Evidence of Genetic Diversity Underlying Rh D−, Weak D (D0), and Partial D Phenotypes as Determined by Multiplex Polymerase Chain Reaction Analysis of the RHD Gene


The human blood group Rh antigens are expressed by proteins encoded by a pair of highly homologous genes located at chromosome 1p34-36. One of the genes (RHCE) encodes Rh CcEe antigens, while the other (RHD) the D antigen. Point mutations in the RHCE gene generate the C/c and E/e polymorphisms, while it has been shown that an RHD gene deletion can generate the D-negative phenotype. We have analyzed intron 4 of the RHCE and RHD genes and have defined the site of an RHD-specific deletion located in this intron. Using a multiplex RHD typing assay, which combines a reverse polymerase chain reaction (PCR) primer, which straddles this RHD-specific sequence, and a pair of primers located in exon 10 of the RHD gene, we have analyzed 357 different genomic DNA samples derived from individuals expressing D0, D0, weak D, and partial D phenotypes. Of these, we have noted a significant discordance with our multiplex PCR assay in the D− phenotypes DcEe and DccEe (which have been previously described) and weak D phenotypes.

Our results suggest that in five serologically D− individuals we have identified an apparently intact RHD gene. Sequence analysis of transcripts obtained from one of these individuals (of phenotype DcEe) illustrates the presence of full-length RHD transcripts, which have a point mutation at nucleotide 121 (C→T), which generates an in-frame stop codon (Gln41Stop). Thus, we describe a different molecular basis for generating the D− phenotype to the complete RHD gene deletion described previously. We also show that there are discordances with serotype and the multiplex assay in weak D and partial D phenotypes, indicating that the underlying molecular basis can be heterogeneous. Existing Rh D PCR assays assume the complete absence of the RHD gene in D− phenotypes. We describe a different molecular basis for generating the D− phenotype to the complete RHD gene deletion described previously.

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MATERIALS AND METHODS

Sources of erythrocytes and extraction of genomic DNA (gDNA) from peripheral blood (PB) and fetal amniocytes. gDNA was extracted from 5 to 10 mL of PB and 5 mL amniotic fluid as described by Avent and Martin.25 The Rh DCeE status of all erythrocytes was established by using routine D and CcEe typing antibodies: English samples: MAD-2 (BioProducts laboratory, Elistree, Hert); and Seraclone anti-D blend (BS 221, H41-1187, BS 232; Biostest, Dreieich, Germany); Scottish samples: LMD1 plus blended anti-D ESD1 and LDM3 (Scottish National Blood Transfusion Service National reagents programme, Edinburgh, Scotland). Weak D phenotypes were established by demonstration of weakened D antigen expression by reaction profiles with MAD-2 and LDM1, and confirmed using the blended anti-Ds. D-variant erythrocytes were made available from either frozen cells or fresh samples referred to IBGRL, while others were made available through the international workshop on monoclonal antibodies against red cell and related antigens (September 1996, Nantes, France) and were classified by typing using monoclonal anti-D into distinct D categories.

Cloning and DNA sequence analysis of RHD and RHCE intron 4 PCR products. A 1,200-bp PCR product containing intron 4 of the RHD gene was amplified by using an exon 4 sense primer (5'-ACTTCTACGTGTTCGCAGCCTATTT-3') and an exon 5 antisense primer (5'-ACTTCTACGTGTTCGCAGCCTATTT-3'), and an exon 5 antisense primer (5'-ACTTCTACGTGTTCGCAGCCTATTT-3') of the RHD and RHCE genes with gDNA derived from lymphocytes obtained from an individual of Rh phenotype dce. A 600-bp PCR product containing intron 4 of the RHD gene was obtained from lymphocytes derived from an individual of Rh phenotype DcE with the same primer pair as above. PCR conditions were as follows: 94°C/1 min; 60°C/1 min, 30 s; 72°C/2 min, 30 s for 30 cycles. Fifty microliters of PCR reaction mix contained 2.5 mmol/L MgCl2; 10 mmol/L Tris-Cl pH 8.3; 500 mmol/L KCl; 1 μmol/L each primer, 1.25 mmol/L each dNTP and 2.25 U Taq DNA polymerase (Perkin Elmer, Warrington, UK). Both PCR products were gel purified on Low-Melt point gels (Flowgen Instruments Ltd, Licheld, UK), and cloned into pCR II (TA cloning kit, Invitrogen, San Diego, CA) using amplimers complementary to the 5' and 3' noncoding regions of the RHD and RHCE genes. The location of the RHD specific deletion or RHCE specific insertion are highlighted on the figure.

