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Gene conversion-like events cause steroid 21-hydroxylase deficiency in congenital adrenal hyperplasia

(inborn error of metabolism/cytochrome P-450/multigene family/pseudogene/homologous recombination)

FUMIKI HARADA†, AKINORI KIMURA†, TOMOHISA IWANAGA†, KAZUHIKO SHIMOZAWA‡, JUNICHI YATA‡, AND TAKEHIKO SASAZUKI†§

†Department of Genetics, Medical Institute of Bioregulation, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812, Japan; and ‡Department of Pediatrics, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113, Japan

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ABSTRACT **Genomic DNAs from twelve Japanese patients with steroid 21-hydroxylase [21-OHase; steroid 21-monooxygenase; steroid, hydrogen-donor:oxygen oxidoreductase (21-hydroxylating); EC 1.14.99.10] deficiency were analyzed by Southern blot hybridization. A 3.7-kilobase (kb) *Taq* I and a 1.7-kb *Pvu* II restriction endonuclease fragment that correspond to a *21-OHase B* gene were absent from the DNA of two unrelated patients with the salt-wasting form of the disease. However, a 10.5-kb *Bgl* II fragment corresponding to the region encompassing the *21-OHase B* gene was still present in these two patients. The genes encoding 21-OHase were cloned from one of these two patients, who was homozygous by descent for *HLA-A26;B39;C4A3;C4B1;DR4*. Restriction endonuclease mapping as well as partial nucleotide sequencing analysis revealed that the *21-OHase B* gene of the patient has been converted to the pseudogene, *21-OHase A*, as far as the critical 0.5-kb sequence was concerned. Thus, the defect was due to both chromosomes each carrying two copies of *21-OHase A* pseudogene and lacking functional *21-OHase B* gene.**

Steroid 21-hydroxylase [21-OHase; steroid 21-monooxygenase; steroid, hydrogen-donor:oxygen oxidoreductase (21-hydroxylating); EC 1.14.99.10] deficiency is a major cause (95%) of congenital adrenal hyperplasia (CAH), one of a group of disorders of adrenal steroidogenesis. The disease affects about one in 5000–15,000 births in Caucasian (1) and one in 10,000–20,000 births in Japanese (2) populations, and thus is one of the most common inborn errors of metabolism. These patients are clinically subdivided into three types—the salt-wasting form, the simple-virilizing form, and the nonclassical late-onset form, or cryptic variant. The disease is inherited as a monogenic autosomal recessive trait closely linked to genes in the major histocompatibility complex (*HLA*) (3). There are two 21-OHase-encoding genes, *21-OHase A* and *21-OHase B*, each located to the 3′ side of one of the two genes encoding the fourth component of complement, *C4A* and *C4B*, which reside in the class III region of *HLA* (4, 5). The *21-OHase B* gene is functional, whereas the *21-OHase A* gene is a pseudogene that has become defunct in the recent past (6, 7). This pseudogene has been deleted together with the *C4A* gene in *HLA-A1;B8;C4A*Q0(null); C4B1;DR3* haplotype in Caucasians (8). Deletion of the *21-OHase B* gene was seen in Caucasians with salt-wasting CAH who were homozygous for *HLA-Bw47* (9). We performed Southern blot analysis of the genomic DNA from a number of Japanese patients with the disease, using a 21-OHase cDNA probe. Indeed, some of the affected haplotypes did not yield certain restriction fragments corresponding to the *21-OHase B* gene. This apparent absence was not due to deletion of the *21-OHase B* gene; rather, it was due to conversion of the functional *21-OHase B* gene into the nonfunctional *21-OHase A* gene. Cloning and sequencing analysis¶ of the 21-OHase genes from a patient was done to obtain evidence for this conversion.

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MATERIALS AND METHODS

Patients and Healthy Controls. Patients from 12 Japanese families included eight patients with salt-wasting CAH and four patients with simple virilizing CAH. Three families were consanguineous. The hormonally normal controls consisted of 17 Japanese, including 7 homozygotes for typical Japanese *HLA* haplotypes and 2 Caucasians homozygous for the *HLA-B8* haplotype. *HLA* typing and C4 allotyping were done as described (10, 11).

DNA Preparation. High-M_r DNA was prepared from peripheral blood leukocytes or lymphoblastoid cell lines, according to the established method (12).

