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Molecular cloning and primary structure of the human blood group RhD polypeptide

(erythrocyte membrane/RhD antigen/cDNA/gene analysis/PCR)

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ABSTRACT **The RH (rhesus) blood group locus from RhD-positive donors is composed of two homologous structural genes, one of which encodes the Cc and Ee polypeptides, whereas the other, which is missing in the RhD-negative condition, encodes the D protein that carries the major antigen of the RH system. Recently, different splicing isoforms transcribed from the *CcEe* gene were isolated. We report now the characterization of two other Rh clones, RhII and RhXIII, generated by alternative choices for poly(A) addition sites that were identified as the RhD gene transcripts. That these cDNAs represented the RhD messenger and that the previously described Rh clones were derived from the *CcEe* gene was demonstrated by amplification of RhII/XIII sequences only from D-positive genomes and by cloning and sequencing of *D*- and *CcEe*-specific gene fragments. The predicted translation product of the RhD mRNA is a 417-amino acid protein (M_r = 45,500) that exhibited a similar membrane organization with 13 bilayer-spanning domains compared with the polypeptide encoded by the *CcEe* gene. The D and Cc/Ee polypeptides differ by 36 amino acid substitutions (8.4% divergence), but the NH_2- and COOH-terminal regions of the two proteins are well conserved. Similarly, five of the six cysteine residues of the Cc/Ee proteins were conserved in the D protein, including the unique exofacial cysteine, which is critical for antigenic reactivity. The sequence homology between the Cc/Ee and D proteins supports the concept that the genes encoding these polypeptides have evolved by duplication of a common ancestor gene.**

The RH blood group system is important in transfusion and clinical medicine because it is involved in the hemolytic disease of the newborn, transfusion reactions, autoimmune hemolytic anemias, and hemolytic reactions of nonimmune origin (1–3). This system is recognized as one of the most complex polymorphisms in man (4), but until recently the structural basis of blood group antigens has remained poorly understood (for review, see ref. 5). Currently, individuals are classified as Rh-positive and Rh-negative according to the presence or the absence of the major D antigen on the surface of their erythrocytes, but >46 other antigens, including those of the Cc and Ee series, have been identified (6). The absence or the severe reduction of all of these antigens on erythrocytes from Rh-deficient individuals (Rh_{null}, Rh_{mod}) is associated with morphological and functional abnormalities and a mild hemolytic anemia (3).

The D, c, and E epitopes are carried by at least three distinct homologous unglycosylated integral membrane proteins of apparent M_r 30,000–32,000 that share a common NH_2-terminal sequence (reviewed in ref. 5). Using this structural information, the same Rh mRNA has been cloned independently by our group (7) and others (8) from a human bone marrow cDNA library (clones RhIXb and Rh30A, respectively). It has been shown subsequently by Southern blot analysis that the RH locus is composed of two structural homologous genes, one encoding the RhD polypeptide and the second encoding the Cc and Ee polypeptides (9), most likely by a mechanism of alternative splicing of a primary transcript (10). These findings are consistent with a two-locus model of Rh inheritance (11) and suggest that the two *Rh* genes have evolved through duplication of a common ancestor gene. In the genome of Rh-negative individuals the *RhD* gene is missing, either as a result of a gene deletion or possibly as a survival of the ancestral RH locus before the duplication occurs (9).

The Rh antigen specificity of the protein encoded by the cDNAs cloned previously (7, 8) has not been formally established. Partial RhD protein sequence analysis suggested that neither clone encoded the postulated D polypeptide (8), but recent studies with polyclonal antibodies raised against synthetic peptides indicated that such a clone more likely encodes the E or e antigens (P. Hermand, I.M., M. Huet, C. Bloy, K. Suyama, J. Goldstein, J.-P.C., and P. Bailly, unpublished data). This report describes the characterization of the RhD gene product that encodes the major antigen RhD of the RH system.†

MATERIALS AND METHODS

cDNA Library Screening. A human bone marrow λgt11 cDNA library (Clontech) was screened with the previously isolated RhIXb cDNA (7).

