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Hereditary hypertension caused by chimaeric gene duplications and ectopic expression of aldosterone synthase

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Patients with glucocorticoid-remediable aldosteronism (GRA) from 12 kindreds possess chimaeric gene duplications arising from unequal crossing-over, fusing regulatory sequences of steroid 11β-hydroxylase to coding sequences of aldosterone synthase. These chimaeric genes are specific for GRA and explain the biochemistry, physiology and genetics of this form of hypertension. Sites of crossing over range from intron 2 to intron 4. Most mutations have arisen independently from either sister or non-sister chromatid exchange between these genes, which are only 45 kilobases apart. The possibility of a susceptibility allele for GRA of Irish origin is suggested. These findings indicate the utility of a direct genetic test for this disorder.

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Hypertension is a common disorder of largely unknown cause contributing to over 200,000 deaths in the United States alone each year due to myocardial infarction, stroke and end-stage renal disease[1]. Evidence from twin studies, population-based epidemiologic and adoption studies has demonstrated a strong genetic component to this trait, suggesting that mutations contributing to the pathogenesis of hypertension can be found (reviewed in ref. 2).

Several mendelian hypertensive syndromes have been described in humans. Among these, glucocorticoid-remediable aldosteronism (GRA) is characterized by autosomal dominant transmission of hypertension, variably elevated aldosterone levels with suppressed plasma renin activity[3,4] and high levels of abnormal adrenal steroids, 18-hydroxycortisol and 18-oxocortisol[5-7]. These aberrant steroids and aldosterone, which is normally under control of angiotensin II, are all under positive control of adrenocorticotrophic hormone(ACTH), and are consequently suppressible by exogenous glucocorticoids[1,2,8]. Penetrance of the biochemical features and hypertension is high, with the hypertension often severe and dating from childhood[9].

The genes encoding steroid 11β-hydroxylase (11-OHase)[10] and aldosterone synthase (AldoS)[11,12] are 95% identical in nucleotide sequence[10], are both present on chromosome 8[10,13] and show evidence of linkage[14]. The 11-OHase gene is normally expressed in both the adrenal fasciculata and glomerulosa, where it is involved in the biosynthesis of cortisol and aldosterone, respectively; in the fasciculata, this gene is regulated by ACTH[15]. The AldoS gene is normally expressed only in adrenal glomerulosa[16] where its product catalyses the final two steps in aldosterone biosynthesis.

We have recently described a large GRA pedigree in which the disease co-segregates with a chimaeric gene duplication arising from unequal crossing-over between steroid 11-OHase and AldoS genes[14]. We have proposed that this duplication is a neomorphic regulatory mutation which accounts for the known biochemistry, physiology and genetics of GRA by fusing the ACTH-responsive regulatory sequences of 11-OHase to coding sequences of aldosterone synthase. This results in ectopic expression of AldoS activity in the adrenal fasciculata under control of ACTH[14] (Fig. 1).

A critical requirement of this model is that analysis of the mutation in this kindred should reveal fusion of 11-OHase and aldosterone synthase genes at the DNA sequence level. Also, these findings raise the possibility that these mutations will prove to be the common cause of GRA. We now address these issues by the characterization of the molecular lesions responsible for GRA in 12 kindreds.

5' 11-OHase/AldoS 3' gene duplications in GRA

The structure of the chimaeric gene in 12 GRA patients from pedigree K2061[14] suggests the use of a simple screening test for similar chimaeric gene duplications arising via unequal crossing over between 11-OHase and AldoS genes. Digestion of genomic DNA with either *Bam*HI or *Eco*RI will yield characteristic signatures of chimaeric gene duplications upon hybridization with appropriate probes (Fig. 2*a*).

We have analysed DNA samples from an additional 17

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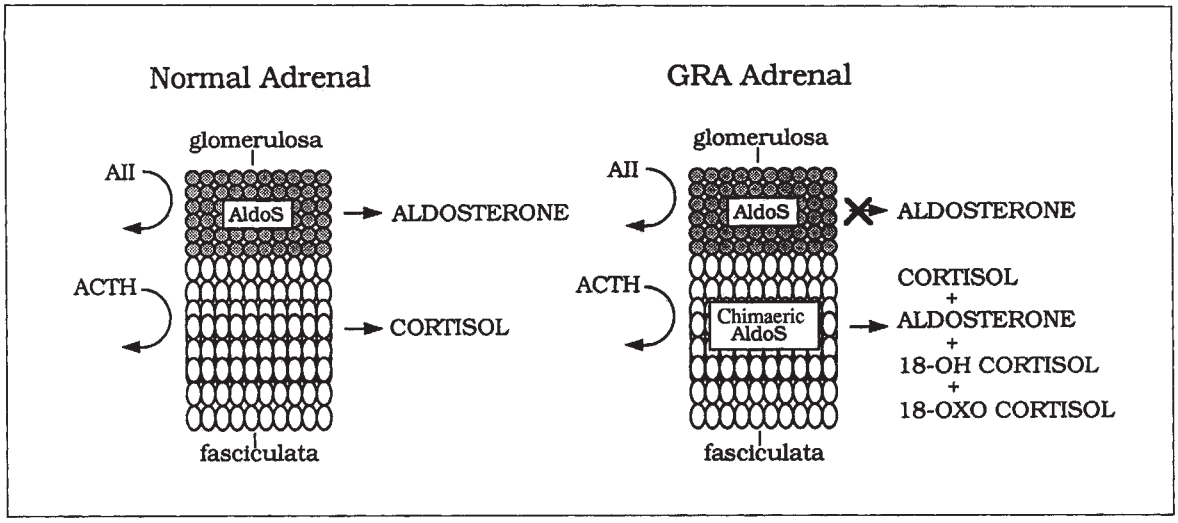

Fig. 1 Model of the physiologic abnormalities in the adrenal cortex in GRA. In the normal adrenal gland, AldoS activity is present only in the adrenal glomerulosa. Aldosterone is produced in the glomerulosa under regulation of angiotensin II, and cortisol is secreted from the adrenal fasciculata under regulation of ACTH. Steroid 11β-hydroxylase is involved in the biosynthesis of both of these hormones, and is expressed in both tissues. This gene is under positive control of ACTH in fasciculata[15]. In the GRA adrenal gland, the chimaeric gene is expressed in the fasciculata under control of ACTH by virtue of the 5' regulatory sequences of 11-OHase, but has AldoS enzymatic activity due to coding sequences of the AldoS gene. Ectopic activity of this enzyme in fasciculata results in metabolism of cortisol to 18-hydroxycortisol and 18-oxocortisol, as well as production of aldosterone from high levels of corticosterone present in fasciculata. These mineralocorticoids are under control of ACTH, and can consequently be suppressed by exogenous glucocorticoids. This dysregulation of mineralocorticoid secretion results in mineralocorticoid excess and consequent hypertension. Long-term suppression of the renin-angiotensin system results in unresponsiveness of the adrenal glomerulosa to physiologic or pharmacologic stimulation with angiotensin II[31].