DNA Probes. The 21-OHase probe was a human 21-OHase cDNA clone (pC21/3C) (5), provided by P. C. White (Memorial Sloan–Kettering Cancer Center); the C4 probe was a human C4B cDNA clone (pAT-F) (13), provided by M. C. Carroll (Oxford University).

Southern Blot Analysis. High-M_r DNA (10 μg) was digested with restriction endonucleases *Taq* I, *Pvu* II, and *Bgl* II (3 units/μg of DNA). DNA fragments were separated on 0.8% or 0.5% agarose gels and transferred to a nitrocellulose filter, according to Southern (14). The filters were baked and hybridized with a nick-translated cDNA probe at 65°C for 16 hr. Hybridized filters were washed twice for 10 min each at room temperature in 0.3 M sodium chloride/0.03 M sodium citrate, pH 7.0/0.1% $NaDodSO_4$ and three times for 1 hr each at 65°C in 0.15 M sodium chloride/0.015 M sodium citrate, pH 7.0/0.1% $NaDodSO_4$. The filters were exposed to Kodak XAR films using an intensifying screen at −70°C.

Construction of the Genomic Library. Genomic DNA was partially digested with *Eco*RI, following the procedure of Maniatis *et al.* (12). Fragments 15–20 kilobases (kb) long were isolated by sucrose gradient sedimentation and cloned in the phage vector Charon 4A.

Screening the Library and Analysis of the Cloned DNA. The genomic library was screened by plaque hybridization with the C4 and the 21-OHase probes, as described (12). The DNAs of positive clones were prepared by the plate lysate

Abbreviations: CAH, congenital adrenal hyperplasia; 21-OHase, steroid 21-hydroxylase; C4, fourth component of serum complement.

§To whom reprint requests should be addressed.

¶This sequence of the gene alteration in 21-OHase deficiency is being deposited in the EMBL/GenBank data base (Bolt, Beranek, and Newman Laboratories, Cambridge, MA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J02981).

method (12) and were analyzed by restriction mapping, as well as by Southern blot hybridization.

DNA Sequence Analysis. *Eco*RI fragment (0.5 kb) from each of the 21-OHase genes was subcloned into pTZ19R and sequenced by the dideoxy nucleotide chain-termination method (15).

RESULTS

Southern Blot Analysis. The *21-OHase B* gene, which includes a 3.7-kb *Taq* I fragment, can be distinguished from the *21-OHase A* gene, which includes a 3.2-kb *Taq* I fragment, on Southern blot analysis using the 21-OHase cDNA probe (5). We first performed Southern blot analysis of 21-OHase genes from both hormonally normal individuals and patients with the disease using *Taq* I restriction endonuclease and pC21/3C as the probe. In all DNAs from 17 normal Japanese individuals, including 24 typical haplotypes (7 homozygotes), two hybridizing fragments (3.2 and 3.7 kb) of equal intensity were seen, whereas only the 3.7-kb fragment was seen in two DNAs from the normal Caucasian individuals (AVL and LHM) homozygous for the *HLA-B8* haplotype. These observations suggested that *HLA-B8*-like haplotypes lacking *C4A* and *21-OHase A* genes are either rare or absent in the Japanese population.

In DNAs from two Japanese patients with salt-wasting CAH, the 3.7-kb fragment was absent, and only the 3.2-kb fragment was observed (Fig. 1*A*). In three DNAs, from two patients with salt-wasting CAH and from a patient with simple-virilizing CAH, the 3.7-kb fragment had a reproducibly decreased hybridization intensity compared with the 3.2-kb fragment, whereas two fragments of equal intensity were observed in the other seven patients (data not shown). These data suggested that 6 of 21 unrelated affected haplotypes (28.6%) lacked the 3.7-kb *Taq* I fragment. Southern blot analysis using *Pvu* II demonstrated that two *Pvu* II fragments (1.9-kb and 1.7-kb) were present in DNAs from normal Japanese individuals, but DNAs from normal Caucasians AVL and LHM with *HLA-B8* haplotype showed the absence of the 1.9-kb fragment, confirming that the 1.9-kb and 1.7-kb fragments, indeed, corresponded to *21-OHase A* gene and *21-OHase B* gene, respectively. These two Japanese patients without the 3.7-kb *Taq* I fragment also lacked the 1.7-kb *Pvu* II fragment (Fig. 1*B*). It first appeared then that the *21-OHase B* gene was deleted in these two patients. Surprisingly, however, Southern blot analysis using *Bgl* II revealed that a 10.5-kb *Bgl* II fragment corresponding to the wider region centered by *21-OHase B* gene was conserved in DNAs from these two patients (Fig. 1*C*). Therefore we concluded that the *21-OHase B* gene must not have been deleted; rather, it was converted to the *21-OHase A* gene in these two patients (Fig. 2).