Amplification of Genomic Sequences. One microgram of human leukocyte DNAs from donors of different Rh phenotypes was used as templates in PCR experiments (40 cycles: 94°C for 1 min, 46°C for 1 min, and 72°C for 1.5 min) as described (10) using a Perkin Elmer amplification kit (containing 2.5 units of Taq polymerase) and 300 ng of each primer. PCR A was performed between amplimers common to the RhIXb and RhXIII cDNAs: A1 (5′-GGTGTTGTAACCGAG-3′; sense primer, positions 941–955; +1 taken as the first residue of the initiator AUG) and A2 (5′-ATCATGCCATTGCCG-3′; antisense primer, positions 1076–1062). Amplification products were analyzed on agarose gel and hybridized at 45°C with an RhXIII-specific probe, pXIII (5′-GGCTCCGACGGTATC-3′; positions 1048–1062), or at 65°C with the Rh cDNA probe (an equimolar mixture of the RhIXb and RhXIII cDNAs). PCR B was performed between amplimers A3 (5′-TAAGCAAAAGCATCCAAGAA-3′; positions 1252–1271, sense primer common to RhIXb and RhXIII) and A-XIII (5′-ATGGTGAGATTCTCCTC-3′; po-

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†The sequences reported in this paper have been deposited to the GenBank data base (accession nos. X63094 and X63097).

sitions 1437–1421, antisense primer specific of the RhXIII cDNAs) and probed with the Rh cDNAs.

Southern Blot Analysis. Human leukocyte DNA was digested with *Eco*RI, resolved by agarose gel electrophoresis, and transferred to a nitrocellulose membrane (12). Hybridizations were performed at 65°C in 5× SSPE (SSPE = 0.18 M NaCl/10 mM sodium phosphate/1 mM EDTA), 3× Denhardt's solution, and 0.1% SDS with Rh exon-specific probes (9). Final washes were performed at 65°C for 15 min in 1× SSC (SSC = 0.15 M NaCl/15 mM sodium citrate) and 0.1% SDS.

Genomic Libraries. Three hundred micrograms of leukocyte DNA from a DCCee donor was digested by *Eco*RI and resolved by 0.6% agarose gel electrophoresis. Gel slices of 1 cm thick were cut, DNA fractions were electroeluted, and an aliquot was hybridized with exon-specific probes. Three hundred nanograms of each positive fraction was ligated with 1 μg of λgt11 *Eco*RI arms and packaged *in vitro*. For each library, 3×10^5 plaques were transferred to nitrocellulose filters and hybridized with PCR probes in standard conditions.

DNA Sequencing. Genomic and cDNA inserts were subcloned into pUC18 vectors and sequenced on both strands by the dideoxy chain-termination method (13).

RESULTS AND DISCUSSION

Preliminary attempts to characterize the RhD mRNA were carried out by an oligonucleotide approach using partial sequence information on the NH_2-terminal 54 amino acids of the immunopurified RhD polypeptide published recently (8). Compared to the already cloned polypeptide presumably produced by the *RhCcEe* gene (7–9), the proposed RhD protein exhibits an identical NH_2-terminal sequence up to residue 41 but diverged beyond this point (although still related). Different oligonucleotides deduced from the postulated RhD NH_2-terminal sequence were used as primers in PCR experiments. Surprisingly, when reticulocyte and bone marrow RNA or leukocyte genomic DNA was used as templates no amplification product could be detected.