GRA patients from 11 pedigrees not known to be related either to each other or K2061. Hybridization of an exon 3–4 probe from 11-OHase to genomic DNA of all these subjects demonstrates the presence of 6.3 kilobase (kb) *Bam*HI or 22 kb *Eco*RI fragments characteristic of gene duplications arising via unequal crossing over between 11-OHase and AldoS genes upstream of intron 5 (Fig. 2*b*). Inspection of the intensity of hybridization indicates equal signal from the normal 11-OHase and AldoS genes, with lower signal from the chimaeric gene. Quantitation (not shown) confirms a ratio of 11-OHase:mutant:AldoS genes of 2:1:2 in all cases, indicative of a single additional gene per diploid genome, and incompatible with gene conversion or simple polymorphism as the origin of the anomalous fragments.

Further evidence supporting gene duplication comes from quantitation of PCR products[17] in pedigree members heterozygous for the single strand conformational polymorphism (SSCP) marker, AldoX9[14], which specifically amplifies a segment of exon 9 of AldoS. If unequal crossing over has resulted in a gene duplication of the structure shown, the 3' end of AldoS including exon 9 should be duplicated and therefore present three times per diploid genome; consequently, heterozygotes for polymorphic markers at the 3' end of this gene should show a 2:1 ratio of intensity of alleles. Figure 3 indicates that this is indeed the case in GRA subjects heterozygous for AldoX9, consistent with 3 copies of this AldoS segment per diploid genome. One hundred and forty-five unaffected heterozygotes show 1:1 ratios of alleles (Fig. 3; ref. 14 and unpublished data).

If unequal crossing over has fused 5' 11-OHase sequences to 3' AldoS sequences, the duplication should carry 5' sequences specific for 11-OHase and not AldoS. This was tested by hybridization of segments specific for the 5' flanking region of either 11-OHase or AldoS to blots of genomic DNA of GRA patients from each of the different pedigrees. In all cases, the 5' flanking sequences of 11-OHase specifically hybridize to the duplicated gene, while 5' sequences of AldoS do not (Fig. 4).

These results demonstrate that all 29 GRA patients studied (12 from K2061, plus 17 herein) harbour chimaeric gene duplications arising by unequal crossing over and fusing 5' 11-OHase sequences to more distal AldoS sequences.

The specificity of these chimaeric genes for GRA has been determined by searching for the presence of chimaeric gene signatures in *Eco*RI- and *Bam*HI- digested genomic DNA of 407 unrelated subjects not known to have a diagnosis of GRA. None of these subjects displayed either the anomalous *Bam*HI or *Eco*RI fragments characteristic of the gene duplication (not shown). These results thus far indicate 100% sensitivity and 100% specificity of chimaeric 11-OHase/AldoS gene duplications for GRA. This association is extremely strong ($p < 10^{-35}$ for association of clinical and genetic diagnosis of GRA, Fisher's exact test). In conjunction with prior linkage data demonstrating co-segregation of this gene with the disease, these results provide compelling evidence that these mutations are the cause of GRA in these pedigrees.

Variable sites of crossing over

We sequenced the mutant gene from 11 of our GRA kindreds to examine the unequal crossing-over model. In each case, exon 2 and a continuous segment from intron 2 to the end of exon 5 was sequenced (see Methodology), and the sequences of the mutant genes were compared to the normal AldoS and 11-OHase sequences.

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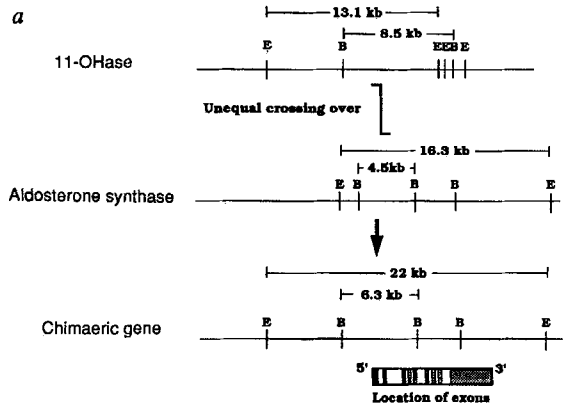
In all 11 pedigrees, the mutant gene displays the predicted chimaeric structure, fusing 5' 11-OHase sequences to more distal AldoS sequences (Fig. 5*a*). The transition from bases specific for 11-OHase to bases specific for aldosterone synthase is generally abrupt. Because of the extremely high homology of 11-OHase and AldoS, the site of crossing over can only be localized to within a few hundred base pairs in most cases (Fig. 5*b*). Three pedigrees show crossover sites within the 300 bp segment between the last base distinguishing the two genes in exon 4 and the first base distinguishing these genes in intron 4. Other sites of crossing over are within exon 4, at the junction of intron 2 and exon 3, and at least two different sites within intron 2. Three different chimaeric proteins are encoded among different pedigrees. The three pedigrees with the most distal breakpoint encode proteins with exons 1-4 specific for 11-OHase and exons 5-9 specific for AldoS, indicating that there are no AldoS residues encoded in exons 1-4 that are essential for the GRA phenotype and by inference, AldoS activity.



