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Pulsed field gel electrophoresis identifies a high degree of variability in the number of tandem 21-hydroxylase and complement C4 gene repeats in 21-hydroxylase deficiency haplotypes

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The human steroid 21-hydroxylase gene, CYP21B, and its closely homologous pseudogene, CYP21A, are each normally located centromeric to a complement C4 gene C4B and C4A respectively, in an organization suggesting tandem duplication of a CYP21 + C4 unit. Such an organization has been considered to facilitate gene deletion and addition events by unequal crossover between the tandem repeats. However, the large size (~30 kb) of the individual CYP21 + C4 repeat units together with the difficulty in identifying reliable CYP21A- and CYP21B-specific markers has prevented direct monitoring of gene organization on individual haplotypes by conventional Southern analyses. In the present investigation we have sought to clarify the CYP21 and C4 gene organization in members of 32 British 21-hydroxylase deficiency families by employing additional experimental approaches, notably a long-range restriction mapping approach, which permits assessment through a VNTR type of analysis, of the number of CYP21 and C4 units on individual haplotypes. Our results show that there is a very high frequency (33%) of 21-hydroxylase deficency haplotypes where functional CYP21B gene sequence has been removed as a consequence of CYP21 + C4 gene deletion while several haplotypes show evidence of gene addition. In each case that we have investigated the gene deletion and gene addition haplotypes differ in length from conventional haplotypes by integral multiples of ~30 kb, which strongly supports the involvement of unequal crossover mechanisms. Additionally, the comparatively frequent occurrence of CYP21 fusion genes which contain both CYP21A- and CYP21B-associated markers is suggested by the combined data from Southern analyses, long-range restriction mapping and characterization of selected regions of CYP21 genes which have been amplified *in vitro*.

Key words: 21-hydroxylase gene/crossover/tandem repeats/21-hydroxylase deficiency haplotypes/RFLP analysis

Introduction

Congenital adrenal hyperplasia is a recessively inherited disorder of cortisol biosynthesis which, in >90% of cases, is attributable to deficiency in steroid 21-hydroxylation [for recent reviews see White *et al.* (1987a,b) and Miller (1988a)]. The disorder is clinically heterogeneous but is always characterized by excessive androgen production which may lead to marked virilization. Classical congenital 21-hydroxylase (21-OH) deficiency occurs with a frequency of about one in 5000 - 10 000 births and affected females are born with ambiguous genitalia. Approximately two out of the three classical 21-hydroxylase deficiency patients show an additional inability to conserve dietary sodium ('salt-wasting') as a consequence of defective aldosterone production, while the remainder have been classified as 'simple virilizers'. Non-classical forms, including late onset development of symptoms, are less severe but have been found to be extremely common.

Family studies have revealed that the disorder is closely linked to the HLA complex on the short arm of chromosome 6 (Dupont *et al.*, 1977; Price *et al.*, 1978), thereby permitting prenatal diagnosis using DNA probes that map to this region (Strachan *et al.*, 1987). Molecular genetic approaches have identified steroid 21-OH genes in the class III region of the HLA complex and two highly homologous 21-OH gene loci have been distinguished, CYP21A and CYP21B. Of these, only the CYP21B gene is normally functional, while the CYP21A gene is a pseudogene (White *et al.*, 1986; Higashi *et al.*, 1986; Rodrigues *et al.*, 1987). The CYP21A and CYP21B genes are positioned ~2 kb centromeric to the complement C4A and C4B genes respectively (Carroll *et al.*, 1985a; White *et al.*, 1985; Dunham *et al.*, 1987) (see Figure 1) and the extant organization of these genes is presumed to reflect tandem duplication of an ancestral DNA segment ~30 kb long and including a compound unit of a 21-OH gene + C4 gene.

Notwithstanding the high degree of sequence homology between the two 21-OH + C4 repeat units, certain restriction enzymes reveal restriction fragment length variants that can permit distinction between the homologous CYP21 loci and between the homologous C4 loci. Consequently, 21-OH and C4 DNA probes have been utilized in Southern analyses to infer gene organization at the CYP21 and C4 loci and the results have been interpreted to suggest a high frequency of gene deletion in 21-OH deficiency patients (e.g. Boehm

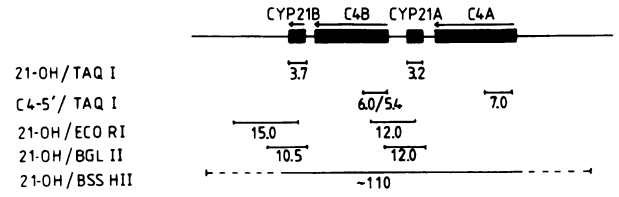

Fig. 1. Diagnostic restriction fragments used in short- and long-range restriction mapping of the CYP21 and C4 genes. Genes are shown in the conventional order for genes in the HLA complex, with the centromere to the left. Arrows mark the direction of transcription of the expressed genes. Numbers refer to the size in kilobases of restriction fragments generated by the indicated restriction enzymes and detected by using a 21-OH-specific DNA probe, 21A-1.8, or a C4-5' probe, C4B550.

et al., 1986; Schneider et al., 1986; Werkmeister et al., 1986; Jospe et al., 1987; Rumsby et al., 1988; White et al., 1988). The great majority of 21-OH deficiency haplotypes which have been inferred, on the basis of short-range restriction mapping, to lack a CYP21B gene also appear to lack a C4 gene. Consequently, the genesis of deletions has been widely assumed to occur by unequal crossover.

Recently, however, conventional short-range restriction mapping (Donohoue et al., 1986; Jospe et al., 1987; Matteson et al. 1987; Rumsby et al., 1988; White et al., 1988), DNA probing analyses using locus-specific oligonucleotides (Higashi et al., 1988a) and direct DNA sequencing studies (Harada et al., 1987; Higashi et al., 1988b) have suggested that the situation is complicated by inter-locus sequence transfer. Haplotypes have been observed, at high overall frequencies in some of these studies, where there appears to have been prior sequence transfer between the CYP21A and CYP21B loci as a consequence of gene-conversion-like mechanisms. On the basis of these observations the evidence for gene deletion in the molecular pathology of 21-OH deficiency has recently been strongly challenged (Matteson et al., 1987; Miller, 1988b). In the present study we have addressed the controversy about the authenticity of gene deletion in 21-OH deficiency by using a variety of experimental procedures, especially a long-range restriction mapping analysis which greatly facilitates the interpretation of gene organization at the CYP21 and C4 loci.