Isolation and Nucleotide Sequence of Putative RhD cDNA Clones. As the previously isolated RhIXb cDNA cross-hybridized with the two homologous *Rh* genes (*D* and *CcEe*) (7, 9), we have characterized all of the clones detected in the human bone marrow cDNA library with this probe. Two clones, RhVI and RhVIII, were clearly related to splicing isoforms of the RhIXb mRNA transcribed from the *CcEe* gene (10). Two other clones, RhII and RhXIII, were analyzed. Sequence analysis (Fig. 1) indicates that these cDNAs [1522 and 2789 base pairs (bp), respectively] differed only by the length of their 3′ noncoding region (237 bp and 1504 bp, respectively). This is consistent with the detection of a faint 3- to 3.5-kilobase (kb) band in addition to a major 1.5- to 1.7-kb species on Northern blot of erythroblast RNAs (7). Two AATAAA poly(A) signals and a class IV *Alu* sequence were identified in the RhXIII 3′ untranslated sequence. The AATAAA sequence at positions 2747–2752 precedes the poly(A) tail found at the 3′ end of the RhXIII cDNA, whereas the other AATAAA signal at positions 1462–1467 is used to generate the RhII cDNA. These data indicate that the RhII and RhXIII represent two mRNA isoforms generated by alternative poly(A) site choices. Examination of the 3′ noncoding sequences of the RhII and RhXIII clones reveals that they are identical to the 3′ untranslated region of the Rh cDNAs previously published (7–10) until nucleotide 1358 but surprisingly differ after this position.

The coding sequence of the RhXIII and RhIXb cDNAs exhibited 3.5% divergence, which results in a 8.4% divergence at the amino acid level (Fig. 1). Such polymorphism in the coding region and the difference in the 3′ end sequence suggest that the RhXIII mRNA is transcribed from a distinct *Rh* gene rather than from an allelic form of the *CcEe* gene coding for RhIXb. We assumed that the RhXIII-encoded polypeptide was a likely candidate for the RhD protein, although residues 42–54 were different from those attributed to the specific RhD protein by Avent *et al.* (8). Because of this difference, further evidence was needed to establish unambiguously whether the RhXIII cDNA corresponded to the *RhD* gene transcript.

Amplification of RhXIII Sequences from RhD-Positive DNA. To determine whether the RhXIII cDNA derived from the *RhD* gene, genomic DNA extracted from RhD-positive (DCCee, DccEE, Dccee) or RhD-negative (ddccee and ddccEE) donors was analyzed in different PCR experiments. At first, primers were selected to amplify identical size products from the *CcEe* and *D* genes (or cDNAs), which could be subsequently distinguished by hybridization to a gene-specific oligonucleotide located in a region of greater sequence divergence. Accordingly, using the A1 and A2 amplimers flanking nucleotide positions +956 to +1061 that show as much as 30% divergence between RhIXb and RhXIII (Fig. 1), a 136-bp product was amplified and detected in all DNA preparations with the Rh cDNA probe (Fig. 2, PCR A). However, when the pXIII oligonucleotide probe specific for the RhXIII clone was used, only the 136-bp product from RhD-positive DNAs or from the RhXIII cDNA used as control was detected and no hybridization to the 136-bp product amplified from RhD-negative samples occurred. Thereafter, primers were chosen to amplify selectively a *D* gene fragment in RhD-positive genomes. Accordingly, oligonucleotides A3 and A-XIII flanking nucleotides 1272–1420 of the RhXIII cDNA were used as primers. Since the A-XIII amplimer is specific for the 3′ noncoding sequence of RhXIII, an expected fragment of 186 bp could be amplified only in the RhD-positive DNA samples and in the control RhXIII cDNA, but no product was detected in RhD-negative samples (Fig. 2, PCR B).

These experiments demonstrated that specific RhXIII sequences are present in the RhD-positive DNA only, providing proof that this mRNA is transcribed from the *RhD* gene. To add further evidence, sequencing of *D* and *CcEe* gene fragments was performed.

Cloning and Sequencing of RhD Genomic Fragments. Although strongly related, the *RhD* and *RhCcEe* genes can be distinguished by restriction analysis of RhD-positive (two genes) and RhD-negative (one gene) genomic DNAs (9). Southern hybridization with probes specific for nucleotides 486–635 and nucleotides 1227–1347 of the RhIXb cDNA, which are complementary to exon 4 and exon 10 of the *RhCcEe* gene, respectively (B.C.-Z., C.L.V.K., V.R., J.-P.C., and Y.C., unpublished data), each revealed two fragments in the RhD-positive genomes but only one in the RhD-negative DNAs (Fig. 3). The 5.6- and 3.0-kb fragments detected in RhD-positive and -negative DNAs with the exon 4 (Fig. 3*A*) and exon 10 (Fig. 3*B*) probes correspond to the *RhCcEe* gene. The 3.0- and 5.2-kb fragments are D specific since they are revealed only in RhD-positive genomes.