Haplotypes support independence of GRA mutations

The power to distinguish independent cross-overs can be increased by determining alleles of polymorphic markers tightly linked to the duplication; different haplotypes of

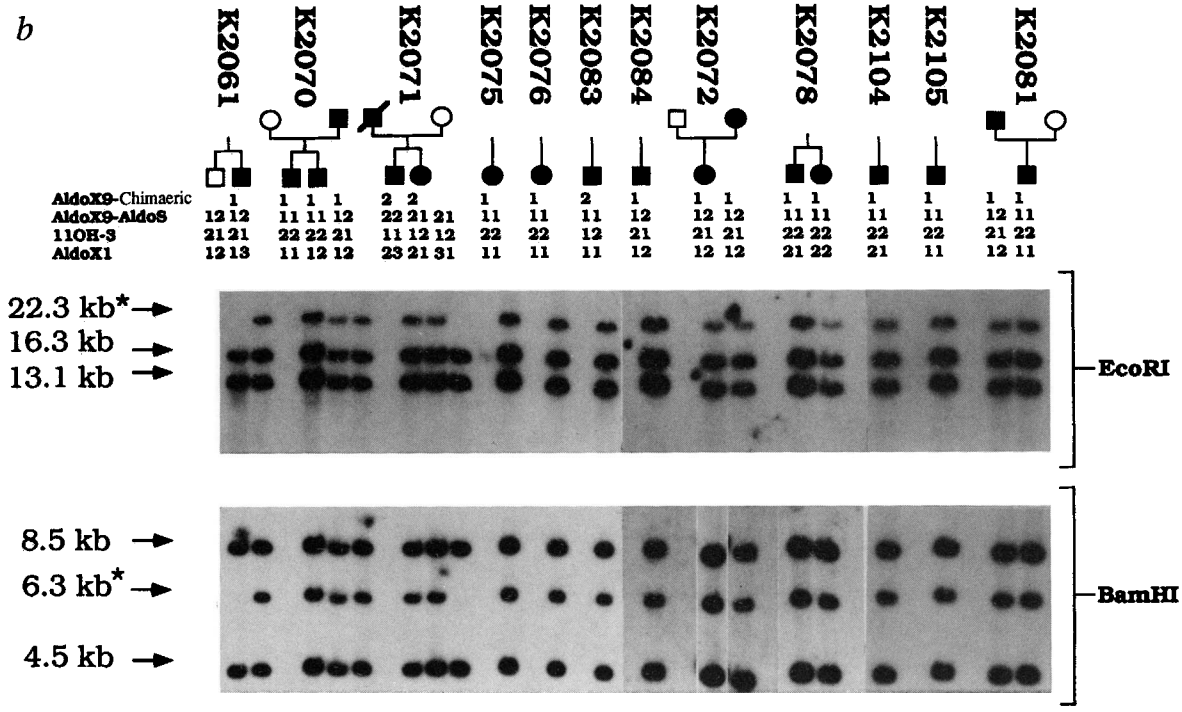


Fig. 2 Genotyping and Southern blotting of individuals from 12 GRA kindreds. *a*, Map of genomic DNA of 11-OHase and AldoS synthase genes and chimaeric gene duplications resulting from unequal crossing over between them[14]. Cleavage sites for the *Eco*RI and *Bam*HI are shown, and the length of the fragments detected by hybridization with the exon 3–4 probe is shown. The products of *Bam*HI digestion can detect chimaeric genes arising from unequal crossing-over anywhere between the 5' end of the genes up to intron 5; *Eco*RI digestion can detect chimaeric genes arising from unequal crossing over anywhere between the 5' end of the genes and intron 8. Importantly, the size of the resulting fragments from the duplicated gene will always be 6.3 kb (*Bam*HI) and 22 kb (*Eco*RI), regardless of the exact site of crossing over within these intervals. A diagram of the recombining chromosome is shown in Fig. 6*a*. *b*, Hybridization of exon 3–4 of 11-OHase to *Eco*RI- or *Bam*HI-digested genomic DNA of individuals from 12 GRA kindreds and genotypes for AldoS and 11-OHase markers. At the top of the panel family relationships of GRA pedigrees is shown. Individuals with GRA are indicated by black symbols. Below each symbol, genotypes for SSCP markers AldoX9 (in the 3' end of AldoS and chimaeric genes) 11OH-3 (in exon 5 of 11-OHase) and AldoX1 (in the 5' flanking region of AldoS) are shown. The allele of AldoX9 present on the chimaeric gene in GRA patients is shown separately, and in each case the haplotype on the GRA allele is shown in the left-most column. The lower panel shows results of hybridization of exon 3–4 of 11-OHase to *Eco*RI-cut and *Bam*HI-cut genomic DNA of these patients. Genomic DNA was digested with the indicated enzyme, fractionated via agarose gel electrophoresis (0.5% agarose gel for *Eco*RI blots, 0.7% agarose gel for *Bam*HI blots), transferred to nylon membranes and hybridized with the exon 3–4 probe. Subjects with GRA all have both the 6.3 kb *Bam*HI fragment and the 22 kb *Eco*RI fragment (both denoted by an asterisk) characteristic of chimaeric gene duplications. Eleven additional GRA patients from K2061 all have identical chimaeric gene signatures (10 subjects in reference 14, plus 2 additional subjects; R.P.L. unpublished).

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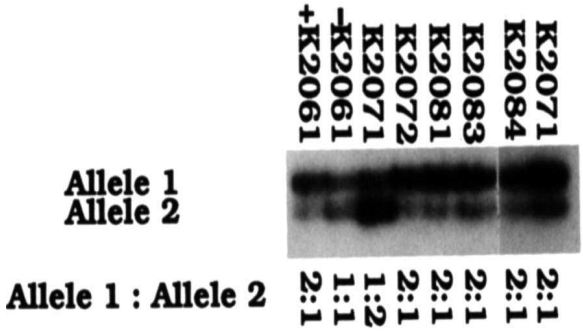

Fig. 3 Three copies of the 3' end of AldoS per genome in GRA patients. Marker AldoX9 (ref. 14) was genotyped by single strand conformational polymorphism[32]. Results from one affected heterozygote (+K2061) and one unaffected heterozygote (-K2061) from K2061 are shown, and one heterozygote from each other pedigree with at least one heterozygote is shown. Products were quantitated by densitometry of a film exposed in the absence of intensifying screens, and the ratios of allele 1:allele 2 are shown at the bottom of the figure; the ratios are all based on 3 independent amplifications of DNA of each subject. For GRA heterozygotes, the mean of these determinations ranged from 1.84–2.12; for unaffected heterozygotes the ratio ranged from .95–1.09. All GRA patients who are heterozygotes for this 2-allele system show an approximate 2:1 ratio of alleles, indicating 3 copies of the 3' end of AldoS per diploid genome. All 145 heterozygotes for this marker from CEPH and disease pedigrees who do not have GRA show a 1:1 ratio of alleles.

these markers will indicate mutations which arose independently. We have determined genotypes at 4 sites on GRA chromosomes using polymorphic SSCP markers which flank the site of unequal crossing-over[14]. AldoX1 detects a polymorphism in the 5' end of AldoS; AldoX9 detects a polymorphism in exon 9 of both the normal AldoS and the chimaeric gene; 11OH-3 detects a polymorphism in exon 5 of 11-OHase. These markers are all in strong linkage disequilibrium (see below), and demonstrate 4 different haplotypes on GRA chromosomes (Fig. 2*b*).