RESULTS

Molecular cloning of intron 4 of the RHD and RHCE genes. Genomic clones corresponding to intron 4 of the RHD and RHCE genes were isolated as described in Materials and Methods. One RHD and three RHCE clones were isolated and fully sequenced on both strands. The DNA sequences obtained were identical for all RHD clones, and the RHD intron 4 was found to be composed of 426 nucleotides.
Fig 2. Nucleotide sequences of cloned RHCE and RHD gene Intron 4. The complete nucleotide sequences of the RHCE and RHD gene intron 4 are displayed. The sequences are derived from cloned PCR products, the sequences of exon 4 and 5 are displayed in uppercase. Intron 4 sequence is displayed as lowercase, and the first nucleotide of the intron is numbered as position 1. The RHCE intron extends to 1077 nts, while the RHD intron to 426 nts. Sequence differences between the two genes are denoted by a cross on the shadow sequence. The two nucleotide sequences have been submitted to Genbank/EMBL databases and have the following accession numbers: Intron 4 RHCE: Y10604; intron 4 RHD: Y10605.

The RHCE intron 4 was composed of 1077 nucleotides, and contains an Alu repeat element. The extent of the Alu repeat element (including both poly A rich tracts located at ends of the head to tail dimeric repeats) is underlined in Fig 2. The aligned sequences of both RHD and RHCE intron 4 are illustrated in Fig 2. Analysis of the DNA sequence indicated that the exon 4/intron 4 and intron 4/exon 5 boundaries differed to those described by Cherif-Zahar et al., (Fig 3) in all clones analyzed (five). DNA sequence electropherograms obtained on the ABI-373A DNA sequencer for the intron/of the head to tail dimeric repeats) is underlined in Fig 2. The aligned sequences of both RHD and RHCE intron 4 are displayed in Fig 3. Analysis of the DNA sequence indicated that the exon 4/intron 4 and intron 4/exon 5 boundaries differed to those described by Cherif-Zahar et al., (Fig 3) in all clones analyzed (five). DNA sequence electropherograms obtained on the ABI-373A DNA sequencer for the intron/exon boundaries are displayed in Fig 3.

**Organization of the RHD gene in individuals different Rh**
Fig 3. Nucleotide sequences of exon 4/Intron 4 and Intron 4/exon 5 boundaries. Nucleotide sequences obtained at the intron/exon boundaries of both cloned RHCE and RHD genes are displayed. Exonic sequences are shown in uppercase, intronic sequences in lowercase. The last nucleotide of exon 4 and the first of exon 5 are numbered. The numbering corresponds to the Rh cE and D cDNAs with nucleotide 1 being the first base of the ATG triplet. A representative sequence electropherogram generated during sequence analysis of these regions is displayed in this figure.

phenotypes as determined by the RHD exon 10/intron 4 multiplex assay. The multiplex assay was performed on a number of genomic DNA samples derived from lymphocytes and amniocytes isolated from individuals of known Rh phenotype. Three bands were obtained in D+ samples (531 bp; 299 bp and 196 bp) and only one band was obtained in D− samples (531 bp) (see Figs 1 and 4). These results are tabulated in Table 1. A typical PCR using the multiplex PCR is displayed in Fig 4. Significantly, the assay showed concordance with RhD serotype in all D+ samples tested, except in partial D and weak D phenotypes (Tables 1 and 2). However, the assay was discordant in some D− phenotypes (Tables 1 and 2). The most common D− phenotype (dce) was concordant with both the exon 10 and intron 4 bands, but D− haplotypes dCe and dcE showed marked discordance (299 bp and 196 bp) and only one band was obtained in D− samples (531 bp) (see Figs 1 and 4). These results are tabulated in Table 1. A typical PCR using the multiplex PCR is displayed in Fig 4. Significantly, the assay showed concordance with RhD serotype in all D+ samples tested, except in partial D and weak D phenotypes (Tables 1 and 2). However, the assay was discordant in some D− phenotypes (Tables 1 and 2). The most common D− phenotype (dce) was concordant with both the exon 10 and intron 4 bands, but D− haplotypes dCe and dcE showed marked discordance (Table 2). Of 33 dCcee samples, 8 were discordant with one or more aspect of the multiplex assay. Of these, 6 were exon 10 positive intron 4 negative, one was exon 10 negative


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and numbers of samples that are positive or negative with the two
been previously established. This table also illustrates the phenotypes
genomic DNAs of which the Rh phenotypes of the erythrocytes had
results of screening genomic DNAs with the multiplex assay with 357
viduals expressing different Rh phenotypes. This table illustrates the
serotype are highlighted in bold (further de®ned in Table 2).