Accordingly, four partial genomic libraries were constructed. Libraries CE4 and D4 were enriched in the exon 4-specific *CcEe* and *D* genomic fragments, respectively, whereas libraries CE10 and D10 were selectively enriched in the exon 10 complementary fragments derived from the *CcEE* and *D* genes, respectively. Sequence analysis indicated that the CE4 and CE10 genomic fragments contain sequences identical to those present in the RhIXb cDNA between positions 486 and 801 (exons 4 and 5) and 1227 and 1488 (exon 10), whereas the D4 and D10 genomic fragments contain the homologous but not identical exonic sequences found in the RhXIII cDNA, including the entire 3′ noncoding region (not shown).

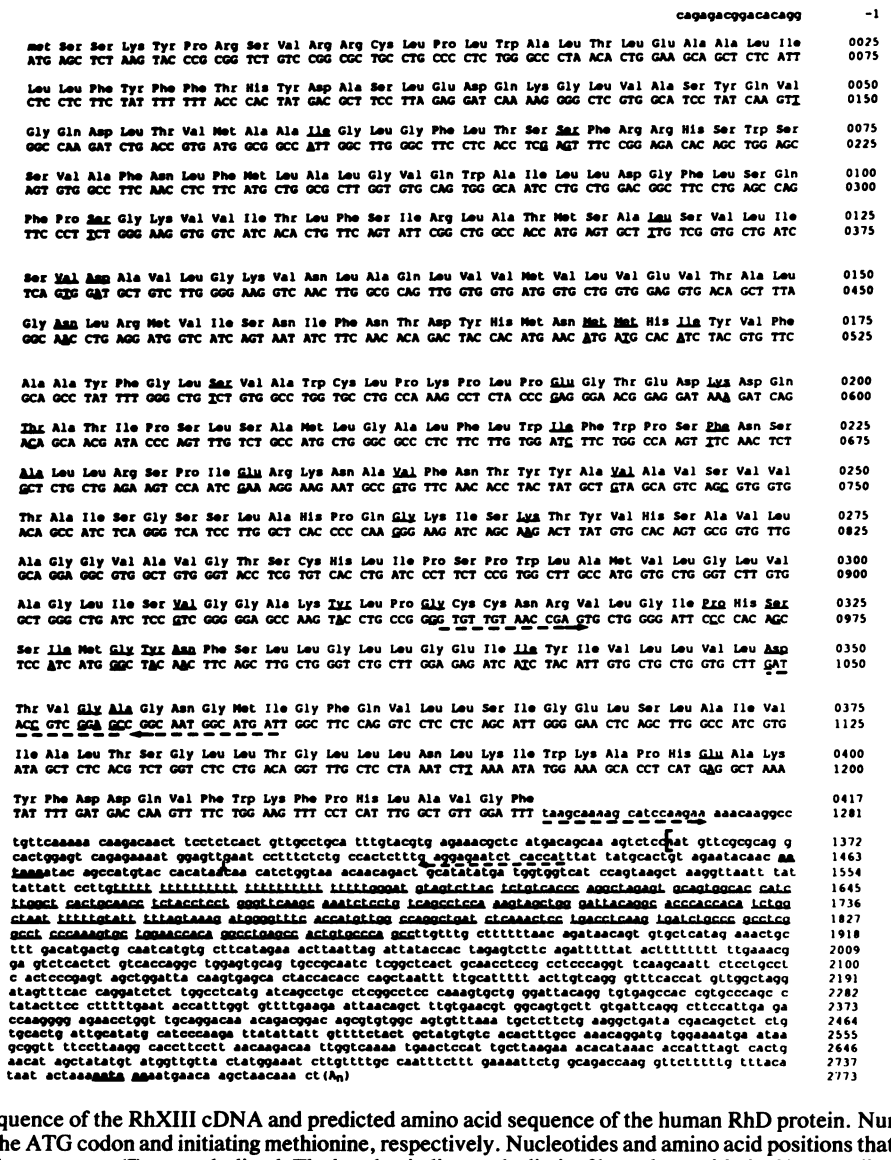

FIG. 1. Nucleotide sequence of the RhXIII cDNA and predicted amino acid sequence of the human RhD protein. Numbering of nucleotides and amino acids starts at the ATG codon and initiating methionine, respectively. Nucleotides and amino acid positions that differ from the RhIXb cDNA and deduced protein sequences (7) are underlined. The bracket indicates the limit of homology with the 3′ noncoding sequence of a splicing isoform of RhIXb (10). The poly(A) signals used in the RhII and RhXIII cDNAs and the *Alu* sequence present in the 3′ region of RhXIII are underlined. The vertical arrow (at nucleotide 1488) indicates the end of RhII cDNA. Positions of the oligonucleotides used as amplimers or as probe in PCR experiments (see Fig. 2) are indicated by dashed lines; from 5′ to 3′: A1, pXIII, A2, A3, and A-XIII.

These findings conclusively established that the RhIXb cDNA, as well as its splicing isoforms (10), derive from the *RhCcEe* gene and that the RhXIII mRNA is transcribed from the *RhD* gene. No spliceoform of the RhD cDNA could be identified, either by the screening of the cDNA libraries or after PCR amplification of various RNA preparations (not shown). Elucidation of the *D* and *CcEe* gene structures might allow determination of whether polymorphisms in the intronic splicing