When these haplotypes are analysed in conjunction with sequence data, some pedigrees which cannot be distinguished from the site of crossing over have different haplotypes (Fig. 5*b*). For example, K2104 and K2083 show the same site of crossing over; the markers linked to the mutation, however, are discordant in 3 of 4 positions. From the combination of sequence and haplotype data, it is apparent that at least 8 of these 11 sequenced mutations arose independently.

The finding of independent mutations specifically associated and linked with GRA provides conclusive evidence that these mutations cause GRA, and are not merely in linkage disequilibrium with the true causal mutation.

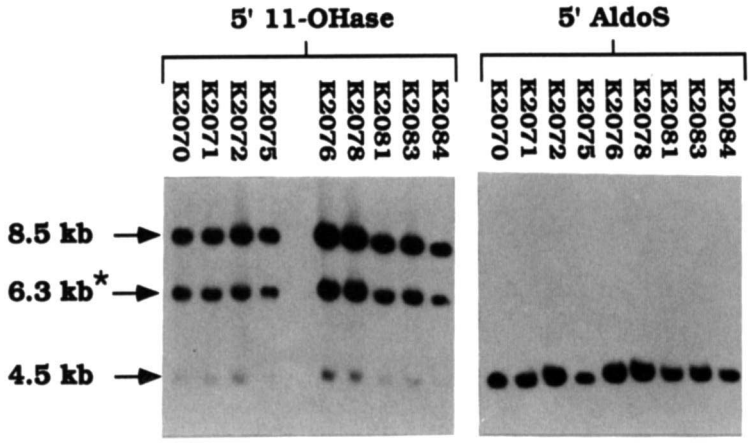

Fig. 4 Hybridization of fragments specific for the 5' end of either 11-OHase or AldoS to *Bam*HI-digested genomic DNA of patients with GRA. Genomic DNA of one affected subject from each GRA pedigree was digested with *Bam*HI and Southern blotting and hybridization with the indicated probes performed. In each case, the 6.3 kb *Bam*HI fragment derived from the chimaeric gene duplication hybridizes to the 5' end of 11-OHase but not the 5' end of AldoS. Results in the remaining three GRA pedigrees are identical (not shown). Specific hybridization of the probes to either the 8.5 kb fragment from the 11-OHase gene or the 4.5 kb fragment from the AldoS gene provides an internal control for the specificity of the probes; faint signal from 4.5 kb size fragments with the 5' 11-OHase probe is due to a 40 bp segment of homology between the probe and the AldoS gene.

Linkage disequilibrium of 11-OHase and AldoS

The occurrence of unequal crossing over between these genes suggests they are closely linked on chromosome 8 (ref. 14). If these genes were so close to one another that meiotic recombination in the interval between them rarely occurred, markers at these loci might prove to be in linkage disequilibrium. Three SSCP markers specific for either 11-OHase or AldoS were genotyped in 70 unrelated subjects from the CEPH pedigrees. The very strong linkage disequilibrium of these three markers indicates that native 11-OHase and AldoS genes must indeed lie very close to one another (Table 1).

Distance between 11-OHase and AldoS genes

The tight genetic linkage of 11-OHase and AldoS suggests that examination of the pulse-field gel products of GRA patients can be used to determine the distance between the two genes (Fig. 6*a*). In normal subjects, the 11-OHase and AldoS genes are both present on a single fragment of 130 kb after digestion of genomic DNA with *ClaI*; in contrast, GRA patients show two hybridizing fragments of 130 and 180 kb in length (Fig. 6*b*). As the length of the duplicated gene is approximately 6 kb, the distance between 11-OHase and AldoS is approximately 45 kb.

Origin of GRA duplications

Unequal crossing-over could occur either as recombination between sister or non-sister chromatids. By the mechanism of unequal crossing over, the duplicated 3' end of AldoS in the chimaeric gene and the 3' end of the normal AldoS gene in *cis* are derived from the two recombining chromosomes. Consequently, if unequal crossing-over has resulted from sister chromatid exchange, alleles of AldoX9, present in both the 3' end of AldoS and the chimaeric gene, should always be identical; conversely, if crossing over has occurred between non-sister chromatids, these alleles should be randomly associated. These results can be further supported by analysis of the alleles of the flanking markers AldoX1 and 11OH-3. We determined the prevalence of alleles of AldoX9 in 162 unrelated subjects of the CEPH pedigrees, demonstrating 172 copies of allele 1 (53%) and 152 copies of allele 2 (47%).

In GRA pedigrees, identity of AldoX9 alleles in *cis* on the duplication-bearing chromosome were determined (Fig. 5*b*; see Methodology). In one of the 12 pedigrees, K2083, the AldoX9 alleles in *cis* are different, indicating an origin via unequal exchange between non-sister chromatids. The flanking markers show the appropriate

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alleles from the known linkage disequilibrium relationships, providing support for this interpretation. In contrast, in the remaining 11 pedigrees, the AldoX9 alleles in *cis* on the GRA chromosome are identical. In all cases, the flanking markers show no violation of the expected linkage disequilibrium, making it unlikely that gene conversion has led to identity of alleles in *cis*. The likelihood of obtaining 11 out of 12 pedigrees with identical alleles in *cis* if all cross-overs were between non-sister chromatids can be calculated from the observed allele frequencies for AldoX9. The results reject non-sister chromatid exchange as the sole source of crossing-over (χ^2 1 df = 8.33, $p < 0.005$), and are most compatible with unequal sister chromatid exchange being the predominant mode in the genesis of GRA chromosomes. This result is not merely due to differences in allele frequencies in CEPH and GRA pedigrees, since in GRA patients studied, the prevalence of AldoX9 alleles on the non-GRA chromosome is virtually identical to that in CEPH controls (15/29 allele 1, 52%; 14/29 allele 2, 48%).