Results

Gene deletions do contribute very significantly to 21-hydroxylase deficiency

As outlined in the Introduction there has been considerable recent controversy over the authenticity of apparent deletions of the CYP21B gene on 21-OH deficiency haplotypes. Hitherto the method of scoring such gene deletions, short-range restriction mapping by Southern blot analysis using 21-OH-specific or C4-specific DNA probes, has necessarily been an indirect one (see Discussion). We have therefore sought to facilitate the investigation of gene organization on 21-OH deficiency haplotypes by two methods: (i) extended short-range restriction mapping in families where the affected child is HLA homozygous as a consequence of parental consanguinity; and (ii) long-range restriction mapping of DNA fragments that have been size fractionated by pulsed field gel electrophoresis.

In the case of short-range restriction mapping, distinction between the two homologous CYP21 loci and the two homologous C4 loci is possible using certain restriction enzymes. In each case, the enzyme is one which generates restriction fragment length variants (RFLVs), by which we mean non-allelic restriction fragments which show size differences that distinguish between closely homologous but distinct loci. Thus, using a 21-OH-specific DNA probe, one can differentiate the CYP21A locus (normally characterized by a 3.2 kb *Taq*I RFLV, a 12 kb *Eco*RI RFLV and a 12 kb *Bgl*II RFLV) from the homologous CYP21B locus (normally associated with a 3.7 kb *Taq*I RFLV, a 15 kb *Eco*RI RFLV and a 10.5 kb *Bgl*II RFLV) (see Figure 1). Similarly, a C4−5′ DNA probe allows the C4A locus to be distinguished from the C4B locus, e.g. by *Taq*I RFLVs (7.0 kb for C4A, either 5.4 or 6.0 kb for C4B). As a first approach to short-range restriction mapping we have scored *Taq*I RFLVs representing the CYP21 loci and also the adjacent C4 loci in all available members in 32 British 21-OH deficiency families. Our results suggest that 43% (25/58) of the disease haplotypes appear to lack a CYP21B gene, on the basis of the absence of the CYP21B-associated 3.7 kb *Taq*I RFLV, or a reduction in intensity of the 3.7 kb *Taq*I hybridization band compared with the CYP21A-associated 3.2 kb *Taq*I hybridization band.

Of the 21-OH deficiency haplotypes that appear to lack a CYP21B gene, 76% (19/25) appear to show concomitant absence, or diminished comparative intensity, of the 6.0 kb/5.4 kb *Taq*I RFLVs characteristic of the C4B gene. Figure 2 illustrates two families which show evidence of such haplotypes. In each case parental DNA samples show a ratio of ~2:1 betweeen the intensity of the CYP21A-associated 3.2 kb *Taq*I hybridization bands and that of the CYP21B-associated 3.7 kb band (Figure 2, panels B and G; also densitometric data not shown). Similarly, the parental samples show a 2:1 ratio between the intensity of the C4A-associated 7.0 kb *Taq*I hybridization band and that of the C4B-associated 6.0 kb *Taq*I band (both parental samples in family 6 and paternal sample in family 18) or 5.4 kb *Taq*I band (maternal sample in family 18). The affected children exhibit only the 3.2 and 7.0 kb band while the unaffected sibling in family 18, who has inherited the two non-disease parental haplotypes, b and c, shows equivalence in intensity of the CYP21-specific 3.2 and 3.7 kb *Taq*I bands and a 2:1:1 ratio for the intensities of the C4-specific 7.0, 6.0 and 5.4 kb *Taq*I bands respectively. Consequently, it would appear that the disease haplotypes in both families carry a deletion of a compound CYP21 + C4 gene unit, including CYP21B and C4B-associated markers, while both non-disease haplotypes show no evidence for gene deletion.

When the short-range mapping analyses on such families, where the affected child is HLA-homozygous, are extended to include RFLVs generated by *Eco*RI or by *Bgl*II, a similar pattern emerges with parental samples showing a 2:1 ratio between the intensities of two CYP21-specific RFLVs (the lower *Eco*RI RFLV in the paternal sample from family 18 is 9 kb in length as a consequence of differences in the extent to which the CYP21-associated *Eco*RI sites are cleaved) (see White et al., 1988). Also, the unaffected sibling in family 18 shows equivalence between the two RFLVs, and affected children in both families show only a single RFLV (Figure 2, panels C, D, H and I). In the latter case, the single remaining *Eco*RI and *Bgl*II RFLVs are ones normally characteristic of the CYP21B gene (see Discussion).

In order to monitor the gene organization on individual haplotypes and thereby permit a more direct detection of gene deletions at the CYP21 and C4 loci, we have also employed a long-range restriction mapping procedure. The latter method relies on the ability of the rare cutter enzyme *Bss*HII to cleave genomic DNA at well-conserved sites that flank the DNA region which encompasses the array of CYP21 and C4 genes (our unpublished results; D.Campbell, personal communication). Thus it is possible to examine length variation as a consequence of loss or gain of CYP21 + C4 repeat units on individual haplotypes. In the case of normal haplotypes, digestion with *Bss*HII will produce a fragment of ~110 kb that contains two CYP21 + C4 repeat units (see Figure 1). However, deletion haplotypes that lack