sequences could account for this difference in the expression patterns of the two *Rh* genes. The strong homology between the CcEe and D cDNAs is in agreement with Southern blot analysis indicating that the two genes composing the RH locus of RhD-positive donors are related and may be derived by duplication from a common ancestral gene (9). As expected, the tissue-specific expression of the *D* gene explored by Northern blot analysis was found to be identical to that previously reported for the *CcEe* gene [represented by the RhIXb cDNA, (7)]—i.e., restricted to tissues or cell lines expressing erythroid/megakaryocytic characters (not shown).

Amino Acid Analysis of the RhD Gene Product. The predicted translation product of the *RhD* transcript (RhXIII) is a protein of 417 amino acids (M_r = 45,500) that exhibited 8.4% divergence compared with the RhIXb-encoded polypeptide. Hydropathy plot analysis suggests that the two proteins are organized similarly, starting with an intracellular NH_2 terminus (14) followed by 13 hydrophobic transmembrane domains and terminating with a predicted exofacial COOH end (Fig. 4; ref. 7). Among the 36 amino acid substitutions between the RhIXb and the RhXIII proteins, only 2 are within the first 100 residues and only 1 is within the 60 last amino acids, indicating a high degree of conservation of the NH_2- and COOH-terminal regions of the Rh proteins. Five of the six cysteine residues of the RhIXb polypeptide are conserved at the same positions in RhXIII, particularly the unique exofacial cysteine at position 285 (Fig. 4). Another difference includes a potential N-glycosylation site on Asn-331, which may not be used since the RhD protein is unglycosylated and because this residue is predicted to be located within a hydrophobic domain (Fig. 4).

Which among the 36 amino acid substitutions are essential for antigenic expression of RhD is still undetermined. Remarkably, however, <10 of these substitutions occur on the predicted extracellular hydrophilic loops connecting the