Evidence for an allelic bias for GRA

We have begun extending these 12 GRA pedigrees in order to identify all affected individuals in each. So far, we have not identified a *de novo* mutation, suggesting that in general many affected subjects will be found for each index case. Extension of pedigrees has permitted inference of the ethnic origin of GRA alleles; an unexpectedly high

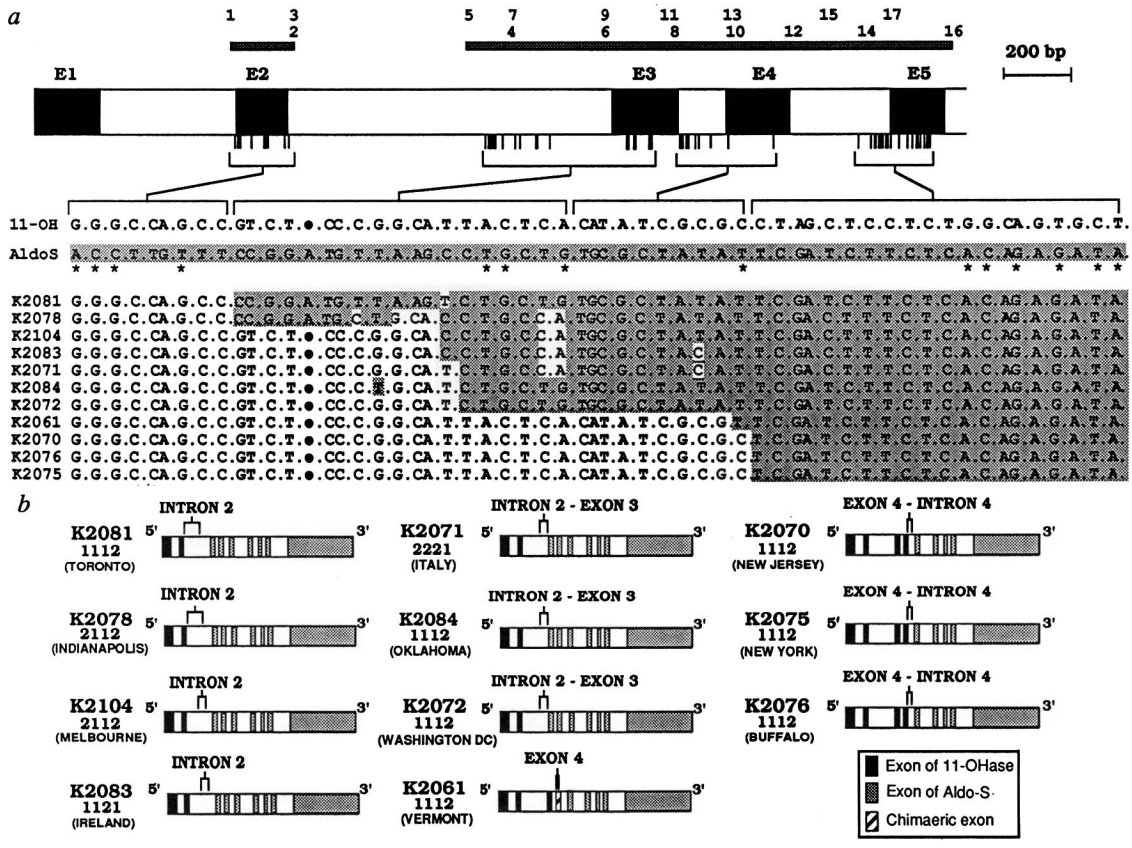

Fig. 5 Identification of cross-over breakpoints by sequence analysis in 11 GRA pedigrees. *a*, Sequence differences among 11-OHase, AldoS, and GRA genes. The genomic segment encoding the first 5 exons of 11-OHase or AldoS is shown, with the exons represented by dark black boxes labeled E1 through E5. The regions subjected to DNA sequence analysis, including exon 2 and a continuous segment from intron 2 to exon 5, are indicated above by the shaded bars. The numbers above these bars indicate the positions of the corresponding primers (table 2) used in DNA sequencing; odd-numbered primers have 5' to 3' orientation from left to right as drawn, while even-numbered primers have 5' to 3' orientation from right to left. The regions sequenced encompass 1.7 kb per pedigree. Beneath the genomic map are vertical bars indicating the locations of nucleotide substitutions and/or insertions which distinguish the normal 11-OHase and AldoS genes. The sequence differences at these locations are shown below; only the nucleotides which distinguish these genes are shown, with the variable length of DNA sequence lying between adjacent substitutions indicated by dots. A single base which is absent in 11-OHase is indicated by a large dot. Brackets relate the locations of the indicated substitutions to the genomic map. Nucleotide substitutions which result in changes in the encoded amino acid are denoted by an asterisk. The sequence of the mutant gene in 11 different GRA pedigrees was determined (see Methodology) and is shown below. Residues which are specific for AldoS genes are shaded. In all cases, the mutant genes display a chimaeric structure. The full 1.7 kb sequence of each gene has been submitted to GenBank. *b*, Inferred cross-over sites in chimaeric genes from different GRA pedigrees. Cross-over sites were inferred from the DNA sequences shown in *a*. Marker haplotypes on the GRA chromosome and the geographic location of the index case of each pedigree are shown below the kindred number. The order of markers shown in each haplotype is in the chromosomal order of the markers on GRA chromosomes: 5' end AldoS (Aldox1); 3' end AldoS (Aldox9); 3' end chimaeric GRA gene (Aldox9); exon 5 11-OHase (11OH-3).

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Table 1 Linkage disequillibrium of markers in 11-OHase and aldosterone synthase genes

a		Genotypes, AldoX9		
		11	12	22
Genotypes, 11OH-3	11	0	1	15
	12	1	32	0
	22	21	0	0

χ^2 1df = 66.3, $p < 10^{-8}$

b		Genotypes, AldoX9		
		11	12	22
Genotypes, AldoX1	11	16	0	0
	12	6	24	0
	22	0	9	15

χ^2 1df = 49.9, $p < 10^{-8}$

prevalence of Irish ancestry of the GRA allele has been observed, with a documented Irish origin of the GRA allele in six pedigrees (K2083, K2072, K2061, K2070, K2076, K2105), a likely Irish origin in an additional two (K2075, K2084) and a English origin in one (K2104). Non-Irish ancestry has been documented in three pedigrees, with rural Italian (K2071), Dutch (K2078) and Ukrainian (K2081) ancestry. The unexpectedly high prevalence of Irish ancestry in these pedigrees raises the possibility that an allele exists in this population with an increased likelihood of undergoing unequal crossing over to produce a gene duplication with the GRA phenotype.

