Transcript Analysis of D Category Phenotypes Predicts Hybrid Rh D-CE-D Proteins Associated With Alteration of D Epitopes


The Rh blood group locus from RhD-positive donors is composed of two closely related genes, RHCE and RHD, encoding the Cc/Ee and D antigens, respectively. The major Rh antigen, D, is serologically defined as a mosaic of at least nine determinants (epD1 to epD9), and the lack of expression of some of these D epitopes at the surface of variant red blood cells defines the D category phenotypes. In this report, we have analyzed the Rh transcripts from reticulocytes of different D category phenotypes (Dm, Dv, Dve, and Dfr). Although Southern blot analysis did not show obvious deletions within the RHD gene, sequence analysis of the RhD transcripts indicated that, in all cases studied, the lack of D epitopes is associated with substitutions, in the deduced polypeptides, of amino acids specific of the RhD protein by those encoded at the equivalent position by the RHCE gene. These results strongly suggested that the D category phenotypes resulted from segmental DNA replacement between RHD-specific fragments and their equivalents in the RHCE gene. The regions involved in the Dm, Dv, Dve, and Dfr phenotypes were shown to encompass all or part of the exons 3 and 7, exons 7 to 9, exon 5, and exon 4, respectively. All protein variants encoded by these rearranged RH genes represent new CE-DE-C hybrid molecules that retain only some of the nine D epitopes. Because segmental DNA replacements have been previously identified in other Rh variant genomes, we postulate that such genomic rearrangements between different regions of the RHCE and RHD genes should be one of the most frequent events involved in the extreme polymorphism of the Rh blood group system.

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MATERIALS AND METHODS

Materials. Restriction enzymes, bacterial alkaline phosphatase, and pUC vectors were from Appligene (Strasbourg, France). T4 polynucleotide kinase, DNA polymerase I Klenow fragment, and radiolabeled nucleotides were from Amersham (Bucks, UK). Avian myeloblastosis virus (AMV) reverse transcriptase was obtained from Promega Biotec (Madison, WI) and Thermus aquaticus polymerase (Taq polymerase) was from Perkin-Elmer Cetus (Norwalk, CT). Random priming labeling kits were from Boehringer Mannheim (Mannheim, Germany) and pUC sequencing kits were from Pharmacia (Upsala, Sweden).

Blood samples. Blood samples from D category donors were collected in EDTA. Dm (Bel.) and Dv (Hus.) samples were provided by the Centre National de Référence pour les Groupes Sanguins (CNRGS; Paris, France). Other samples (Dfr (Da.), Dve (Kou.), and Dfr (Ri.)) were identified and collected at the Centre Régional de Transfusion Sanguine (CRTS) of Lille (France).

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Antibodies and agglutination techniques. Polyclonal antibodies directed against RhD (D⁴), Rh30 (Go⁴), and Rh50 (FPUTT, serum Mol) antigens were provided by Drs. J. Moulds (University of Alabama, Houston, TX), Dr. P.Y. Le Pennec (CNRS, Paris, France), and Dr. M. Bizot (CRTS, Montpellier, France), respectively. Monoclonal anti-D antibodies specific for the nine D epitopes were those reported at the First and Second International Workshop on Monoclonal Antibodies Against Human Red Cells and Related Antigens and elsewhere. Agglutination studies were performed using the gel test system with native or papain treated cells by the antiglobulin test according to the manufacturer (DiaMed SA, Morat, Switzerland).

Southern blot analysis. Human genomic DNAs extracted from peripheral leukocytes were digested by HindIII and BamHI restriction enzymes (100 U/μg DNA), resolved by electrophoresis in 9.8% agarose gels, and transferred as described by Southern to a Zeta film. DNA probes (10⁷ cpm/mL) was performed for 24 hours at 65°C in 7% sodium dodecyl sulfate (SDS), 0.5 mol/L NaHPO₄. Final washes were performed at 65°C for 45 minutes in 5% SDS, 40 mmol/L NaHPO₄, and for 30 minutes in 1% SDS, 40 mmol/L NaHPO₄.

Reverse transcription coupled with PCR amplification (RT-PCR). Total RNAs were extracted from 10 mL of whole blood by the acid-phenol-guanidium method. RNAs (1 μg) were reverse-transcribed at 42°C for 60 minutes in a reaction mixture (25 μL) containing 50 mmol/L Tris HCl (pH 8.3), 50 mmol/L KCl, 10 mmol/L MgCl₂, 20 mmol/L DTT, 0.5 mmol/L spermidine, 1 mmol/L of each deoxy-nucleotide triphosphate (dNTPs), 5 U of ribonuclease inhibitor (Promega), and then subcloned in pUC vector.