<table>
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<tr>
<th>Phenotypes</th>
<th>DcCee (n = 39)</th>
<th>DccEe (n = 27)</th>
<th>DccEe (n = 6)</th>
<th>DcCee (n = 1)</th>
<th>DcCee (n = 5)</th>
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<td>4</td>
<td>9</td>
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* DHR: D variant due to point mutation in exon 5 of the RHD gene
+ DNU: D variant due to point mutation in exon 7 of the RHD gene.

Significant progress has been made in the last 6 years in
defining the molecular basis of human blood group antigen

<table>
<thead>
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<th>Sample</th>
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Discordant samples with the multiplex RHD assay. All genotypically
discordant samples are illustrated. The donor identification number,
serological phenotype and result of analysis with the two RHD-specific
aspects of the assay are shown. Some discordant samples are also
illustrated in Fig 4.
Fig 5. Nucleotide sequence of Rh D transcripts isolated from donor 538150. The nucleotide sequence obtained after DNA sequence analysis of 538150 RHD transcripts is shown. The sequence obtained from two different full-length cDNA clones was found to be identical.
expression. Many human blood group antigens result from point mutations generating allelic forms of the genes encoding blood group active proteins. However, the Rh D positive/negative polymorphism is thought to be generated by the complete absence of the Rh D protein, initially shown to result from a complete RHD gene deletion. This finding shows that the absence of the Rh D protein is not physiologically detrimental to D- individuals. Therefore, it follows that RHD gene rearrangements (gene conversions, point mutations) which do not alter the Rh D protein structure significantly may be expressed at the erythrocyte surface. These RHD gene alterations generate partial D (D-like) phenotypes, many of which have been defined at the molecular level. We have investigated the gross organization of the RHD gene in all commonly occurring Rh phenotypes including partial D and weak D (D') phenotypes. Our findings suggest genetic diversity among dCe, dcE, and weak D phenotypes.

The molecular determination of blood group antigen expression has allowed the use of antenatal PCR-based detection of fetal blood groups in cases of maternal alloimmunization. We have optimized a multiplex RHD typing assay (analyzing intron 4 and exon 10 of the RHD gene), which we routinely use for prenatal Rh D determination, and have used this assay in this study. Our findings illustrate that a multiplex approach is essential for RHD typing; there are many situations where the exclusive use of a single RHD typing assay would generate both false negatives and positive results which confirms the findings of others.

DNA sequences of RHD and RHCE intron 4. We report here the entire sequence of both the RHD and RHCE gene intron 4. The RHD gene intron is composed of 426 nucleotides, while the RHCE gene of 1077 nucleotides (Fig 2). The RHCE intron contains an Alu repeat element. These elements are widely dispersed in mammalian genomes. The absence of such a repeat from the RHD gene could potentially be why this gene serves as the acceptor of gene conversion events. Inhibition of gene conversion events by Alu repeat elements has been suggested for α1-α2 and β-γ globin genes, and this may explain why the RHCE gene serves as the donor in the majority of Rh gene conversion events generating partial D phenotypes involving RHD exons 4 and 5. One exception to this occurs in the RhCE phenotype, where an RHCE gene has an RHD-like exon 5. It is possible that a double crossing over event rather than gene conversion generates this phenotype. The sequence of a hybrid RHD gene intron 4 derived from an individual expressing the Rh phenotype DCe has been recently determined. Analysis of Rh transcripts from this individual reveals that a hybrid RHC-D gene comprising of exons 1 from a C-D gene, exons 2-10 from an RH gene. The RHD intron 4 sequence is identical to that described here except for two nucleotides, 320 and 339 (T → A and A → G, respectively).

We have determined the intron/exon boundaries of the exon 4/intron 4 and intron 4/exon 5 junctions of cloned PCR products from three different individuals (Fig 3). These sequences differ to those described by Cherif-Zahar et al. We have shown that exons 4 and 5 of both genes are composed of 150 and 195 nucleotides, respectively. We assume that the sequences derived from a single cosmid clone for the exon 4/intron 4 and intron 4/exon 5 boundaries reported by these workers are erroneous, possibly caused by band compression of these GC-rich sequences. The intron 4/exon 5 splice boundary described here agrees with that determined by Huang from 15 different individuals.

We have cloned and sequenced intron 4 of the RHCE and RHD genes primarily to identify the site of the RHD-specific deletion identified originally by Arce et al. Primers complementary to this RHD-specific site have been incorporated into a multiplex PCR-based assay for determination of RHD type. The intron 4 assay initially described by Arce et al, involved the use of amplimers which are common to both RHCE and RHD genes, and amplified two products (600 and 1,200 bp) from D' genomes and one product (1,200 bp) from D-. As a consequence, in D- genomes often the only product observed under certain reaction conditions (eg, low concentrations of DNA, frequently obtained from amniotic fluid samples) is the 600-bp RHD-specific band (N.D.A. and P.G.M.; S.S.A.F. and Susan Cochrane, independent unpublished observations, September 1995). We have shown that by using an entire RHD-intron 4 specific primer that a more robust assay is obtained, and is controlled with the incorporation of a RHCE-specific amplimer. Exon 10 of the RHD gene is also amplified using an RHD-specific reverse amplimer (Fig 1).