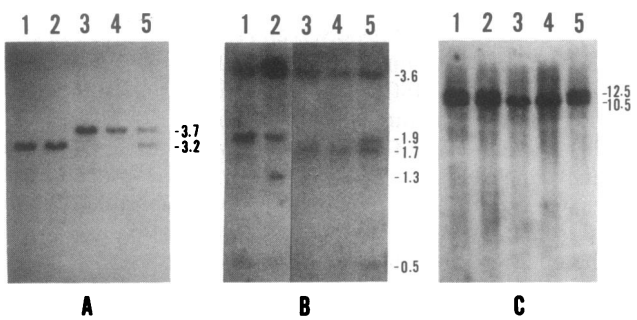

FIG. 1. Southern blot analysis of DNA from two Japanese patients with salt-wasting CAH and hormonally normal individuals. DNAs were digested with *Taq* I (*A*), *Pvu* II (*B*), and *Bgl* II (*C*), separated by electrophoresis in 0.8% agarose gels (*A*, *B*) and in a 0.5% agarose gel (*C*), and transferred to nitrocellulose before hybridization with pC21/3C. The order of genomic DNA samples is as follows: lanes 1, patient homozygous by descent for *HLA-A26;B39;C4A3;C4B1;DR4*; lanes 2, patient with *HLA-A2;B35;DR5/-A24;Bw61;DRw9*; lanes 3 and 4, Caucasian individuals homozygous for *HLA-A1;B8;C4A*Q0;C4B1;DR3*—AVL and LHM, respectively; and lanes 5, normal Japanese individual.

In one of these two patients, the 1.3-kb *Pvu* II fragment was present (Fig. 1*B*, lane 2). This fragment has not been reported previously but during this study it was also found in normal individuals with *HLA-A24;Bw54;C4A3;C4B5;DR4*, a typical Japanese haplotype, in addition to the four *Pvu* II fragments—3.6 kb, 1.9 kb, 1.7 kb, and 0.5 kb. This finding suggests the possibility of a third locus for the 21-OHase in certain Japanese haplotypes.

Cloning and Partial Sequencing Analysis. To obtain direct evidence for the gene conversion-like events, we isolated 21-OHase-encoding genes from one of these two patients, homozygous by descent for *HLA-A26;B39;C4A3;C4B1;DR4*. Four different clones were isolated from 10^6 recombinant phages using 21-OHase and C4 cDNA probes. A molecular map of these clones is shown in Fig. 3. Clones 105 and 227 contained a 2.0-kb *Eco*RI–*Bgl* II fragment that hybridized to the C4 probe and weakly to the 21-OHase probe. Clone 110 contained a 0.5-kb *Eco*RI fragment and a 10.0-kb *Eco*RI–*Bgl* II fragment, both of which hybridized to the 21-OHase probe. Clone 216 contained a 0.5-kb *Eco*RI fragment and a 8.0-kb *Eco*RI–*Bgl* II fragment, both of which hybridized to the 21-OHase probe. The restriction mapping and hybridization analysis of these clones revealed that the affected haplotype carried two 21-OHase genes, one was a *21-OHase A* gene in the 12.5-kb *Bgl* II fragment and another was a *21-OHase B* gene in the 10.5-kb *Bgl* II fragment. Most recently, Higashi *et al.* (6) and White *et al.* (7) independently reported that two 21-OHase genes were highly homologous (98%), but several mutations render the *21-OHase A* gene nonfunctional. We subcloned 0.5-kb *Eco*RI fragments of two 21-OHase genes in this patient and determined the nucleotide sequences. Interestingly, within the 504-base pair (bp) *Eco*RI fragment of *21-OHase B* gene from the patient, 24 base substitutions, one 3-bp insertion, two 1-bp insertions, and a deletion of 8 bp were observed, compared with the sequence of normal *21-OHase B* gene (6) (Fig. 4). This base sequence of 0.5-kb *Eco*RI fragment of *21-OHase B* gene from the patient was completely identical to that of 0.5-kb *Eco*RI fragment of *21-OHase A* gene from the patient. The 8-bp deletion in the third exon renders the *21-OHase B* gene nonfunctional. Thus, the *21-OHase B* gene of the patient became a pseudogene identical to the *21-OHase A* gene. These observations suggest that the *21-OHase B* gene was converted to a nonfunctional

