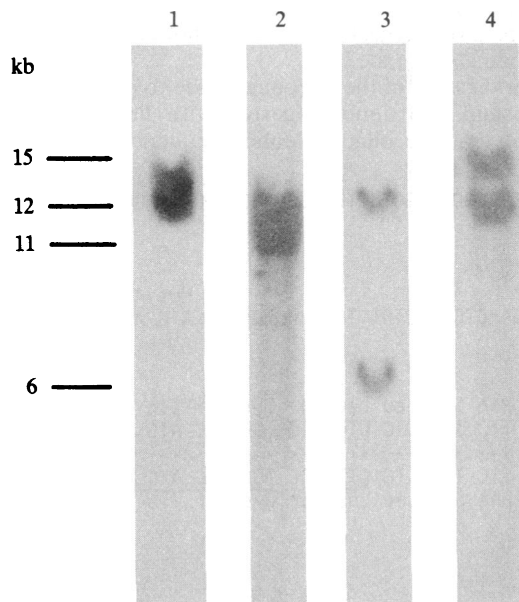

FIG. 6. Southern analysis of uncloned genomic DNA showing that there are two 21-hydroxylase genes. Approximately 10-μg samples of human genomic DNA were digested to completion with the indicated restriction endonuclease, separated in a 0.7% agarose gel, and treated for Southern blotting as described. The restriction endonucleases used were *Bgl* II (lanes 1 and 2) and *Eco*RI (lanes 3 and 4). Probe A hybridizes to 14.5- and 12.0-kb *Bgl* II fragments (lane 1) and to 6- and 12-kb *Eco*RI fragments (lane 3), representing 5′ ends of *C4A* and *C4B*, respectively. The 21-hydroxylase-specific genomic probe C hybridizes to the 12- and 15-kb *Eco*RI fragments which contain the 21-hydroxylase A and B genes, respectively. The 12-kb *Bgl* II and 12-kb *Eco*RI fragments seen with probes A and C show that the fragments overlap the 5′ ends of *C4B* and the 21-hydroxylase A gene. The sizes of the fragments were estimated from standards included in the agarose gel.

Further evidence that the human sequence represents the gene for 21-hydroxylase and not another cytochrome P-450 came from comparison of the derived amino acid sequences (human and bovine 21-hydroxylase and rat liver cytochrome P-450_{pb}) and a peptide sequence of porcine 21-hydroxylase (Fig. 5). The human amino acid sequence was 70% homologous to the bovine enzyme but only 30% homologous to the rat liver enzyme.

Determination of the coding sequence also showed that the orientation of transcription of the human 21-hydroxylase gene was the same as for the *C4A* and *C4B* genes.

Duplication of Human 21-Hydroxylase Genes. Evidence for duplication of the 21-hydroxylase genes 3′ to *C4A* and *C4B* was shown by Southern analysis of uncloned genomic DNA digested with restriction enzymes *Bgl* II or *Eco*RI and hybridized with either 5′ C4 cDNA (probe A) or 21-hydroxylase (probe C). The C4-specific probe A hybridized to two *Bgl* II fragments of 14.5 and 12.0 kb, which represent the 5′ ends of *C4A* and *C4B* respectively (Fig. 6). When probed with the 21-hydroxylase probe C, the same 12-kb band hybridized as well as an 11-kb band. These two fragments contain the genes for 21-hydroxylase A and 21-hydroxylase B, respectively. Hybridization of probe A to genomic DNA digested with *Eco*RI showed 6- and 12-kb bands, which represent the 5′ end of *C4A* and *C4B*, respectively (Fig. 6). When the same filter was probed with probe C, again the 12-kb band was seen representing 21-hydroxylase A, and a second band of 15 kb was seen representing 21-hydroxylase B.

DISCUSSION

Two genes, 21-hydroxylase A and 21-hydroxylase B, coding for 21-hydroxylase have been localized to a 6-kb region flanking the 3′ end of the *C4A* and *C4B* genes. The 21-hydroxylase gene was estimated as a minimum of 4.5 kb, approximately twice the length of the 2.4-kb mRNA identified in adrenal RNA.

This 6-kb region containing the 21-hydroxylase gene was probably duplicated together with the *C4* genes. The *C4* genes have been shown by restriction mapping (3) and nucleotide sequencing of the respective cDNAs to be highly conserved (21), and it is probable that the 21-hydroxylase genes are equally conserved. Restriction mapping of uncloned genomic DNA by using several enzymes recognizing common four-base sequences did not distinguish between the two genes (unpublished data).

The significance of the close association of the 21-hydroxylase and *C4* genes is uncertain. The *C2* and factor B genes, 30 kb away from the *C4A* genes, are less than 1 kb apart but are related both structurally and functionally and, presumably, arose by gene duplication. The limited sequence data on 21-hydroxylase gene shows no homology with the *C4* sequence.

Injection of glucocorticoid steroids represses several aspects of the immune response, and their rate of synthesis may be influenced by the catalytic activity of 21-hydroxylase. There is little conclusive evidence, however, that the glucocorticoid steroids have any physiological role in immunity in man (22). This is in contrast to the other known products of the *HLA* complex. The assumption, for the present, must be that the close association of the 21-hydroxylase and *C4* genes is fortuitous.

Note Added in Proof. The results given to use by P. C. White and colleagues prior to this work are now published (23) and in press (24).

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