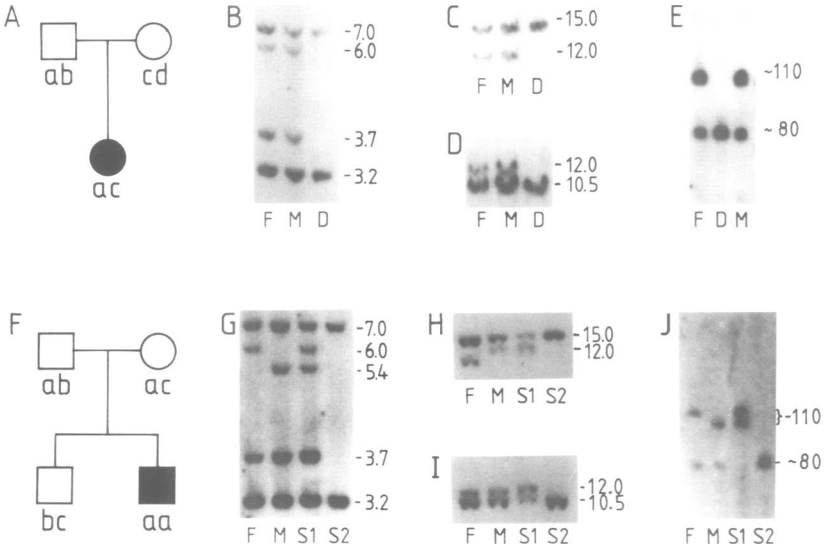

Fig. 2. Identification of 21-OH deficiency haplotypes that lack a CYP21 + C4 gene repeat unit. **Panels A** and **F**: pedigrees of family 6 (**panel A**) and family 18 (**panel F**). The affected child in both families has the salt-wasting form of 21-OH deficiency and is HLA homozygous (as a consequence of known parental consanguinity in the case of family 18). HLA haplotypes in family 6 are: a and c, DR7 Bw47 A3; b, DR7 B44 A2; d, DR2 B7 A30. HLA haplotypes in family 18 are: a, DR3 B7 A24; b, DR4 B44 A3; c DR4 B44 A2. **Panels B–D** and **G–I**: Southern blot analysis of genomic DNA from individuals in family 6 (**panels B–D**) and family 18 (**panels G–I**). Lanes represent DNA samples from the following family members: **F**, father; **M**, mother; **D**, daughter; **S1** elder son; **S2**, younger son. DNA samples from indicated family members were digested with *Taq*I (**panels B** and **G**), *Eco*RI (**panels C** and **H**), or *Bgl*II (**panels D** and **I**). DNA blots were probed with a 21-OH-specific DNA probe, 21A-1.8 (**panels C, D, H** and **I**) or with a combination of the 21A-1.8 probe and a complement C4-specific probe, C4B550 (**panels B** and **G**). Figures represent the size in kilobases of indicated bands. **Panels E** and **J**: pulsed field gel electrophoresis analysis of genomic DNA from individuals in family 6 (**panel E**) and family 18 (**panel J**). Lanes represent DNA samples from the following family members: **F**, father; **M**, mother; **S**, son; **D**, daughter. DNA samples from indicated family members were digested with *Bss*HII prior to size fractionation by pulsed field gel electrophoresis (see Materials and methods). Pulsed field gel electrophoresis gels were dried down prior to direct probing with the 21-OH-specific probe, 21A-1.8.

one of the ~30 kb CYP21 + C4 repeat units would be expected to exhibit a corresponding *Bss*HII RFLP of ~80 kb. *Bss*HII mapping analyses on parental samples in the families above reveal two CYP21-specific hybridization bands, one of ~110 kb and one of ~80 kb (Figure 2, panels E and J). The affected child in each case demonstrates a single band at ~80 kb while the unaffected sibling in family 18 exhibits two hybridization bands in the 110 kb region. The minor length variation between the paternally and maternally inherited bands in the latter individual represents a difference of ~6.5 kb between different C4B genes: long C4B gene variants are characterized by the 6.0 kb *Taq*I RFLV and short C4B genes by the 5.4 kb *Taq*I RFLV (Palsdottir *et al.*, 1987).

From the above analyses, we conclude that the families in Figure 2 carry 21-OH deficiency haplotypes where there has been a deletion of a CYP21 + C4 gene unit which leads to elimination of functional 21-OH gene sequence. Equivalent analyses on other families in the study reveal a total of 19 deletion haplotypes of this type in patients (see Table I, mutation category 1), which represents 33% of the disease haplotypes studied.

21-OH deficiency haplotypes where the CYP21B gene has been replaced by a gene resembling CYP21A

In contrast to the deletion haplotypes demonstrated in the above families, disease haplotypes in some other families lack the CYP21B-associated 3.7 kb *Taq*I RFLV without showing any evidence of the companion C4 gene deletion which would be expected in the case of deletions arising as a consequence of unequal crossover. For example, *Taq*I mapping analyses on DNA samples from parents of families 1 and 8 reveal reduced representation of the 3.7 kb RFLV when compared with the 3.2 kb RFLV, while the combined intensities of the C4B-associated 6.0 and 5.4 kb RFLVs equal that of the C4A-associated 7.0 kb band (Figure 3, panels B and F). In both families the affected child lacks the 3.7 kb band but shows equality in intensity between the C4A-associated 7.0 kb and C4B-associated 6.0 kb RFLVs. The affected child in family 1 is the daughter of first cousin parents and is HLA homozygous but demonstrates both a C4A3 and a C4B1 allotype, confirming the absence of C4 gene deletion on her disease haplotypes. However, the apparent deletion of a CYP21B gene alone on the disease haplotypes in families 1 and 8 is not substantiated by the results of densitometric quantitation of parental *Taq*I bands: the ratio between the intensities of the 3.2 and 3.7 kb *Taq*I bands is ~3:1 rather than the 2:1 expected of simple CYP21B deletion (data not shown). Also, extended short-range mapping analyses on the affected child in family 1 reveal CYP21-specific 15 and 12 kb *Eco*RI RFLVs and analogous 12 and 11 kb *Bgl*II RFLVs (Figure 3, panels C and D). Because of the identity by common descent of the two disease haplotypes in this child, such a result verifies the presence of two CYP21 genes on the disease haplotype, both of which resemble the CYP21A gene in possessing a 3.2 kb *Taq*I RFLV and in presumably being non-functional.

The disease haplotypes in the affected child in family 8 are non-identical and consequently extended short-range restriction mapping may be subject to ambiguity in interpretation (see Discussion). We have therefore employed the *Bss*HII mapping procedure to score gene deletions directly

Table I. 21-OH deficiency haplotypes

Mutation category	Haplotype						No. of CYP21 + C4 units	Frequency
	HLA-DR	C4B	C4A	Bf	HLA-B	HLA-A		
1. CYP21B deletion	7	Qo	1	F	47	3	1	8
	2	Qo	1	F	47	2	1	1
	4	Qo	1	F	47	3	1	1
	7	n.d.	n.d.	n.d.	38	24	1	1
	4	Qo	3	S	17	2	1	1
	2	n.d.	n.d.	n.d.	21	2	1	1
	1	Qo	3	S	60	3	1	1
	2	n.d.	n.d.	n.d.	51	28	1	1
	3	Qo	3	S	7	24	1	1
	7	Qo	1	S	44	29	1	1
	n.d.	n.d.	n.d.	n.d.	40	3	1	1
	1	Qo	3	S	44	2	1	1
2. CYP21B point mutation/deletion?	1	Qo	3	S	35	2	1	1
	7	Qo	3	F	51	26	1	1
	2	n.d.	n.d.	n.d.	47	3	1	1
	n.d.	n.d.	n.d.	n.d.	39	2	1	1
3. CYP21B point mutation	3	1	Qo	S	8	1	1	1
	4	6	4	S	55	11	2	4
	2	n.d.	n.d.	n.d.	5	24	2	2
	2	1	3	F	7	3	2	1
	2	n.d.	n.d.	n.d.	60	3	2	1
	6	6	4	S	7	24	2	1
	5	1	3	S	44	2	2	1
	2	n.d.	n.d.	n.d.	51	7	2	1
	7	1	3	S	44	3	2	1
	11	n.d.	n.d.	n.d.	35	24	2	1
	6	5	4	S	22	3	2	1
	4	Qo	3	S	44	2	2	1
	4	1	3	S	60	30	2	1
	4	n.d.	n.d.	n.d.	51	11	2	1
	6	1	3	S	7	29	2	1
	5	1	3	S	18	2	2	1
	2	n.d.	n.d.	n.d.	5	3	2	1
	7	1	3	S	27	2	2	1
	4	1	3	S	17	1	2	1
	6	1	3	S	21	3	2	1
	4	Qo	3	S	52	1	2	1
	1	n.d.	n.d.	S	14	3	3	1
	2	n.d.	n.d.	S	5	2	3	1
	7	n.d.	n.d.	n.d.	50	29	3	1
	2	n.d.	n.d.	n.d.	61	2	3	1
4. CYP21B replaced by 'CYP21A'	4	Qo	3	S	44	2	2	1
	5	1	3	S	17	1	2	1
	3	n.d.	n.d.	n.d.	35	3	2	1
	4	1	3	F	7	31	2	1
	7	n.d.	n.d.	n.d.	17	32	2	1
	4	n.d.	n.d.	n.d.	51	31	4	1