RESULTS

Serologic confirmation of the D category cells. D category phenotypes of RBCs from donors Bel., Da., Kou., Hus., and Ri. were determined by agglutination studies with anti-D monoclonal antibodies (MoAbs) and with polyclonal antibodies against low-frequency antigens Rh23 (D⁴), Rh30 (Go⁴), and Rh50 (FPUTT, serum Mol). The results obtained in the papain gel test (Diamed) are summarized in Table 1. Each sample investigated was characterized by a typical pattern of D epitope expression that correlated perfectly with the subcategory classification previously assigned to D⁴ (Bel.), D⁶⁵ (Da.), D⁵⁸ (Kou., Hus.), and DFR (Ri.) phenotypes. As expected, RBC samples from D⁴ (Bel.), D⁵⁸ (Kou., Hus.), and DFR (Ri.) were found positive with polyclonal antibodies directed against Rh30, Rh23, and Rh50 antigens, respectively (Table 1).

Southern blot analysis of the RH locus in D category phenotypes. Genome DNAs from the D category variants were digested with HindIII and BamHI restriction enzymes and subjected to Southern blot analysis with the full-length RhXb cDNA probe. DNAs from RhD-positive (DCee) and RhD-negative (dccee) donors were included as controls. Because the coding region of the two RH genes share 96% sequence similarity, the RhXb cDNA probe detected both RHCE and RHD gene fragments in Rh-positive genotypes (Fig 1). In the Rh-negative DNA, which carries only the RHCE gene, the 19- and 4-kb BamHI bands; and the 7.5- and 2.2-kb HindIII bands specific of the RhD gene were missing.

HindIII and BamHI hybridization patterns of the D⁴ (Hus.) and DFR genotypes did not differ from that of the DCee DNA, suggesting that the lack of expression of epitopes D1 and D5 and epitopes D2, D5, and D8, respectively, are not associated in these variants with any obvious deletion within the RHD gene (Fig 1). In contrast, the BamHI pattern of the D⁶⁵ (Da.) and D⁵⁸ (Kou.) genotypes exhibited one additional fragment of 20 and 17 kb, respectively, whereas two new bands of 16 and 3.5 kb were observed in the HindIII digest of the D⁴ DNA. Because the abnormal fragments were seen with only one of the two restriction enzymes used, it cannot be determined from this analysis whether these restriction fragment length polymorphisms (RFLPs) are caused by rearrangement of the RHD gene or by point mutations not associated with the D⁴, D⁶⁵, and D⁵⁸ phenotypes. It was previously shown that the tygosity for RHD could be determined by comparing the signal intensities, measured by densitometry, of CE- and D-specific fragments generated by HindIII digestion. As shown in Fig 1, the 2.0- and 2.2-kb fragments carrying exon 1 of the RHCE and RHD genes, respectively, were detected with the same intensity in the homozygous D-positive control DNA and in the D⁴ (Kou.) (D/C ratio: 0.95 and 1.1, respectively), whereas a 1.2 gene dosage effect was observed in all the other samples (D/C ratio: 0.45 to 0.55). These results indicated that D⁴ (Bel.), D⁶⁵ (Da.), D⁵⁸ (Hus.), and DFR (Ri.) donors are heterozygous (Dd) and carry only one copy of the RHD gene, whereas the D⁴ (Kou.) donor carries two copies of the RHD gene and is homozygous for the D⁴ haplotype.

Isolation and nucleotide sequence of RhD category transcripts. Total RNA extracts from peripheral blood of the D variants and D-positive individuals were converted to cDNAs and enzymatically amplified between two sets of primers designed to generate a 5' fragment (expected size, 669 bp) specific for exons 1 to 4 and a 3' fragment (expected size, 806 bp) specific for exons 4 to 10 from both the D and non-D cDNAs. Hybridization with D-specific oligonucleo-
Table 1. Characterization of D Epitopes on D Category Cells