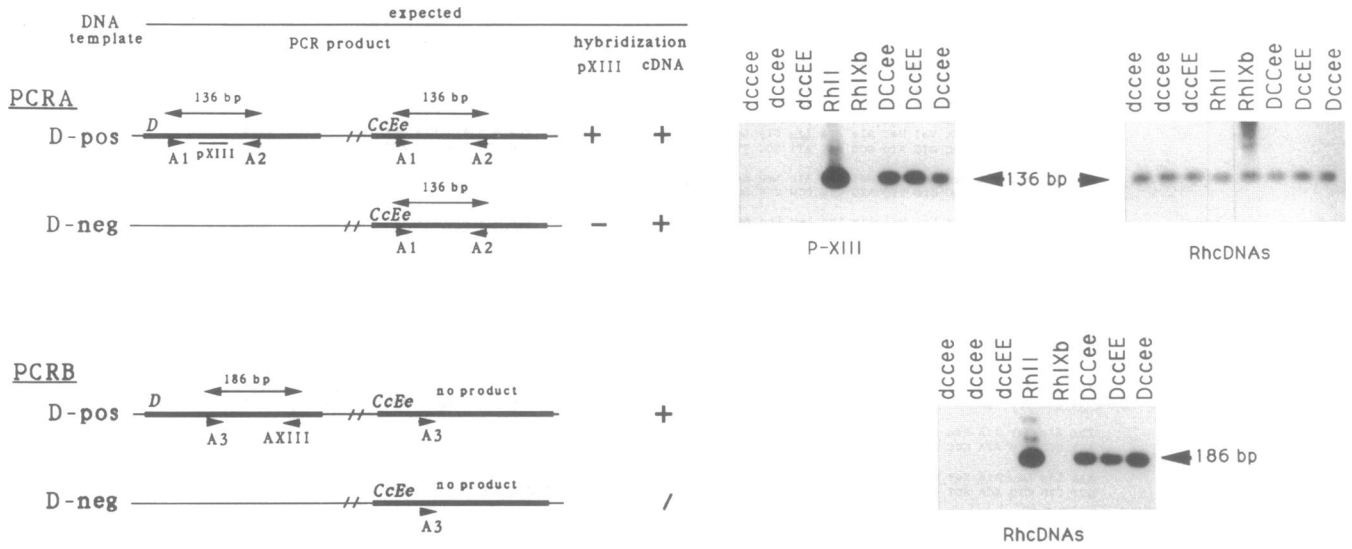

FIG. 2. Amplification of RhXIII-specific sequences from RhD-positive genomes. (*Left*) Diagram of D-positive and D-negative RH loci based on Southern blot analysis (9). Amplimers (arrows) and oligoprobe pXIII positions on the *D* and *CcEe* genes are indicated as are the size and the hybridization pattern of the expected PCR products. (*Right*) DNA from donors with the indicated Rh phenotypes were used as templates in PCR experiments. PCR A: amplification between A1 and A2 primers and hybridization with the Rh cDNA probe (RhIXb plus RhXIII) or with the oligoprobe pXIII specific for the RhXIII coding sequence. RhXIII and RhIXb cDNAs were used as control. PCR B: amplification between primers A3 and A-XIII (specific for the 3′ noncoding sequence of RhXIII) and hybridization with the cDNA probe. See Fig. 1 for the exact locations of the oligoprobes and amplimers.

transmembrane domains of the RhD protein or in close vicinity to the cell surface (Fig. 4), and it is postulated that these positions might be critical for the antigenic properties of the Cc/Ee and D proteins. These few amino acid substitutions are spread along the whole molecule (except for the first and fifth external loops, Fig. 4) and it is of interest that other investigations carried out with a panel of human monoclonal antibodies specific for the D antigen have suggested that at least eight RhD epitopes could be distinguished (16). Whether there may be some correlation between the amino acid exchanges and such RhD epitopes is purely speculative, but the possibility that the monoclonal antibodies may define discontinuous epitopes on the RhD protein is open to analysis. However, the folding and assembly of the loops and transmembrane domains of the RhD protein is not known, but if the Rh proteins are approximately globular in shape with a diameter of 50–60 Å, it is likely that the D epitopes may represent overlapping and conformation-dependent structures (17). Moreover, the lipid composition of the membrane and possibly also the fatty acylation of the RhD protein itself may modulate the D antigenic expression (18–20). Such variations are thought to affect the cell surface exposure of antigenic motifs, particularly among those that are specifically present on the RhD molecule. In addition, as the Rh proteins most likely associate with other glycoproteins that are deficient in Rh_{null} cells to form a membrane complex of relatively large size (5, 21), it cannot be excluded that interactions with other proteins of the Rh protein cluster (5) may also account for D antigen expression.