Out of the 64 disease haplotypes in the 32 21-OH deficiency families investigated in the present study a total of 58 disease haplotypes are counted as a consequence of known parental consanguinity in six families where affected children are HLA homozygous. Mutation category 2 includes haplotypes where the associated 21-OH deficiency is either attributable to loss of functional sequence as a consequence of a deletion event, or to mutation that is independent of the deletion event (see Discussion). Mutation category 4 includes haplotypes where the normal CYP21B gene has been replaced by a gene that resembles CYP21A on the basis of exhibited CYP21-specific *TaqI* RFLVs (see text).
n.d., Not determined.

on haplotypes in this family. As can be seen in panel G of Figure 3, there is no evidence in members of family 8 for an 80 kb RFLP signifying a haplotype with a CYP21 + C4 deletion. Instead both parental samples demonstrate *Bss*HII RFLPs of ~110 kb but differing by somewhat less than ~10 kb. Of these, the larger RFLP is transmitted on both disease haplotypes to the affected child. As mentioned before, the variation in size between the two *Bss*HII RFLPs of approximate size 110 kb is due to C4B gene length heterogeneity. Consequently, both disease haplotypes in this family again contain two CYP21 + C4 repeat units in each of which the CYP21 gene resembles CYP21A in bearing

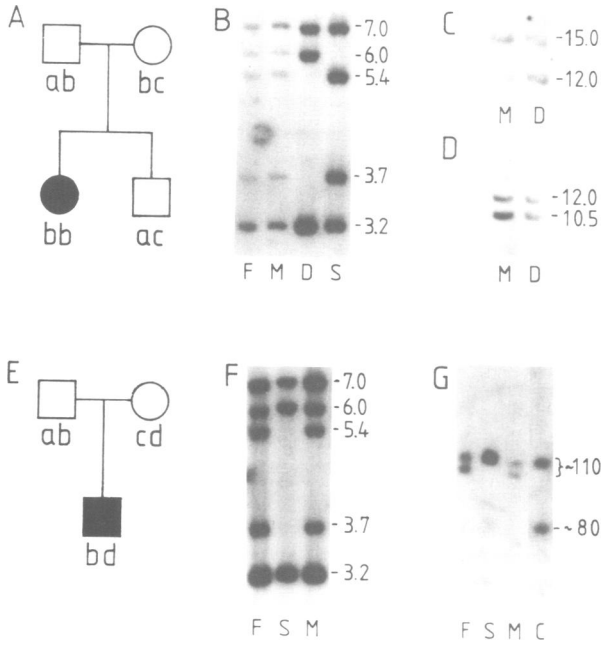

Fig. 3. Identification of 21-OH deficiency haplotypes where the normal CYP21B gene has been replaced by a gene resembling CYP21A. **Panels A** and **E**: pedigrees of families 1 and 8. The affected children in both families have the salt-wasting form of 21-OH deficiency. HLA haplotypes in family 1 are: a, B8 A2; b, DR4 B7 A31; c, DR4 B45 A31. HLA haplotypes in family 8 are: a, DR4 B35 A24; b, DR4 B44 A2; c, DR1 B35 A2; d, DR5 B17 A1. **Panels B–D** and **F**: Southern blot analysis of genomic DNA from individuals in family 1 (**panels B–D**) and family 8 (**panel F**). Lanes represent DNA samples from the following family members: **F**, father; **M**, mother; **S**, son; **D**, daughter. DNA samples from indicated family members were digested with *Taq*I (**panels B** and **F**), *Eco*RI (**panel C**) or *Bgl*II (**panel D**). DNA blots were probed with a 21-OH-specific DNA probe, 21A-1.8 (**panels C** and **D**) or with a combination of the 21A-1.8 probe and a complement C4-specific probe, C4B550 (**panels B** and **F**). Figures represent the size in kilobases of indicated bands. **Panel G**: pulsed field gel electrophoresis analysis of genomic DNA from individuals in family 8. Lanes represent DNA samples from the following family members: **F**, father, **M**, mother; **S**, son. In addition, a DNA sample from a known deletion heterozygote was provided as a control (**C**). DNA samples from indicated individuals were digested with *Bss*HII prior to size fractionation by pulsed field gel electrophoresis (see Materials and methods). Pulsed field gel electrophoresis gels were dried down prior to direct probing with the 21-OH-specific probe, 21A-1.8.

a 3.2 kb *Taq*I RFLV and in presumably being defective in expression. A total of six patient haplotypes were found to demonstrate this pattern, accounting for 10% of all disease haplotypes (see Table I, mutation category 4).

21-OH deficiency haplotypes that carry three or more CYP21 + C4 units

If the genesis of 21-OH deficiency haplotypes is attributable to unequal crossover, haplotypes with three CYP21 + C4 units should be expected. The HLA-DR1 B14 haplotype that is strongly associated with non-classical 21-OH deficiency has been inferred from short-range mapping and complement allotyping studies to have three CYP21 + C4 units (Carroll *et al.*, 1984; Raum *et al.*, 1984; Uring-Lambert *et al.*, 1984; Werkmeister *et al.*, 1986). Of these, two seem to contain CYP21A-like genes and one contains a CYP21B-like gene that appears to have a point mutation which renders gene expression defective (Speiser *et al.*, 1988). In addition, two of the C4 genes are short C4B genes (characterized by

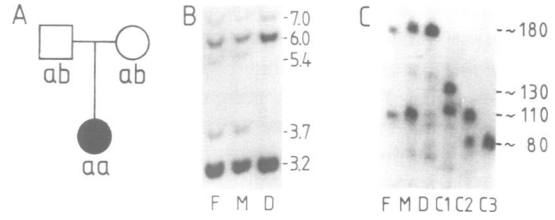

Fig. 4. Identification of a 21-OH deficiency haplotype with four CYP21 + C4 gene repeat units. **Panel A**: pedigree of family 28. The affected child has the salt-wasting form of 21-OH deficiency. HLA haplotypes are: a, DR4 B51 A31; b, DR8 B57 A29. **Panel B**: Southern blot analysis of genomic DNA from individuals in family 28. Lanes represent *Taq*I-digested DNA from the following family members: **F**, father; **M**, mother; **S**, son. DNA blots were probed simultaneously with a combination of a 21-OH-specific DNA probe, 21A-1.8, and a complement C4-specific probe, C4B550. Figures represent the size in kilobases of indicated bands. **Panel C**: pulsed field gel electrophoresis analysis of genomic DNA from individuals in family 28. Lanes represent DNA samples from the following family members: **F**, father; **M**, mother; and **D**, daughter. In addition, control samples **C1–C3** represent an individual that is heterozygous for a triplication haplotype, HLA-DR1 B14 (**C1**), a deletion heterozygote (**C2**) and a deletion homozygote (**C3**). DNA samples from indicated individuals were digested with *Bss*HII prior to size fractionation by pulsed field gel electrophoresis (see Materials and methods). Pulsed field gel electrophoresis gels were dried down prior to direct probing with the 21-OH-specific probe, 21A-1.8.