If this were true, such an allelic association might be reflected in the non-random distribution of alleles in the region of the duplication. The expected frequencies of genotypes for AldoX9 on GRA chromosomes was compared to the frequencies of these alleles on the chromosomes contributing to either the 5' side of the cross over (scoring the AldoX9 allele of the normal AldoS gene) or the 3' side of the cross-over (scoring the AldoX9 allele of the chimaeric gene). All 9 pedigrees of Irish/UK ancestry have allele 1 of AldoX9 in the aldosterone synthase gene of the GRA chromosome ($p = 0.04$, Fisher's exact test); 8 of 9 have allele 1 in the chimaeric gene. Among these 9 pedigrees, 4 different sites of crossing over and three different haplotypes are seen, indicating that even within this subgroup independent duplications have occurred.

Discussion

Chimaeric gene duplication as the cause of GRA

We studied a total of 29 GRA patients from 12 pedigrees and have demonstrated that affected subjects in these pedigrees all have chimaeric gene duplications fusing 5' 11-OHase to distal AldoS sequences. This group includes the first two reported cases of GRA[3]. All lines of evidence, including sequence analysis of mutant genes, indicates that these duplications arise from unequal crossing over between 11-OHase and AldoS genes. Extremely close linkage of normal 11-OHase and AldoS genes, permitting such recombination, has been demonstrated.

These chimaeric duplications show high specificity for GRA, as similar mutations are not present in subjects who do not have this disorder. Importantly, DNA sequence analysis in conjunction with marker analysis has shown that most of these mutations must have arisen independently. This evidence in sum provides conclusive evidence that these mutations cause GRA.

The structure of these mutations explains the biochemical, physiologic and genetic features of GRA on the basis of ectopic expression of a protein with aldosterone synthase activity in the adrenal fasciculata (Fig. 1). The evidence for this ectopic expression is strong: i) The mutant gene carries regulatory elements known to confer expression in the fasciculata[15]; ii) The abnormal steroids secreted in GRA indicate aldosterone synthase activity on cortisol, which is secreted entirely by fasciculata; iii) The positive control of these steroids and aldosterone by ACTH indicates secretion from the fasciculata.

The genetics of GRA is similar to red-green colour blindness[18] and the α-thalassaemias[19] in that unequal crossing-over between homologous genes is the predominant cause of the disorder. In the latter cases, disease alleles are typically null alleles resulting from deletions rather than neomorphic alleles resulting from duplications.

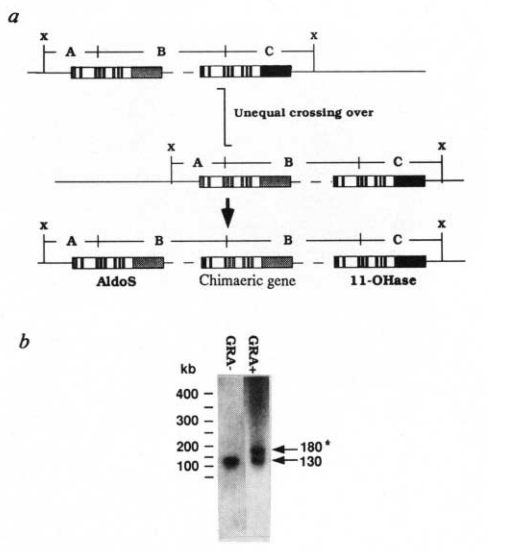

Fig. 6 Pulse-field gel analysis of GRA+ and GRA- patients. *a*, Schematic diagram of gene duplication by unequal-crossing over. The locations of sites for a rare-cutting restriction endonuclease are indicated by X, such that both the 11-OHase and AldoS genes on a normal chromosome are found on the same pulse-field gel fragment. In such a case, after unequal crossing over, the allele carrying the resulting duplication will be larger than the normal allele. This increase in size will correspond exactly to the length of segment B, which comprises the distance between the two genes on the normal chromosome plus the length of the duplicated gene. *b*, Hybridization of exon 3–4 of 11-OHase to pulse field gel of *Cla*I-cut DNA of GRA- and GRA+ subjects. Leukocytes were transformed, grown, plugs prepared, and electrophoresis and Southern blotting performed after digestion with *Cla*I. The location of fragments of known length are shown on the left. The unaffected subject from K2061 shows a single 130kb *Cla*I fragment; in contrast, the GRA patient from K2061 shows two fragments of 130 and 180 kb. Identical results (a single 130 kb fragment) were obtained for 7 additional, unrelated normal subjects; 4 additional GRA patients (3 from K2061 and 1 from K2070) showed 130 and 180 kb fragments.

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Despite the independence of many of the mutations, there is an apparent bias for Irish ancestry with a concomitant allelic bias of many of the GRA duplications. Although the number of observations is relatively small, they raise the possibility of a susceptibility allele with increased likelihood of unequal crossing-over resulting in gene duplication and GRA. Almost all western reports of GRA come from the United Kingdom, Australia and the northeastern United States, with few cases from France, Germany, Scandinavia or Israel, where positive cases might have been identified. The finding of at least 3 kindreds of diverse non-Irish ancestry with chimaeric gene duplications indicates that GRA is not restricted to one group and that the molecular basis of GRA is thus far uniform regardless of ethnic origin. Further investigation will be required to establish whether this allelic susceptibility is genuine, and if so to establish the molecular basis for this phenomenon. These observations may be of practical significance, raising the possibility that the prevalence of hypertension due to GRA will prove to be higher in Ireland and individuals of Irish descent.

It is noteworthy that the reciprocal product of unequal crossing over between 11-OHase and AldoS would result in a chromosome with only a single gene bearing 5' AldoS sequences and 3' 11-OHase sequences. This gene would likely behave as a complete or partial null allele, and heterozygotes would likely be phenotypically normal. Homozygotes or compound heterozygotes for this allele in combination with an 11-OHase deficiency allele might be expected to show the phenotype of 11-OHase deficiency. Few 11-OHase deficiency alleles have been characterized so far[20]; it might be of particular interest to search for this mutation in individuals of Irish descent with 11-OHase deficiency.