Recently, it was proposed that a 17-kDa chymotryptic fragment radiolabeled from intact erythrocytes and containing the NH_2-terminal domain of the RhD protein could form an immune complex with a polyclonal anti-D antibody (22). That such fragment was radiolabeled is surprising since

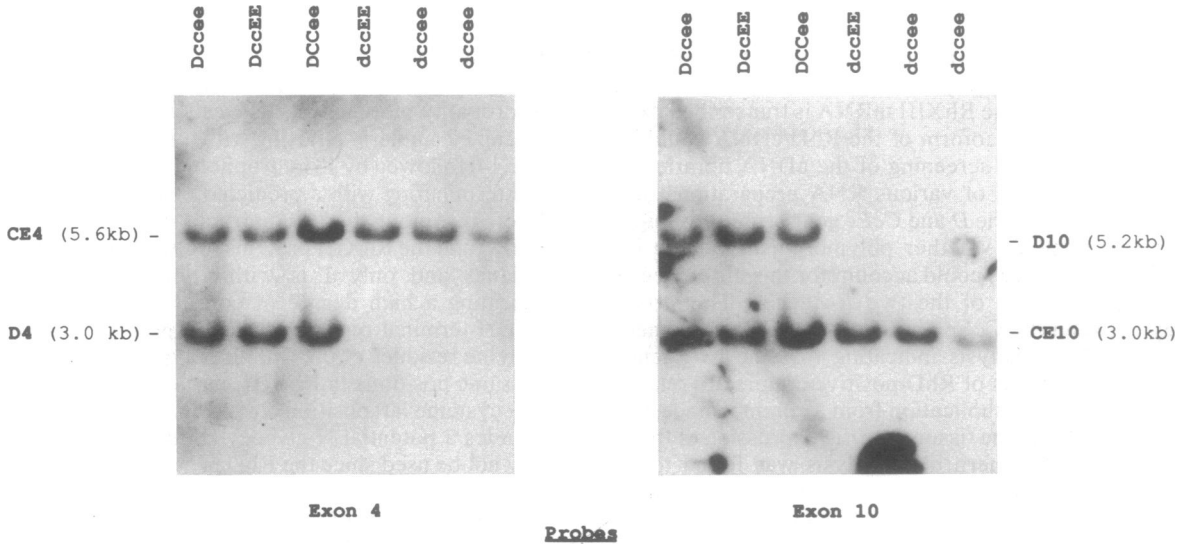

FIG. 3. Identification of RhD and RhCcEe *Eco*RI genomic fragments. RhD-positive or RhD-negative DNAs were digested by *Eco*RI and hybridized on Southern blot with exon 4- and exon 10-specific probes of the *RhCcEe* gene. Fragments of the DccEE DNA identified as *D* specific (D4 and D10) or *CcEe* specific (CE4 and CE10) were subcloned.