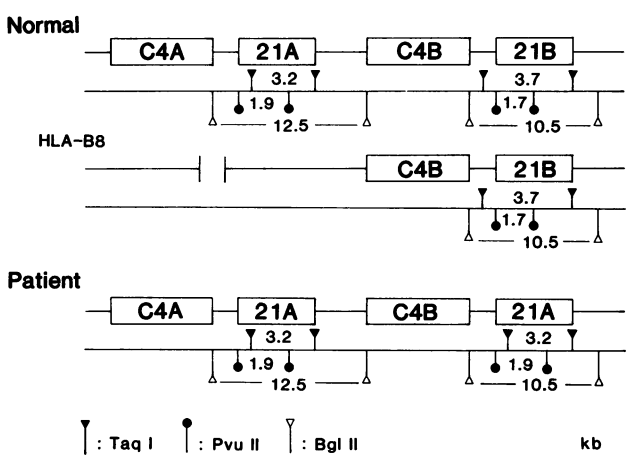

FIG. 2. Schematic comparison of the C4 and 21-OHase genes of patient and hormonally normal individual, based on the data in Fig. 1.

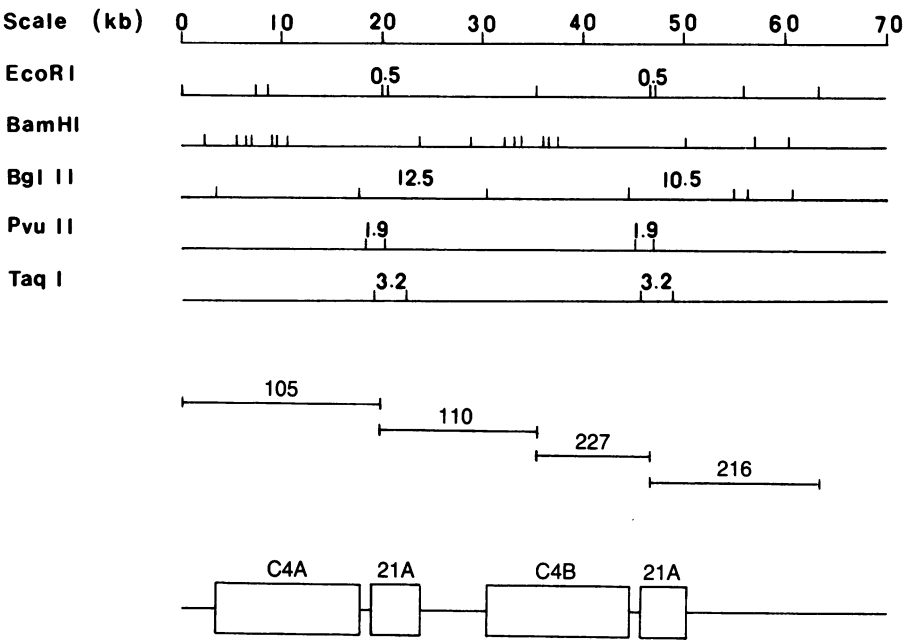

FIG. 3. Molecular map of *HLA* region containing genes for C4 and 21-OHase in a patient with salt-wasting CAH. The patient is homozygous by descent for the *HLA-B39* haplotype and has neither the 3.7-kb *Taq* I nor the 1.7-kb *Pvu* II fragment, as demonstrated in Fig. 1, lane 1. The region was mapped by Southern analysis of genomic DNA as well as recombinant phage clones probed with pC21/3C and pAT-F. The 0.5-kb *Eco*RI fragments of clone 110 and that of clone 216 were hybridized to pC21/3C, and the nucleotide sequences were determined. The regions corresponding to 12.5-kb *Bgl* II, 10.5-kb *Bgl* II, 1.9-kb *Pvu* II, and 3.2-kb *Taq* I fragments are shown.

21-OHase A gene, resulting in the salt-wasting CAH of this patient.