5′ 5.4 kb *Taq*I RFLVs) and one is long (C4A). When we have examined HLA-DR1 B14 haplotypes by *Bss*HII mapping, there is a correlation in all cases we have investigated with a 130 kb B*ss*HII RFLP, as expected of a haplotype with three CYP21 + C4 units of the type described above (e.g. Figure 4, lane C1).

In addition to the HLA-DR1 B14 haplotype we have identified four other patient haplotypes that carry more than two CYP21 + C4 repeat units (see Table I). Two of these have the same triplication organization, as on the B14 DR1 haplotypes, while a third haplotype, DR7 B50 A29, shows a closely similar structure but with two CYP21B-like genes and one CYP21A-like gene, as inferred from *Taq*I RFLVs. The fourth haplotype is exceptional in possessing four CYP21 + C4 units and was observed in a family where there is a very high degree of parental consanguinity (Figure 4, panel A). In this family the parents are HLA identical through common descent and the affected child is HLA homozygous as a consequence of inheriting the same disease haplotype from both parents. When members of this family were investigated by the *Taq*I mapping approach, the affected child appeared to show a similar pattern to those in families 1 and 8: lack of the CYP21B-associated 3.7 kb RFLV but presence of both a C4A-associated 7.0 kb and a C4B-associated 6.0 kb RFLV (Figure 4, panel B). However, densitometric analysis suggests that the 6.0 kb band is approximately three times as intense as the 7.0 kb band. Also, both parental samples show ratios of ~5:1 between the 3.2 and 3.7 kb bands and a ratio of ~2:3:1 for the 7.0:6.0:5.4 kb comparison (data not shown). Investigations using the *Bss*HII mapping procedure reveal two RFLPs in the parents of ~110 and 180 kb. In the affected child there is a single major *Bss*HII hybridization band of 180 kb (minor *Bss*HII hybridization bands, especially visible in the DNA samples from the affected child, represent infrequently

cutting *Bss*HII sites which are known to occur within the CYP21 gene sequences) (see Discussion). Consequently, the possibility is suggested that the disease haplotype in this family bears four CYP21 + C4 gene units. All the C4 genes in the affected individual are of the long variety (one C4A gene plus three long C4B genes each accompanied by the 6.0 kb *Taq*I RFLV). Accordingly, the disease haplotype would be expected to generate a *Bss*HII fragment ~46 kb longer than that exhibited by DR1 B14 haplotypes (one long C4A gene and two short C4B genes), which agrees well with the observed size difference between the two *Bss*HII RFLPs (see Figure 4, lanes D and C1).

All four CYP21 genes in the disease haplotype above appear to resemble CYP21A in carrying the 3.2 kb *Taq*I RFLV and in presumably being defective in gene expression. Accordingly, this haplotype may be viewed as an extreme example of mutation category 4 (see Table I). Within this category, four haplotypes in particular were amenable to further investigation: the two haplotypes found in individuals that are HLA homozygous as a consequence of parental consanguinity (patients in families 1 and 28) (see Figures 3 and 4) and two independent haplotypes found in the same individual (the patient in family 8) (see Figure 3). In order to investigate further the CYP21 genes of such haplotypes, we have amplified *in vitro* selected regions of the CYP21 genes of patients in families 1, 8 and 28. In particular we have focused on two intragenic regions. One of these includes a sequence equivalent to the beginning of exon 3 in CYP21B genes. In the corresponding CYP21A gene sequence there is an 8 bp deletion which introduces a frameshift mutation (Higashi *et al.*, 1986; White *et al.*, 1986; Rodrigues *et al.*, 1987). Oligonucleotide primers were designed to hybridize specifically to well-conserved sequences which closely flank the vicinity of the 8 bp deletion. In this way it is possible to score directly for the presence of the 8 bp deletion by simple length variation: the amplified product in genes lacking the deletion would be expected to be 64 bp long, and that in genes containing the deletion to be 56 bp long (Figure 5, upper left panel). When this analysis was performed on DNA samples from patients in families 1, 8 and 28, a single amplification band of 56 bp was observed in all cases, suggesting the presence only of CYP21 genes which resemble CYP21A in carrying the 8 bp deletion (Figure 5, upper right panel, lanes a, e and f). Probing of dot blots of amplified products using allele-specific oligonucleotides (pm1a and pm1b—see Materials and methods) has confirmed that the 64 and 56 bp bands correspond to the desired amplified products (data not shown).

The second intragenic region that we have investigated includes sequence equivalent to the beginning of exon 8 in CYP21B genes. The corresponding sequence in CYP21A genes is considered to be defective in expression as a consequence of a specific C→T transition which introduces a premature termination codon, TGA (Higashi *et al.*, 1986; White *et al.*, 1986; Rodrigues *et al.*, 1987). The nucleotide substitution also has the effect of altering a restriction site, i.e. a *Pst*I recognition site found in the CYP21B gene sequence is mutated in the corresponding CYP21A sequence (Figure 5, lower left panel). Oligonucleotide primers representing conserved sequences that flank the mutation site permit *in vitro* amplification to generate a product that is either resistant to *Pst*I digestion (314 bp) or which is cleaved