RHD genes and gene fragments in D' genomes. Using the RHD intron 4/exon 10 multiplex assay and genomic DNA derived from individuals expressing D- phenotypes, we have found that the dce haplotype is completely concordant with the assay, supporting the hypothesis that a complete RHD gene deletion generates this phenotype. However, in the D' haplotypes dCe and dEc we have illustrated that a significant proportion of these individuals carry portions of the RHD gene, and probably an intact but dysfunctional RHD gene in five individuals (phenotypes 2 × dCee, dCCEe and 2 × dCcEe; Table 2). Of the 33 individuals studied who express the products of the dCe gene complex, 7 elements has been suggested for Rh active proteins. However, the Rh D position of fetal blood groups in cases of maternal alloimmunization.

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The existence of such individuals is problematic when one considers that RH D typing assays for use in prenatal determination were initially based on the tenet that all Dnegative chromosomes lack the RH D gene. Our findings and of others 17,18,20 indicate that caution should be applied in prenatal determination with the clear understanding that, although rare, false positive results are unavoidable with DcE and DcE haplotypes. Therefore, it is essential that laboratories performing RH D typing assays use the amplification of at least two different regions of the RH D gene. Intron 4 and exon 10 are sufficiently distant to be good candidate regions. It is also essential that laboratories wishing to perform RH D prenatal diagnosis should consider the ethnic group of the population they will be potentially screening as the genetic basis (or bases) underlying the D phenotype in such populations may well be different to that described for the Caucasian DcE chromosomes described initially. 1 Further investigation of the genetic mechanisms which generate the D phenotype in individuals of different ethnic groups is necessary.

RH D phenotype organization. In individuals expressing normal D phenotype (representing the majority of Dphenotype) the multiplex assay is concordant (Table 1). However, in D-variant and weak D phenotypes, the assay highlights many of these individuals would be negative using single RH D typing assays based on exon 10 or intron 4 of the RH D gene (Tables 1 and 2). In one instance the assay was negative with both intron 4 and exon 10 components in a D-variant (D). In the phenotype, a segmental exchange between the RHCE gene and RH D genes resulting in essentially a RHCE gene with a RH D exon 5 occurs (Fig 7). We also confirm that all RH D genes in individuals expressing the D phenotype appear to lack intron 4 of the RH D gene. A hybrid RH D-RHCE-RH D gene, resulting from a probable gene conversion event is known to generate this genotype 9,40 (Fig 7). The multiplex assay could be applied in instances of fetomaternal alloimmunization in mothers expressing the D phenotype. 40 In two individuals expressing the DFR variant phenotype we show that these appear to occur on two different genetic backgrounds, one individual being RH D intron 4 positive, the other negative (Table 1). As this phenotype appears to arise from a gene conversion where exon 4 of RH D is replaced by RHCE equivalents (Fig 7), we assume that intron 4 can be either RH D or RHCE-derived.

Weak D phenotypes are not partial D phenotypes, but represent any D-positive erythrocyte sample with a depression in the apparent numbers of D antigen sites. The molecular basis of the weak D phenotype is poorly defined but has been found to be due to a depression of Rh D mRNA levels in three different individuals. 44 Our studies indicate there is genetic heterogeneity in the weak D phenotype individuals examined in this study. It is possible that those weak D phenotype individuals who type as RH D intron 4 negative are uncharacterized partial D phenotypes. However, it is important to discount the possibility that these are D VI phenotypes, as these erythrocytes typed positive with an anti-D that does not react with these erythrocytes. It is important to
to identify partial D-phenotype individuals (especially women of child bearing age), as they may be alloimmunized on exposure to normal D⁺ blood.

The discordance of any PCR-based Rh D typing assay in D⁻ phenotypes is concerning. However, we show that discordance is confined to weak D and D-variant phenotypes, and there has been only one published example of fetomaternal alloimmunization due to a fetus expressing these phenotypes. This is most likely due to the significantly depressed D antigen site numbers on these erythrocytes. Alloimmunization has been described in mothers expressing D-variant phenotypes and carrying normal D⁻ fetuses. As with D⁻ phenotypes, the use of a multiplex assay minimizes the risk of false negative results that may be obtained when using a single RHD gene assay. It is possible that multiplexing using more than two different regions in the RHD gene may be necessary.

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