DISCUSSION

In two unrelated patients with 21-OHase deficiency the *21-OHase B* gene was converted to a *21-OHase A* gene. This DNA segment exchange could be explained either by gene conversion or by homologous recombination. Gene conversion-like events, including both gene conversion and homologous recombination, have been considered to play an important role in the evolution of multigene families and are among the major mechanisms involved in intrachromosomal sequence homology (16). On the other hand, it has recently become apparent that the same mechanism also generates further diversity among the closely related members of a multigene family, such as the major histocompatibility class I gene (17). The 21-OHase gene is a member of the cyto-

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AAATTCCTAGTCTCAAATGATCCTCCCACCTCAGCCTCAAGTGTGAGCCACCTTTGGGG-CATCCCCAATCC---AGGTC
G      G      G              G         G         C   G T         TCC


CCTGGAAGCTCTTGGGGGGCATATCTGGTGGGGAGAAAGCAGGGGTTGGGGAGGCCGAAGAAGGTCAGGCCCTCAGCTGC
A                        TCA  A  AG       T    A      A                 G   T

                                                  yr Lys Leu Val Ser lys Asn Thr Pro
CTTCATCAGTTCCCACCCTCCAGCCCCCACCTCCTCCTGCAG   AC AAG CTG GTG TCT AAG AAC TAC CCG
   GG                          G

Asp Leu Ser Leu Gly Asp Tyr Ser Leu Leu Trp Lys Ala His Lys Lys Leu Thr Arg Ser
GAC CTG TCC TTG GGA GAC TAC TCC CTG CTC TGG AAA GCC CAC AAG AAG CTC ACC CGC TCA
         G      ●● ●●● ●●●   T

Ala Leu Leu Leu Gly Ile Arg Asp Ser Met Glu Pro Val Val Glu Gln Leu Thr Gln Glu
GCC CTG CTG CTG GGC ATC CGT GAC TCC ATG GAG CCA GTG GTG GAG CAG CTG ACC CAG GAG


Phe Cys Glu
TTC TGT GAG   GTAAGGCTGGGCTCCTGAGGCCACCTCGGGTCAGCCTCGCCTCTCACAGTAGCCCCCGCCCTG-CC
                                                                              C

                                                  Arg Met Arg Ala Gln Pro Gly Thr Pro
GCTGCACAGCGGCCTGCTGAACTCACACTGTTTCTCCACAG   CGC ATG AGA GCC CAG CCC GGC ACC CCT


Val Ala Ile Glu Glu Glu Phe
GTG GCC ATT GAG GAG GAA TTC
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FIG. 4. Partial nucleotide sequence of the *21-OHase B* gene from the patient. Sequence of the 0.5-kb *Eco*RI fragment of clone 216 was determined, and sequence of the normal *21-OHase B* gene with the deduced amino acid sequence determined by Higashi *et al.* (6) is also shown; only differences from normal *21-OHase B* gene are shown below each line of the normal sequence. Eight bp (closed circles) are missing in the third exon of the *21-OHase B* gene from the patient. This 8-bp deletion causes a frame shift, which brings a termination codon into the reading frame (underlining).

chrome P-450 multigene family. DNA sequence data of the genes for closely related phenobarbital-inducible P-450 isoenzymes suggested that gene conversion represented an important evolutionary mechanism for the generation of functional diversity of P-450 enzymes (18). Moreover, one 21-OHase gene and one C4 gene formed a closely linked pair that is duplicated as a single set. Although both C4-encoding genes are functional, one of the genes encoding 21-OHase has, in the recent past, become defunct and joined the rank of pseudogene. The presence of duplicated and highly homologous sets of the C4 and 21-OHase gene pair in humans as well as in mice (19) suggests the possibility that DNA sequence homology between the two pairs has been maintained by gene conversion-like events. We obtained evidence for the conversion of the functional *21-OHase B* gene to the nonfunctional *21-OHase A* gene in two Japanese patients. Affected *HLA* haplotypes of these two patients were not identical, thereby suggesting that conversion of the *21-OHase B* gene to the *21-OHase A* gene had occurred as independent events. Some of the other three haplotypes with the 3.7-kb *Taq* I fragment deleted might also be explained by yet additional gene conversion-like events. Donohoue *et al.* (20) reported that two siblings without C4B protein lacked certain restriction fragments corresponding to a *21-OHase B* gene, and they suggested that a contiguous gene-conversion event would result in a nonfunctional *21-OHase B* gene and in a *C4B* null allele. Our patient was typed as *C4A3,3;C4B1,1*, so that the gene conversion in the patient would not include sequences that differentiate *C4B* from *C4A*.