<table>
<thead>
<tr>
<th>MoAb-D</th>
<th>D Category Cells</th>
<th>D Epitopes</th>
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</thead>
<tbody>
<tr>
<td>Reg-A</td>
<td>IV* (Bel.)</td>
<td>+</td>
</tr>
<tr>
<td>23W3</td>
<td>IV* (Da.)</td>
<td>+</td>
</tr>
<tr>
<td>HD7</td>
<td>V* (Kou.)</td>
<td>+</td>
</tr>
<tr>
<td>H27</td>
<td>V* (Hus.)</td>
<td>+</td>
</tr>
<tr>
<td>Form-1</td>
<td>V* (type II)</td>
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</tr>
<tr>
<td>Fog-B</td>
<td>H* (Bel.)</td>
<td>+</td>
</tr>
<tr>
<td>RUM-1</td>
<td>H* (Da.)</td>
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<td>105</td>
<td>H* (Hus.)</td>
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<td>MS26</td>
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<tr>
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<td>-</td>
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</table>

Presence (+) or absence (-) of epitopes on variant RBCs treated with papain was deduced from agglutination studies in the gel test system (see Materials and Methods) using specific anti-D MoAbs (MoAbs-D) for the nine D epitopes presently identified and polyclonal antibodies specific for the low-frequency antigens Rh23, Rh30 and Rh50.

- The D* (type II) condition has been studied elsewhere.
- Other examples of MoAbs with apparently the same epitope specificity gave positive reactions.

tide probes (see Materials and Methods) showed that the sizes of the PCR products were identical with the D-positive control and all D category samples (5' fragment, 669 bp; 3' fragment, 806 bp, not shown). These results indicated that the D category phenotypes under study were not caused by a gross deletion within the coding sequence of the RHHD gene. The PCR products were subcloned in pUC18 vectors and the recombinant clones hybridizing with D-specific oligonucleotide probes were sequenced. Several independent clones were analyzed to exclude errors caused by the Taq polymerase activity.

Sequence comparison between the normal RHHD cDNA and...
Table 2. Nucleotide and Amino Acid Polymorphisms of D Category Cells

<table>
<thead>
<tr>
<th>D Variants</th>
<th>Specific Antigens</th>
<th>D Epitopes Lacking</th>
<th>Substitutions</th>
<th>Exofacial Positions</th>
<th>Exon(s) Involved</th>
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<td>TTT</td>
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<td>Ile 172</td>
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Nucleotide and amino acid polymorphisms of the RhD transcripts and deduced RhD proteins characteristic of the five D variant cells compared with the normal RhD (RhXIII) transcripts and proteins.\textsuperscript{12} Mutated nucleotides are underlined. All polymorphisms except that indicated (*) corresponded to D-to-C/E substitutions.

Abbreviation: +, mutations at exofacial positions.
† Only part of the relevant exons are involved in the segmental DNA exchanges.

the different D category cDNAs showed several mutations, which are listed in Table 2. Interestingly, all these polymorphisms, except that at nucleotide 186 of the D^\*4 (Bel.) cDNA, occurred only at sites previously identified as defining the difference between the D and the other RH genes.\textsuperscript{14} The polymorphic nucleotides were located as follows: (1) in exons 2, 3, and 7 for the Bel. (Bel.) cDNA, occurred only at sites previously identified as defining the difference between the introdixon organization of the RH genes,\textsuperscript{14} the polymorphisms identified in the cDNAs of these variants, the Southern blots described in Fig 1 were rehybridized with different exon-specific probes. The PCR probes were designed to detect the genomic fragments carrying exons 2, 3, 5, 7, 8, and 9 of the RH genes (see Materials and Methods) in which the mutations have been located (Table 2). None of the unusual bands shown with the full-length cDNA probe in the HindIII and BamHI patterns of Bel., Da., and Kou. samples are related to the nucleotide polymorphisms identified in the cDNAs of these variants, the Southen blots described in Fig 1 were rehybridized with different exon-specific probes. The PCR probes were designed to detect the genomic fragments carrying exons 2, 3, 5, 7, 8, and 9 of the RH genes (see Materials and Methods) in which the mutations have been located (Table 2). None of the unusual bands shown with the full-length cDNA probe in the HindIII and BamHI patterns of Bel., Da., and Kou. samples (Fig 1) were detected with the PCR probes specific for the relevant mutated exons (exons 2, 3, and 7; exons 7 to 9; and exon 5, respectively; data not shown). These results indicate that the presence of additional fragments in the restriction patterns of the Bel., Da., and Kou. RH loci was caused by nucleotide polymorphisms located in that part of the genomic region not involved in the expression of the D^\*4, D^\*8, and D^\*9 category phenotypes.