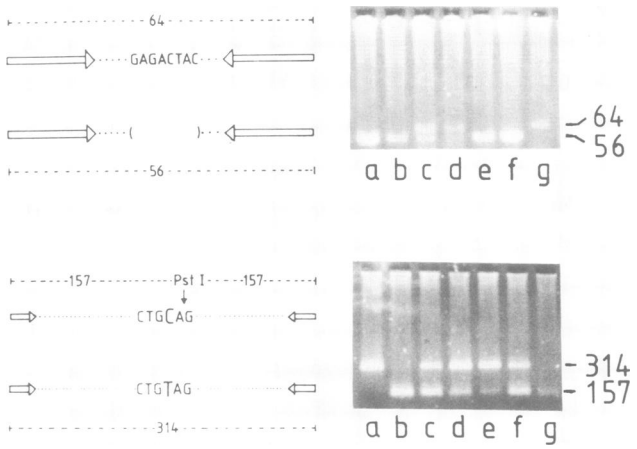

Fig. 5. Mapping of pathological point mutations in CYP21 genes on haplotypes where the CYP21B gene has been replaced by a gene resembling CYP21A. **Left panels**: strategies for identifying CYP21 genes that contain point mutations normally associated with the CYP21A pseudogene. The investigated point mutations are a deletion of 8 bp normally found in exon 3 of the CYP21B gene (**upper panel**) and a substitution of a C residue normally occurring at the beginning of exon 8 in CYP21B genes (**lower panel**). **Right panels**: polymerase chain reaction analysis on genomic DNA samples from individuals that have a 21-OH deficiency haplotype where the normal CYP21B gene has been replaced by a gene resembling CYP21A. **Upper right panel**: direct size fractionation by agarose gel electrophoresis of DNA that has been amplified using oligonucleotide primers as indicated in upper left panel. **Lower right panel**: size fractionation by agarose gel electrophoresis of *Pst*I-digested DNA that has been amplified using oligonucleotide primers as indicated in lower left panel. Individuals with a 21-OH deficiency haplotype where the CYP21B gene has been replaced by a gene resembling CYP21A are represented by the following lanes: **a**, patient in family 28 (see Figure 3); **e**, patient in family 8 (see Figure 2); **f**, patient in family 1 who is homozygous for a haplotype containing a single CYP21 gene resembling CYP21A (see Figure 2). Control samples are as follows: **b**, patient in family 6 (see Figure 1); **c** and **d**, individuals which appear on the basis of *Taq*I and *Bss*HII mapping to contain a CYP21A and a CYP21B gene on both haplotypes; **g**, an individual who is homozygous for the HLA DR3 B8 A1 haplotype which contains a single CYP21 gene resembling CYP21B.

by *Pst*I to generate two fragments of identical size (157 bp). In the case of the DNA sample from the patient in family 28, a single band of 314 bp is observed following complete *Pst*I digestion of amplified product, indicating that all four CYP21 genes on the identical disease haplotypes resemble CYP21A regarding this intragenic region (Figure 5, lower right panel, lane a). However, the patient in family 1 shows equality in intensity between the 314 and 157 bp bands, while there is an ~3:1 ratio between the intensities of 314 and 157 bp bands in the case of the patient in family 8 (Figure 5, lower right panel, lanes e and f). In the former case, we conclude that one of the CYP21 genes on the patient's two identical disease haplotypes shows some characteristics expected of a CYP21B gene. In the latter case, one of the patient haplotypes appears to bear only CYP21 genes that resemble CYP21A in this region, while the other haplotype bears at least one gene that shows some CYP21B-like sequence as well as a CYP21A-like gene. Again, probing of dot blots of amplified products using allele-specific oligonucleotides (pm3a and pm3b—see Materials and methods) has confirmed that the 314 and 157 bp bands correspond to the desired amplified products (data not shown).

Discussion

A new approach to analysing 21-OH gene organization

There has been considerable controversy in recent years over the authenticity of gene deletions that contribute to a 21-OH deficiency (Matteson *et al.*, 1987; Miller, 1988b; White *et al.*, 1988). A major problem has been that the analysis of gene organization at the duplicated CYP21 and C4 loci has been necessarily indirect. The size of the basic CYP21 + C4 gene repeat unit is ~30 kb and deletions or additions of units of this size cannot be detected directly by conventional agarose gel electrophoresis. Consequently 21-OH-specific and C4-specific DNA probes have been widely used to distinguish between the two CYP21 gene homologues and the two C4 gene homologues by identifying locus-specific restriction fragments (RFLVs). There are several problems with this indirect approach. Firstly, individual analyses focus on the combined CYP21 and C4 genes present on two haplotypes. Because of the frequent occurrence of haplotypes showing evidence of gene deletion and gene expansion (see Table I and below), the inference of gene organization at the CYP21 + C4 loci in a single individual is unreliable. Secondly, the locus specificity of RFLVs is not absolute as a consequence of interlocus sequence transfer due to unequal crossover or other gene conversion-like mechanisms (Harada *et al.*, 1987; Higashi *et al.*, 1988a,b). Intragenic unequal crossover would be expected to generate haplotypes with fusion gene products that show characteristics common to both gene homologues (White *et al.*, 1988). Thus, the single CYP21 gene on deletion haplotypes in the families illustrated in Figure 2 bears a CYP21A-associated 3.2 kb *Taq*I RFLV but 15 kb *Eco*RI and 10.5 kb *Bgl*II RFLVs which are associated in both cases with the CYP21B locus. Presumably, in these cases, the unequal crossover event that generated the deletion haplotypes occurred at a position between the variable *Taq*I site which distinguishes the 3.2 and 3.7 kb *Taq*I RFLVs (~200 bp 5′ to the CYP21 genes) and the analogous variable *Bgl*II and *Eco*RI sites which are located several kilobases 3′ to the CYP21 genes.

Confidence in the gene organization inferred from short-range restriction mapping can be increased by means of family studies, whereby individual haplotypes can be compared against a background of several different haplotypes. Additionally, analyses are facilitated by haplotype identity and the families in the present study have been selected to provide a high frequency of HLA homozygosity (the affected children in eight families are HLA homozygous due in six of the families to known parental consanguinity). Because the scope of short-range mapping is limited, however, we have applied a long-range *Bss*HII mapping procedure to permit a more direct anlaysis of gene organization at the CYP21 and C4 loci. The method relies on the presence of well-conserved *Bss*HII sites that flank the array of CYP21 and C4 genes. Although the established DNA sequences of CYP21 genes reveal a *Bss*HII recognition site in the middle of exon 10 in the CYP21B gene, and in the corresponding CYP21A sequence, cleavage of genomic DNA at this site by *Bss*HII is severely inhibited (presumably due to methylation) and generates, at most, weak *Bss*HII hybridization bands. However, cleavage at the flanking *Bss*HII sites appears to be complete and generates one or two strong hybridization bands per individual, representing genuinely allelic restriction fragments. Thus, it is possible to monitor size variation due to variable number of tandem repeats in a manner directly analogous to the non-genic VNTR polymorphism commonly employed in linkage analyses (Nakamura *et al.*, 1987). The value of this method is particularly observed in cases where the *Taq*I mapping analyses gives unexpected ratios between the intensities of different CYP21-specific or C4-specific hybridization bands. Thus, although the *Taq*I analysis in family 28 was already greatly simplified by the sharing of two pairs of parental haplotypes as a consequence of common descent (see Figure 4), the comparatively unusual ratios between the intensities of the various *Taq*I hybridization bands did not lend themselves to a simple interpretation of gene organization. Finally, in addition to detecting length variation due to differences in the number of CYP21 + C4 repeat units, the *Bss*HII mapping analysis is able to detect minor length heterogeneity of individual repeats due to differences of 6.5 kb between the sizes of different C4 genes (see above). The gross gene organization at the CYP21 and C4 loci in individual genomic samples may now be inferred with confidence by a combination of *Taq*I and *Bss*HII mapping.