Implications of location of cross-over breakpoints

The site of crossing-over in different pedigrees is variable; there is an apparent tendency for crossing-over to occur in regions of the genes in which sequence identity is greatest, that is, the intron 2–exon 3 junction, and the exon 4–intron 4 interval (Fig. 5).

The abnormalities in steroid biochemistry coupled with the nature of the mutations indicate that the GRA phenotype is dependent on the chimaeric gene product having AldoS activity. Consequently, the location of cross-over breakpoints has implications for the amino acid residues of AldoS that are essential for enzymatic function. That three GRA pedigrees show sites of crossing over distal to exon 4 with exons 1–4 all encoding 11-OHase- specific residues indicates that none of the AldoS- specific residues encoded in exons 1–4 are essential for AldoS enzymatic function. That none of the pedigrees studied shows crossing-over distal to intron 4 suggests that exon 5 may have AldoS-specific residues critical to aldosterone synthase activity and that gene duplications due to more distal cross-overs consequently might not have the GRA phenotype due to absent or greatly diminished AldoS activity. *In vitro* expression of genes constructed with varying cross-over breakpoints and/or specific amino acid substitutions, can address this issue.

Genetic testing and therapeutic considerations

Our results document the utility of a simple direct genetic test for GRA: the presence of 6.3 kb *Bam*HI and 22 kb *Eco*RI fragments on Southern blotting are sensitive and specific signatures of chimaeric gene duplication. This test overcomes the problems of previous tests for the disorder which require either investigation of patients before and after pharmacologic intervention, or measurement of abnormal steroids in urine collections; these latter measurements are not widely available. Knowledge of the crossing-over sites raises the additional possibility of developing diagnostic tests in which only chimaeric gene duplications can yield polymerase chain reaction (PCR) products.

Screening with such genetic tests will be applicable in three settings: i) as a research tool in order to determine the prevalence of GRA in the hypertensive population. While GRA is thought to be a rare disease, no systematic studies have been performed, and there is reason to suspect the disease is underdiagnosed[9]. ii) As a clinical tool in patients in whom the likelihood of GRA is increased. The definition of GRA used herein was a strict one. The spectrum of patients in whom similar mutations might be found has not been defined. The diagnosis should be considered in patients with strong family histories of early onset of hypertension, particularly if signs of aldosteronism are present. Given that at least some patients with GRA appear to evolve over time from showing complete suppressibility of aldosterone with glucocorticoids to little suppressibility with no rise in renin, a clinical picture that overlaps with idiopathic hyperaldosteronism[21], GRA may prove to account for a subset of such patients. Two of our patients carrying the GRA duplication show this clinical picture (patient K2070-1, and K2104-1). iii) As a clinical tool in at-risk relatives of documented cases of GRA. Since *de novo* mutations have not been identified in the 12 pedigrees studied to date, there are likely to be many more affected relatives for each case identified. Given the often severe hypertension in GRA with attendant early morbidity and mortality and poor response to conventional therapy[9], the expected benefit from identification and treatment of cases is high.

Identification of the mutations causing GRA provides a detailed explanation of the molecular basis and pathophysiology of this disease, indicating the framework within which therapies must operate. It is important to note that none of the standard therapies for GRA, that is, exogenous glucocorticoids, blockade of the aldosterone receptor, or inhibition of the sodium channel activated by aldosterone, is commonly offered to hypertensive patients, further underscoring the importance of making the diagnosis of GRA. Finally, these findings demonstrate the central thesis in the search for genetic factors in human hypertension, namely that discovery of mutations will explain disease pathogenesis and permit development of diagnostic tests which will identify individuals with specific underlying causes of hypertension which can be treated with specific therapy. Approaches using linkage and/or direct search for mutation holds considerable promise for identifying other genetic factors contributing to the pathogenesis of human hypertension.

Methodology

Patients. The clinical characterization of subjects from 10 of the 12 GRA pedigrees studied have been published: K2061[9]; K2070, K2071, K2072 and K2075 are, respectively, Families B, A, C and D in ref.8; K2078[22]; K2081[3]; K2083[23]; K2084[24] and K2104[21]. Affected subjects in

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these pedigrees all have definite GRA on the basis of either the clinical evaluation alone (5 pedigrees: hypertension with suppressed renin and normal to elevated aldosterone at baseline, with suppression of aldosterone to sub-normal levels and rise in renin after administration of exogenous glucocorticoids) or clinical plus biochemical features (5 pedigrees: the above clinical criteria plus diagnositic elevation in 18-hydroxycortisol, 18-oxocortisol and 18-oxocortisol : tetrahydroaldosterone ratio). K2076 and K2105 have not been previously reported. K2076, from Buffalo, New York, (characterized by L.F. and S.U.) has definite GRA on the basis of hypertension plus high levels of 18-hydroxycortisol and 18-oxocortisol and diagnostic ratios of 18-oxocortisol:tetrahydroaldosterone in 24-hour urine samples in a mother and two offspring. Patient 2105-1 from Melbourne, Australia (characterized by J.R.S.) has definite GRA on the basis of hypertension, suppressed plasma renin activity and elevated aldosterone level, with complete suppression of aldosterone and rise in renin after 2 days of dexamethasone therapy.

Unrelated non-GRA patients derive from CEPH pedigrees and predominantly Caucasian patients from pedigrees of diverse origins collected for other genetic studies.

Southern blotting. Total genomic DNA was extracted from 10–20 ml of peripheral venous blood as described previously[25]. Genomic DNA was digested with indicated restriction endonucleases, fractionated on agarose gels by electrophoresis, and transferred to nylon membranes (Hybond-N+, Amersham) after depurination with 2 washes for 5 min in 0.25 M HCl, followed by denaturation in 0.4 M NaOH for 15 min.

Pulse-field gel electrophoresis. Lymphoblastoid cell lines were established by transformation of peripheral blood lymphocytes with Epstein-Barr virus. Resulting lymphoblastoid cell lines were cultured at 37 °C, 5% $C0_2$ in RPMI 1640 medium (Cellgro/Mediatech) with 5% fetal bovine serum (Hyclone) and 50 U ml^{-1} gentamicin sulphate. Plugs for pulse-field gel analysis were prepared in 1% low-melting agarose (Sigma) and digested with enzyme *Cla*I. Products were fractionated via transverse alternating field electrophoresis[26] on a Geneline apparatus (Beckman Instruments). Samples were electrophoresed for 24 h at 250 V with pulse times of 30 s on 1% agarose gels in 0.5 × TBE buffer at 10 °C.