**DISCUSSION**

We have analyzed the organization and expression of the RH blood group locus in rare individuals (phenotypes D^\*4, D^\*8, D^\*9, and DFR) whose RBCs lack several of the known nine epitopes that compose the major RhD antigen. All ex-
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Fig 2. Topological position of amino acid substitutions associated with D variants. The topology of the RH polypeptides is predicted from hydropathy plot analysis and from immunochemical experiments (see Discussion). Specific D-to-CE substitutions within each variant are numbered and indicated (B, I). The only amino acid substitution that did not replace a D-specific amino acid by a CE-specific amino acid, D epitopes lacking in each D category variant are given as well as the low-frequency antigens expressed by DV, DV, and DFR individuals (Rh39, Rh23, and Rh50, respectively).

ccept one of these individuals was heterozygous at the RH locus, i.e., one chromosome carries one copy of the variant RHD gene and a normal RHCE gene (Rhd-positive chromosome) and the other chromosome contains only the RHCE gene (Rhd-negative chromosome). However, the DV (Kou.) individual was found to be homozygous for a chromosome carrying an RHD variant gene and a normal RHCE gene. As expected from the normal expression of the Ce/Ee antigens in these variants, only the RHD gene was affected. Because the transcripts that carry the nucleotide substitutions typical of each D category phenotype were closely similar to the RhXIII transcript described earlier, these data provide a further confirmation that the RhXIII-encoded polypeptide corresponded to the Rhd protein.

Except for one of the three polymorphisms (nt 186) identified in the DV transcript, all of the nucleotide mutations associated with the four variants under study corresponded to substitutions of D-specific by CE-specific residues. This result strongly suggested that the D category individuals carry hybrid RHD-CE-D genes resulting from segmental DNA exchange between the highly related RHD and RHCE genes (96% sequence homology). However, the exact limit of the sequences involved in these recombination events has not been yet characterized. The reading frame of the hybrid transcripts was conserved and each encoded a 417 amino acids polypeptide (Fig 2) whose predicted topology is similar to normal Rh proteins.

Models of the hybrid RHD-CE-D gene organization in each variant are shown in Fig 3. All the D-to-CE substitutions identified in the DV (Kou. and Hus.) and DFR (Ri.) cDNAs were found in a localized section of the sequence with normal D transcripts flanking both sides. It is therefore suggested that a single genomic rearrangement event might account for the production of the hybrid RH gene in these variants. In contrast, the DV (Bel.) gene might result from two independent events, because the D-to-CE substitutions at positions 152 and 350 correspond to residues encoded by exons 3 and 7, respectively. Because there are no polymorphisms between exon 8 of the RHD and RHCE genes, it is not possible to determine whether the hybrid gene of the DVb (Da.) genome carried a large CE genomic region extending from the 3' end of exon 7 to exon 9 (>25 kb) or whether only a limited number of sequences surrounding the substitutions in exons 7 and 9 were involved in two independent recombination events. However, Southern blot analysis favors the latter hypothesis, because no alteration of the HindIII and BamHI patterns of this variant have been observed when compared with a control D-positive sample. Similarly, it is assumed that only limited RHCE gene fragments are included in the DV (Bel.), DV (Kou. and Hus.), and DFR (Ri.) hybrid genes, because no RFLPs were found related to the expression of these D category phenotypes. Thus, as commonly postulated when the length of the DNA fragments involved in segmental DNA replacements is on the order of a few hundred nucleotides, the genomic re-arrangements described in the present study might result from gene conversion events rather than from intergenic double crossing over. Hybrid RHD-CE-D and RHCE-DCE genes have been recently found associated with the lack of expression of epitopes D1, D2, D5, D6, D7, and D8 and of the E/e antigens at the membrane surface of DVI category (type II) and homozygous De- or DC- RBCs, respectively. Because these hybrid genes also resulted from DNA segmental replacements between the RHD and RHCE genes, it is proposed that such genomic rearrangements are mostly involved in the extreme polymorphism of the RH blood group system.

The D and CE polypeptides differ by 31 amino acids and we have shown here that D-to-CE substitutions occur in no less than 20 of these sites, it is a reasonable assumption
that these substitutions are associated with the lack of expression of the known nine epitopes. The membrane topology of the Rh polypeptides deduced from hydropathy plot analysis suggests that only eight of these polymorphic residues (at positions 169, 170, 172, 233, 238, 350, 353, and 354) are clearly exofacial and therefore available for the binding of anti-D antibodies on intact RRCs (Fig 2). We predict that the expression of individual epitopes. However, because the Rh reactivity is quite sensitive to conformation, substitutions at intramembranous or intracytoplasmic locations may also be important by modulating protein conformation over long distances. In addition, the absence of between 3 and 6 of the epitopes in each of samples of these D category cells makes it difficult to obtain a full elucidation of all amino acids involved.