Unequal crossover and interlocus sequence transfer

The tandemly duplicated nature of the CYP21 and C4 genes has been widely assumed to facilitate unequal crossover resulting in the genesis of haplotypes that have a deficiency or surplus of CYP21 + C4 repeat units, each ~30 kb long. However, the evidence for the involvement of unequal crossover at the CYP21/C4 loci has hitherto been based on indirect complement allotyping studies or short-range restriction mapping approaches which do not have the necessary resolution to permit direct size analyses, and whose interpretation has been the subject of much controversy. In contrast, the long-range restriction mapping procedure described in the present report provides a direct means of evaluating the gene organization on individual haplotypes. In each of the gene-deletion haplotypes that we have investigated the haplotype length is ~30 kb less than conventional haplotypes, while the length of the gene-addition haplotypes exceeds that of normal haplotypes also by integral multiples of the CYP21 + C4 repeat unit length. Consequently, the length variation detected by *Bss*HII mapping strongly supports the involvement of unequal crossover in the generation of gene deletions and gene additions at the CYP21 and C4 loci.

The occurrence of haplotypes which show evidence of loss of CYP21 + C4 repeats appears to be frequent. Thus, 13% (8/62) of non-disease haplotypes and 41% (24/58) of disease haplotypes analysed in the present study show evidence for deletion of a CYP21 + C4 repeat unit. In the non-disease group six out of the eight haplotypes show a diagnostic C4-specific 6.4 kb *Taq*I hybridization band and include four examples of the common HLA-DR3 B8 A1 haplotype, an HLA-DR3 B8 A2 haplotype and an HLA-DR3 B15 A1 haplotype. In all six cases the deletion involves loss of CYP21A and C4-associated *Taq*I RFLVs, as has been reported previously in the case of the HLA-DR3 B8 A1 haplotype (Carroll *et al.*, 1985b). The deletions on the remaining two haplotypes lead to loss of CYP21A- and C4B-associated *Taq*I RFLVs. In the case of the disease haplotypes 79% (19/24) of the deletions have resulted in the loss of the

CYP21B-associated 3.7 kb *Taq*I RFLV so that 21-OH deficiency if expected to be a direct consequence of the elimination of functional CYP21B gene sequence. Of the remainder, one is an HLA-DR3 B8 A1 haplotype which conforms to the expected pattern of showing a 6.4 kb *Taq*I RFLV and absence of the C4A- and CYP21A-associated markers. In this case the accompanying association with 21-OH deficiency would not be expected to be due to the gene deletion itself, but rather to a pathological point mutation in the residual CYP21B gene. The origin of the pathological mutation of the other four haplotypes is, however, uncertain (mutation category 2 in Table I). In each case, the deletion has led to loss of C4B- and CYP21A-associated *Taq*I RFLVs. The possibility that the deletion has not compromised the expression of the residual CYP21B-like gene is suggested by the observation of two non-disease haplotypes with apparently the same class of deletion, the difference presumably being that the CYP21B gene on the disease haplotypes has acquired a pathological point mutation. An alternative possibility is that the deletion has encroached into a region upstream of the CYP21B gene so as to eliminate 5′ flanking sequence which may be required for gene expression (Handler *et al.*, 1988).

From our combined analyses, a total of five disease haplotypes showed evidence of gene addition at the CYP21 and C4 loci, although only one of the non-disease haplotypes, DR6 Bw62 A2, fell into this category. The previous evidence for gene expansion haplotypes has been inferred initially from complement allotyping studies (Raum *et al.*, 1984; Uring-Lambert *et al.*, 1984) which have been supported by short-range restriction mapping analyses using C4- or CYP21-specific DNA probes (Carroll *et al.*, 1984; Garlepp *et al.*, 1986; Schneider *et al.*, 1986). There are, however, limitations on the confidence with which gene organization can be interpreted on the basis of densitometric ratios alone, and the long-range mapping procedure is invaluable in permitting accurate assessment of the number of CYP21 + C4 units on individual haplotypes.

The most exceptional of the gene addition haplotypes we have identified is a disease haplotype which has four CYP21 + C4 repeat units and whose genesis would be presumed to necessitate several rounds of unequal crossover. In this haplotype each of the four CYP21 genes resembles CYP21A in at least four respects: (i) possession of a *Taq*I site not found in CYP21B genes at a position equivalent to ~200 bp upstream of the transcriptional initiation site (thereby generating the 3.2 kb *Taq*I RFLV—see Figure 4); (ii) absence of a *Kpn*I site found in CYP21B genes at a position ~100 bp upstream of the transcriptional initiation site (thereby generating a 4.0 kb *Kpn*I RFLV which differentiates CYP21A genes from the CYP21B-associated 2.9 kb *Kpn*I RFLV—data not shown); (iii) absence of an 8 bp sequence normally found in exon 3 of CYP21B genes—Figure 5; and (iv) substitution of a C residue at a position near the beginning of exon 8 in CYP21B genes—Figure 5.

In the present study a minimum of 10% of disease haplotypes have been identified which show no evidence for gene deletion but where the normal CYP21B gene appears to have been replaced by a gene that resembles CYP21A in many characteristics (see Table I, mutation category 4). On the basis of short-range mapping alone, other recent studies have also defined minority disease haplotypes which appear to have a similar organization (Donohoue *et al.*, 1986; Jospe *et al.*, 1987; Rumsby *et al.*, 1988; White *et al.*, 1988). However, the above frequency of such haplotypes is likely to be a considerable underestimate since many of the CYP21B genes in which pathological point mutation is inferred (see Table I, mutation category 3) may represent CYP21A−CYP21B fusion genes which retain the CYP21B-associated 3.7 kb *Taq*I RFLV (Higashi *et al.*, 1988a). In addition to known CYP21A pseudogenes, the mutant CYP21 genes that have been sequenced previously include one which resembles a CYP21A gene, at least in terms of 5′ flanking markers, and also the sequence of a region ~250 bp long corresponding to the 3′ half of intron 2 and the 5′ half of exon 3 (Harada *et al.*, 1987). Also the other mutant CYP21B genes show evidence of interlocus sequence transfer. Candidate sites of pathological point mutation in these genes are mostly represented by sequences that are identical to the equivalent CYP21A gene sequence (Rodrigues *et al.*, 1987; Amor *et al.*, 1988; Speiser *et al.*, 1988; Higashi *et al.*, 1988b). While the occurrence of simple fusion genes is suggestive of interlocus sequence transfer due to unequal crossover, more complex mosaic patterns could be generated by gene conversion or possibly by multiple recombination events. We are currently examining selected regions of mutant CYP21B genes which have been amplified *in vitro* to map the extent of possible interlocus sequence transfer.