It is apparent that one of the duplicated 21-OHase genes has become a pseudogene in the recent past, and this suggests that the presence of two functional 21-OHase genes is deleterious to the host because of the gene-dosage effect. If duplication of the C4 gene had a selective advantage during evolution, that of the tightly linked 21-OHase gene might have occurred as an inadvertent but inevitable consequence. Thus, the silencing of a redundant 21-OHase gene was called for. But the presence of such a pseudogene so closely linked to a functional gene leads to untoward events, as we have shown. Most of the mutations resulting in 21-OHase deficiency have yet to be characterized; one-fourth are deletions of the *21-OHase B* gene in Caucasians with classical CAH (21). We suggest that the gene conversion-like events between 21-OHase genes probably are a relatively common cause of the disease in the Japanese population. This mechanism might also, in part, account for the predominance of CAH due to the 21-OHase deficiency over CAH due to deficiencies of other steroidogenic P-450 enzymes. The gene conversion-like events described above shed light on the mechanism of other monogenic disorders where the related homologous genes reside in tandem array.

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1. New, M. I. & Levine, L. S. (1984) in *Monographs on Endocrinology*, eds. Gross, F. & Labhart, A. (Springer, Berlin), Vol. 26, pp. 44–67.
2. Naruse, H., Suzuki, E., Irie, M., Tsuji, A., Takasugi, N., Fukushi, M., Matsuura, N. & Shimozawa, K. (1985) *Ann. N.Y. Acad. Sci.* **458**, 103–110.
3. Dupont, B., Oberfield, S. E., Smithwick, E. M., Lee, T. D. & Levine, L. S. (1977) *Lancet* **ii**, 1309–1311.
4. Carroll, M. C., Campbell, R. D. & Porter, R. R. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 521–525.
5. White, P. C., Grossberger, D., Onufer, B. J., Chaplin, D. D., New, M. I., Dupont, B. & Strominger, J. L. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 1089–1093.
6. Higashi, Y., Yoshioka, H., Yamane, M., Gotoh, O. & Fujii-Kuriyama, Y. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 2841–2845.
7. White, P. C., New, M. I. & Dupont, B. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 5111–5115.
8. Carroll, M. C., Palsdottir, A., Belt, K. T. & Porter, R. R. (1985) *EMBO J.* **4**, 2547–2552.
9. White, P. C., New, M. I. & Dupont, B. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 7505–7509.
10. Terasaki, P. I., Bernoco, D., Park, M. S., Ozturk, G. & Iwaki, Y. (1978) *Am. J. Clin. Pathol.* **69**, 103–120.
11. Mauff, G., Alper, C. A., Awdeh, Z., Batchelor, J. R., Bertrams, J., Bruun-Petersen, G., Dawkins, R. L., Demant, P., Edwards, J., Grosse-Wilde, H., Hauptmann, G., Klouda, P., Lamm, L., Mollenhauer, E., Nerl, C., Olaisen, B., O'Neill, G., Rittner, C., Roos, M. H., Skanes, V., Teisberg, P. & Wells, L. (1983) *Immunobiology* **164**, 184–191.
12. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
13. Belt, K. T., Carroll, M. C. & Porter, R. R. (1984) *Cell* **36**, 907–914.
14. Southern, E. M. (1975) *J. Mol. Biol.* **98**, 503–507.
15. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
16. Nagylaki, T. & Petes, T. D. (1982) *Genetics* **100**, 315–337.
17. Hayashida, H. & Miyata, T. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 2671–2675.
18. Atchison, M. & Adesnik, M. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 2300–2304.
19. White, P. C., Chaplin, D. D., Weis, J. H., Dupont, B., New, M. I. & Seidman, J. G. (1984) *Nature (London)* **312**, 465–467.
20. Donohoue, P. A., Dop, C. V., McLean, R. H., White, P. C., Jospe, N. & Migeon, C. J. (1986) *J. Clin. Endocrinol. Metab.* **62**, 995–1002.
21. Werkmeister, J. M., New, M. I., Dupont, B. & White, P. C. (1986) *Am. J. Hum. Genet.* **39**, 461–469.