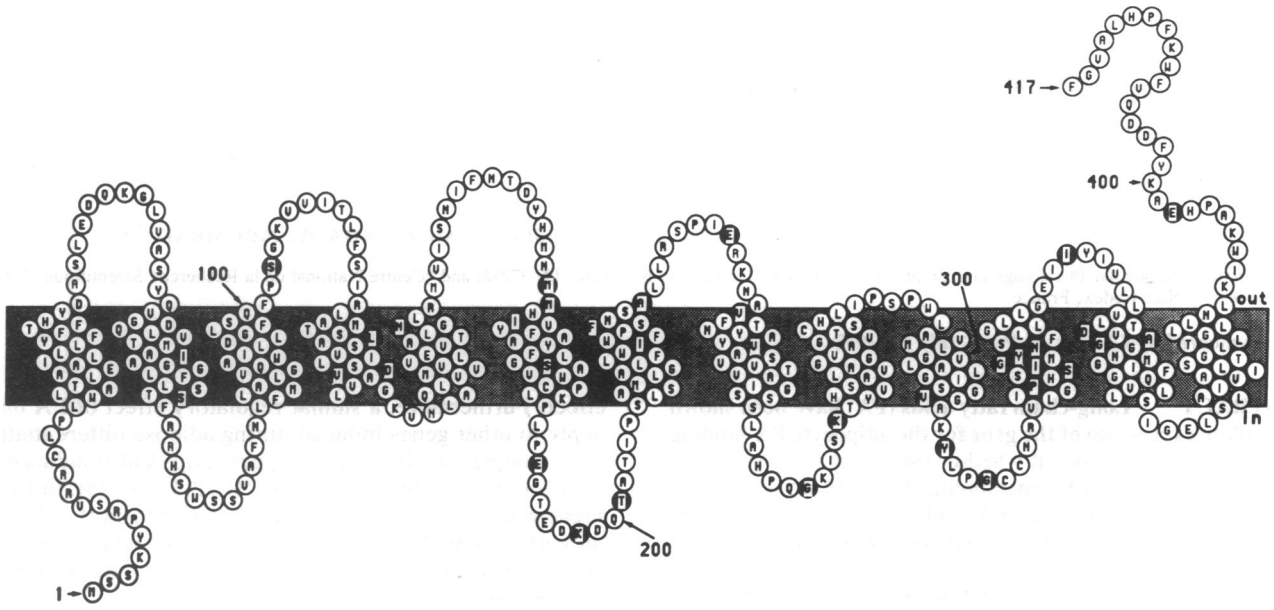

FIG. 4. Topology and protein sequence of the *RhD* gene product. Proposed transmembrane organization of the RhD protein based on hydropathy analysis (15) and on the intracellular orientation of the NH_2 terminus of Rh proteins (14). Open symbols refer to identical positions within the CcEe protein (7) and black symbols refer to substitutions typical of the RhD protein: $^{60}L \rightarrow I$; $^{68}N \rightarrow S$; $^{103}P \rightarrow S$; $^{121}M \rightarrow L$; $^{127}A \rightarrow V$; $^{128}G \rightarrow D$; $^{152}T \rightarrow N$; $^{169}L \rightarrow M$; $^{170}R \rightarrow M$; $^{172}F \rightarrow I$; $^{182}T \rightarrow S$; $^{193}K \rightarrow E$; $^{198}N \rightarrow K$; $^{201}R \rightarrow T$; $^{218}M \rightarrow I$; $^{223}V \rightarrow F$; $^{226}P \rightarrow A$; $^{233}Q \rightarrow E$; $^{238}M \rightarrow V$; $^{245}L \rightarrow V$; $^{263}R \rightarrow G$; $^{267}M \rightarrow K$; $^{306}I \rightarrow V$; $^{311}C \rightarrow Y$; $^{314}V \rightarrow G$; $^{323}H \rightarrow P$; $^{325}I \rightarrow S$; $^{327}V \rightarrow I$; $^{329}H \rightarrow G$; $^{330}S \rightarrow Y$; $^{331}I \rightarrow N$; $^{342}T \rightarrow I$; $^{350}H \rightarrow D$; $^{353}W \rightarrow G$; $^{354}N \rightarrow A$; $^{398}V \rightarrow E$.

previous reports indicated that the ^{125}I label incorporated into tyrosine(s) by cell surface radioiodination was associated with a single chymotryptic peptide following two-dimensional peptide map analysis (23) and was located within the COOH-terminal region of the RhD protein, as it was rapidly removed by carboxypeptidase Y digestion (14, 24). It cannot be excluded, however, that the 17-kDa fragment is weakly labeled under the conditions used, but further investigations are required to clarify this point. Since the number of amino acid residues specific for the RhD protein that are exposed and available to antibodies is extremely low at the NH_2 terminus (Fig. 4), only one of the RhD epitopes might be detected on the 17-kDa fragment.

From the above considerations on the complexity of the RhD epitopes, it is not surprising that preliminary attempts to express the recombinant Rh proteins (D and non-D) alone or together in eukaryotic cells of erythroid or nonerythroid origin have been unsuccessful to date (unpublished data). Determination of whether this is due to the incorrect transport or folding of these proteins to the cell surface awaits further investigation. However, the characterization of various mRNAs and deduced protein sequences encoded by the two genes at the RH locus is a critical step toward the analysis of the Rh polymorphisms and of the molecular defects responsible for the Rh-deficiency syndrome.

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