Clinical, mutational and HLA haplotype heterogeneity

Of the 32 families investigated in the present study, 30 show classical 21-OH deficiency, of which 22 families have affected children with salt-wasting symptoms. Although correlation between clinical phenotypes and specific mutation categories was not significant in the case of compound heterozygotes, the salt-wasting form of the disorder was observed in 7/7 families where the affected children lacked a 3.7 kb *Taq*I RFLV and were homozygous either for CYP21B deletion haplotypes or for haplotypes where the CYP21B gene is replaced by a CYP21A-like gene. This observation would therefore support the idea that the salt-wasting phenotype is associated with mutations that are likely to have a profound effect on normal gene expression (gene deletion, 8 bp deletion in exon 3, etc.).

Correlation between serologically defined polymorphism at the HLA and associated complement loci, with DNA-based polymorphism in this region, reveals a number of features. Firstly, haplotypes that appear to carry a pathological point mutation in the CYP21B gene are generally associated with a diverse set of HLA antigens. However, one particular haplotype, HLA-DR4 C4B6 C4A4 BfS Bw55 A11, was represented in 14% (4/28) of such haplotypes. Also 53% (10/19) of disease haplotypes that carry a deletion of CYP21B gene sequence carry the normally rare HLA-B allele, Bw47, while 42% (8/19) of such haplotypes are characterized by the extended haplotype HLA-DR7, C4BQo C4A1 BfF Bw47 A3. The latter findings are consistent with those of others (White *et al.*, 1984; Boehm *et al.*, 1986; Schneider *et al.*, 1986; Jospe *et al.*, 1987) who have consistently demonstrated apparent absence of the CYP21B gene on Bw47-bearing haplotypes. However, the haplotypic correlation between possession of a Bw47 allele and absence of a CYP21B gene is not absolute, as reported from studies of the Old Order Amish (Donohoue *et al.*, 1987); also in the present study we have detected two Bw47-bearing

haplotypes that show no evidence for gene deletion, one of them being the extended haplotype described above. In addition, we have observed one case where a HLA-DR3 B8 A1 haplotype shows no evidence of CYP21 or C4 gene deletion as inferred from the demonstration in an individual who is homozygous for this haplotype of both 80 and 110 kb CYP21-specific *Bss*HII RFLPs.

Materials and methods

HLA antigen and complement typing

HLA-A, HLA-B, HLA-DR, factor B and C4 typing were performed as described previously (Sinnott *et al.*, 1989).

DNA probes and Southern blot hybridization

The probes used were 21A-1.8, a 21-OH-specific probe, and C4B550, a C4-specific probe. The origin of the probes, their radiolabelling conditions and Southern blot hybridization conditions were as described previously (Sinnott *et al.*, 1989).

Oligonucleotide probes and dot-blot hybridization

The probes used were as follows: pm1a, 5′ TTGGTCTCTGCTCTGGAA-AGC 3′; pm1b, 5′ TTGGGAGACTACTCCCTGCTC 3′; pm3a, 5′ AGCGACTGTAGGAGGAG 3′; pm3b 5′ AGCGACTGCAGGAGGAG 3′. Probes were end labelled using [γ-^{32}P]ATP and T4 polynucleotide kinase in a reaction buffer containing 70 mM Tris−HCl pH 9.2, 10 mM $MgCl_2$, 5 mM spermidine, 20 mM dithiothreitol (DTT). Hybridization was conducted in a 6 × SSC, 0.1% SDS solution containing 5 × 10^5 c.p.m. labelled probe/ml for 2 h at 45°C (pm3a and pm3b) or at 50°C (pm1a and pm1b). Final hybridization washes were conducted in 5 × SSC, 0.1% SDS at 52°C (pm3a and pm3b) or at 60°C (pm1a and pm1b).

Pulsed field gel electrophoresis analysis

Agarose blocks containing ~3 μg of genomic DNA were washed in TE buffer (10 mM Tris−HCl pH 8.0, 0.1 mM EDTA) containing 0.04 mg/ml phenylmethylsulphonyl fluoride (PMSF) and subsequently in TE buffer prior to restriction nuclease digestion. Blocks were incubated with 12 U *Bss*HII for 4 h at 50°C in the buffer recommended by the manufacturers (New England Biolabs). Electrophoresis was conducted in a LKB pulsaphor apparatus using a modification of the contour-clamped homogeneous electric field described by Chu *et al.* (1986). Blocks were inserted in a 1.0% (w/v) agarose gel and DNA fragments were resolved over a period of 40 h at 130 V using a pulse interval of 10−15 s and a running buffer of 0.5 × TBE. Mol. wt markers were provided by concatemers of lambda cI857S7. Dried gels were immersed in hybridization buffer containing radioactively labelled probe as above. Hybridization was conducted overnight at 65°C and hybridization washes were concluded in 0.1 × SSC at 70°C prior to autoradiography as above.

In vitro DNA amplification

Genomic DNA (0.5−1.0 μg) was amplified *in vitro* using the polymerase chain reaction (Saiki *et al.*, 1988). The reaction was conducted in a total volume of 100 μl in a buffer containing 67 mM Tris−HCl pH 8.0, 16.6 mM $(NH_4)_2SO_4$, 6.7 mM $MgCl_2$, 6.7 μM EDTA, 0.017% bovine serum albumin (BSA) and 10 mM β-mercaptoethanol. Oligonucleotide primers were included at a final concentration of 1 μM and the reaction was catalysed using 5 U *Taq*I polymerase. Oligonucleotide primers were as follows: 5′ pm1, 5′ GTCTAA/GGAACTACCCGGACC/TTGTC 3′; 3′ pm1, 5′ GACGAGACCTTTCGGGTGTTCTTC 3′; 5′ pm3, 5′ ACCCTCACT-CAGCTCTGAGCACTGTGC 3′; 3′ pm3, 5′ AATCGGAACGGGGTG-GCGTGGTGTGCCG 3′.

In each case the reaction involved 30 amplification cycles (denaturation at 93°C, reannealing at 55°C, synthesis at 67°C) and 20 μl aliquots were removed for analysis of the amplified DNA. DNA which had been amplified using the 5′ pm1 and 3′ pm1 primers was directly size fractionated on 6% (w/v) NuSieve agarose gels in TBE buffer (4 V/cm). DNA which had been amplified using the 5′ pm3 and 3′ pm3 primers was purified by conventional phenol extraction and ethanol precipitation prior to complete digestion with *Pst*I (usually 20 U, overnight at 37°C) in a buffer recommended by the manufacturers (Boehringer-Mannheim). Terminal digestion was monitored using internal DNA conrols which were selected to reveal *Pst*I cleavage products of known fragment size which did not co-migrate with digested amplified DNA products. Fragments were fractionated by electrophoresis in 2% (w/v) NuSieve agarose gels in TBE buffer (6 V/cm).

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