All the Rh proteins encoded by the D category variants contain the external Cys-285 residue thought to be important for phenotype expression, including the D$^{3}$ type II$^{36}$ that carries a hybrid D-CE-D protein with amino acids 163 to 313 from the RHCE-encoded protein. The only exception is the D$^{5}$ protein type I$^{38}$ that is deleted from amino acids 163 to 313. This observation suggests that some D epitopes (D3, D4, and D9) are not cysteine-dependent structures. However, it is not known whether other cysteines of the truncated protein may become exposed to the cell surface and compensate for the lack of Cys-285.

It is noteworthy that human anti-D MoAbs exhibit the same reactivity with chimpanzee and human D$^{Vb}$ RBCs. The RhD-like antigen of chimpanzee, R$, is therefore more closely related to the D$^{Vb}$ variant of D than to the common form of the human D antigen.$^{38}$ In agreement with the present findings (Table 2), it has been shown recently that chimpanzees carry a RH-like locus$^{39,40}$ that may direct the synthesis of a D-like polypeptide in which residues 350, 353, and 354 (but not 398) correspond, as in the D$^{Vb}$ polypeptide, to CE-specific amino acids$^{41}$.

The expression of the low incidence antigens Rh23 (D$^{+}$), Rh30 (Go$^{+}$), and Rh50 (FPTT) (Table 1) is associated with the presence of CE-specific residues in the D-specific context of the D$^{Va}$, D$^{IVa}$, and DFR polypeptides, respectively. However, synthetic peptides encompassing the putative epitopes Rh23 (residues 228 to 239), Rh30 (residues 343 to 357), and Rh50 (residues 164 to 175) did not inhibit specific antibodies directed against these antigens (our unpublished data). These findings suggest that all these Rh epitopes are also conformation-dependent structures.

Four general conclusions arise from this analysis. First, as expected, all the D epitopes show different patterns of amino acid substitutions. Second, the epitopes overlap each other, as has been previously suggested$^{12}$ from a consideration of the estimated relative sizes of epitopes and the surface area of the D polypeptide. Indeed, there is no example of single amino acid substitution associated with the loss of a single epitope. In the D$^{Va}$ variant, there is a single amino acid change on the exofacial surface at position 350 and two internal mutations (positions 52 and 152) that are associated with the loss of the four epitopes D1, D2, D3, and D9. This suggests that at least some of the amino acids involved (position 350?) must have been common to all the epitopes that were lost. Third, three epitopes (D1, D2, and D5) must contain amino acids present in at least two of the exofacial loops because they are lost by amino acid substitutions in different regions of the polypeptide. It is probable that the other epitopes are similarly constituted but that this is not shown in this analysis because there have been no substitutions to demonstrate it. Fourth, some epitopes (eg, D1 and D2) that are missing in several variants (Fig 2) probably involve amino acids from nonadjacent loops, indicating that the RhD polypeptide is probably folded to form a cylinder.

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<tr>
<th>Epitope</th>
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<th>D2</th>
<th>D3</th>
<th>D4</th>
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Fig 3. Schematic representation of postulated genetic rearrangements associated with D variants. The RH (■) and the RHCE (□) genes share 96% sequence homology in their coding sequence and exhibit a similar intron/exon organization.$^{38,39}$ Gene conversion events (represented by double crosses) between an RH gene and an RHCE gene carried by an Rh-positive or an Rh-negative chromosome (indicated by the D gene between brackets) resulted in hybrid RH-CE-D gene complexes present in D$^{Va}$, D$^{IVa}$, DFR, and D$^{5}$ (type II) individuals, respectively. E3, E4, E5, E7, and E9 denote exons 3, 4, 5, 7, and 9, respectively, involved in the recombination events. *Only part of the relevant exons are involved in the segmental DNA exchanges.
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This folding would bring the respective loops into apposition.

The definitive demonstration that the amino acids identified in the present model are involved in D antigenicity will be provided by expressing the recombinant D category polypeptides as well as D proteins mutated at different positions in eukaryotic cells and observing the reactivity with various monoclonal anti-D antibodies. However, until an efficient expression system can be designed, the analysis of the polymorphisms associated with the expression or the lack of individual Rh antigens may represent an alternative and useful approach to delineate which amino acids are most likely involved in the reactivity with the Rh-specific antibodies. Moreover, they provide key information for the further investigation of the biochemical basis accounting for the high immunogenicity of RhD antigens.

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