Labelling and hybridization of probes. A segment spanning exon 3–4 of the 11-OHase gene was prepared by PCR using specific primers and cosmid c11OH26 as a template[14]. The resulting fragment was purified on an agarose gel, and labelled with α-^{32}P dCTP by random priming[27]. A probe specific for the 5' flanking region of 11-OHase was labelled by PCR using specific primers 11OH-51 and 11OH-54 (Table 2) and cosmid c11OH26 as a template. This probe is 225 bp in length and covers 140–365 bp upstream of exon 1 (ref. 10). A probe specific for the 5' end of AldoS was prepared by amplifying a specific 5' segment from genomic DNA using specific primers AldoX1 and AldoX2b (Table 2), and purifying the fragment by agarose gel electrophoresis followed by GeneClean. This fragment was then re-amplified and labeled in PCR[28] using the same primers. This segment is 234 bp in length and extends from 330 to 554 bp upstream of exon 1 of AldoS[10]. Labelled probes were denatured and hybridized to filters as described[29].

DNA sequence analysis. DNA sequencing of the haploid chimaeric gene duplications was performed on genomic DNA after amplification by PCR. In each case, 10 μg of genomic DNA was digested with *Bam*HI, fractionated on low-melting 0.7% agarose gels, and fragments of size 6.1–6.4 kb excised from the gel using λ-*Hin*dIII size standards as a guide. Additional fragments ranging from 4–9 kbwere also excised. Gel slices were melted and a portion subjected to electrophoresis and Southern blotting, using exon 3–4 of 11-OHase as a probe to ensure that the 6.1–6.4 kb fraction contained the 6.3 kb chimaeric gene fragment free from contamination with either the 8.5 kb 11-OHase fragment or the 4.5 kb AldoS fragment.

Appropriate fragments were purified using GeneClean (Bio101), and segments of the chimaeric gene amplified with appropriate PCR primers. Primary amplifications were performed with primer sets (Table 2; Fig. 5*a*) 1 and 2; 3 and 4; 7 and 10; 13 and 16. PCR was conducted for 30 cycles of 94 °C for 45 s, 60 °C for 45 s and 72 °C for 45 s in the presence of 100 μM dNTP's 1 × PCR buffer (Cetus), and 25 u ml^{-1} *Taq* polymerase. Products were purified by agarose gel electrophoresis followed by isolation using GeneClean. PCR was repeated using appropriate primers; one primer coupled at the 5' end to either M13 universal or reverse primers. Products were purified and subjected to sequence analysis by dideoxy chain termination using fluorescently labeled universal or reverse primers and thermocycling (Applied Biosystems); products were analysed using an ABI model 373A DNA sequence apparatus.

11-OHase and AldoS genes were sequenced by the same approach using the 8.5 kb and 4.5 kb *Bam*HI size fractions excised from agarose gel after electrophoresis. These genes were sequenced from 5 normal and one GRA individual. The sequence differences identified between these two genes are similar to those described[10], although some of the variants previously described from sequence analysis of one cloned allele of each gene[10], particularly those in introns, were not detected in any samples.

Genotyping. SSCP markers AldoX9, 11OH-3, and AldoX1 were genotyped using specific primers in PCR in the presence of α-^{32}P dCTP followed by denaturation and subsequent electrophoresis on non-denaturing polyacrylamide gels as described[14]. That each marker specifically amplifies a segment of either 11-OHase or AldoS was previously shown by amplification from cosmid clones[14] and was confirmed by subjecting the PCR product to DNA sequence analysis and comparing the resulting sequence to the known sequences of 11-OHase and AldoS[10] (data not shown). Where indicated, PCR products were quantitated by use of a Molecular Dynamics densitometer.

In GRA kindreds, markers were haplotyped by linkage when possible, using additional family members not shown when available (K2084). Haplotypes were confirmed by directly genotyping the chimaeric gene in K2083 and 2072. In these cases the 22 kb and 16 kb *Eco*RI size fractions bearing the chimaeric gene and the normal AldoS genes, respectively, were isolated from genomic DNA by gel

Table 2 Oligonucleotides[a]

1.	RL11E21:	CCTCCTGCTTGGCCCCACAG
2.	RL11E22:	ACCCTGCTCCCAGCTCTCAG
3.	RL11B15:	CTGAGAGCTGGGAGCAGGGTG
4.	RL11B16:	CAAGTCTGGCACCACCTCAC
5.	RL11B99:	ATGGAGCCCCCACGCACTCTG
6.	RL11B2A:	CAGCAAGACACAGGCCCTGAC
7.	RL11B1:	GGTGAGGTGGTGCCAGACTTG
8.	RL11E32:	CTGGCCACTCCAGGGTCTCTG
9.	RL11E31:	CAAGCTCTGCCCTGGCCTCTG
10.	RL11B28:	AACCAGGCCCAGCCGCTCTC
11.	RL11B27:	GCCCAGCATCTTCCACTACACC
12.	RL11E42:	GGTGTCCCTTCCCCATAGCACTG
13.	RL11E41:	TTGTGCTCAGCAGTGCATCCTC
14.	RL11B4:	CCTGATAGATTTTCTGGATACAGTTGTC
15.	RL11B3:	CCAGTGGGGAATGGAGGCCAC
16.	RL11E52:	CTCTCTGGGTGGGGCTGGTTG
17.	RL11E51:	CACTGAAGGATGCTTCCCAGCAC
18.	RL11OH51:	TCCTCAGTTCTAGTTTGATTGCAC
19.	RL11OH54:	GGGGTGCATGAGCGTAGACAG
20:	RLALDOX1:	CCCACAGCATGTTGACCACCAG
21.	RLALDOX2B:	GATGAGAGGGCCCCTTGGATTC

[a]The positions of oligonucleotides 1-17 on the genomic sequence are shown in Fig. 5*a*.

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electrophoresis on 0.5% agarose gel, and the alleles present on the chimaeric and normal genes were determined separately by genotyping with marker AldoX9.

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Linkage disequilibrium of markers was determined from the distribution of genotypes in 70 unrelated members of the CEPH reference families as described[30].

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