Structure and Expression of the RH Locus in the Rh-Deficiency Syndrome

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Red blood cell deficiency of Rh proteins is associated with morphologic and functional abnormalities of erythrocytes and with a chronic hemolytic anemia of varying severity. Rh-deficiency may be the result of homozygosity either for a silent allele at the RH locus (Rh_{null} amorph type) or for a recessive inhibitor gene(s) at an autosomal locus unlinked to RH locus (Rh_{null} regulator and Rh_{mod}). In this report, we investigated the RH locus structure of Rh-deficient individuals by Southern analysis using cDNA and exon-specific probes deduced from the recent cloning of *Rh* genes (*CcEe* and *D*). As expected from family studies indicating that Rh_{mod} and Rh_{null} regulator individuals are unable to express Rh antigens but are able to convey functional *Rh* genes from one generation to another, no alteration of the *Rh* genes was detected in these variants. Although Rh_{null} of the amorph type arose by inheritance of a pair of silent alleles at the RH locus, the general organization of the unique *CcEe* gene in the genome of the particular individual under examination was apparently normal and indistinguishable from a Rh-negative chromosome. More surprisingly, no mutation could be detected by sequencing the polymerase chain reaction (PCR)-amplified reticulocyte mRNAs, suggesting that the RH locus of this patient might be altered in its transcriptional activity. Through hybridization with exon-specific probes, we were also able to determine the zygosity for the *D* gene in DNA samples from individuals of known genotypes; using this approach, we found that Rh_{null} regulator variants could be either of the *DD, Dd,* or *dd* genotypes. These findings suggest that the postulated inhibitor gene(s) can negatively suppress the RH locus expression from chromosomes carrying either one or two of the *Rh* genes.

THE Rh DEFICIENCY syndrome[1-3] is a rare hemolytic anemia of varying severity caused by the defect or severe decrease of membrane proteins carrying the Rh blood group antigens. It is associated with morphologic and functional abnormalities of erythrocytes, thus indicating that Rh proteins are important for normal red blood cell (RBC) membrane structure and function.[4] Cells deficient in all Rh antigens are subdivided into Rh_{null} and Rh_{mod}, which totally lack Rh structures or have severely depressed Rh antigens, respectively.[5] Family studies have shown that the Rh_{null} phenotype may arise from two distinct genetic backgrounds. The most common type of Rh_{null}, called the "regulator type" occurs by an inhibition mechanism. According to Levine et al,[6] this phenotype is caused by homozygosity for an autosomal recessive suppressor gene (X^or) that is genetically independent of the RH locus. The second type of Rh_{null}, which was first noted in a Japanese family,[7] is called "amorph" or "silent type" and arose by homozygosity for a silent allele at the RH locus. The Rh_{mod} phenotype was discovered more recently,[8] and was found to arise from the homozygous state of a suppressor gene (X^Q) at an autosomal locus independent of RH. However, the X^or and X^Q nomenclature is inappropriate, because these genes are not located on the X chromosome and because it is not known whether they are alleles at the same locus. The amorph type of Rh_{null} is much less common than other Rh-deficient types. Of 42 recently listed Rh-deficient phenotypes, only 5 are the amorph type of Rh_{null}.[9]

All types of Rh deficiency show the same clinical abnormalities associated with a chronic hemolytic anemia, stomatocytosis and spherocytosis, reduced osmotic fragility, and increased cation permeability. In addition, Rh_{null} membranes have characteristically hyperactive membrane adenosine triphosphatases (ATPases), reduced RBC cation and water contents, and a relative deficiency of membrane cholesterol.[1,2,10,11] Rh_{null} RBCs show an abnormal membrane phospholipid distribution characterized by an increased exchangeability of phosphatidylcholine and phospholipase accessibility of phosphatidylethanolamine.[12] Rh_{null} cells also lack several membrane proteins and glycoproteins (GPs), including the 30- to 32-Kd proteins carrying the Rh antigens[13,14] and the Rh-related GPs recognized by murine monoclonal antibodies.[13,15-19] In addition, glycophorin B, the carrier of Ss antigens, is reduced to approximately 30% of the normal level;[20] certain other blood group antigens, including LW, Duffy (Fy5), U, and Duclos antigens, are also missing or severely decreased in these cells.[21] One important and still unresolved question is to explain how a single genetic alteration at the RH locus itself or at an independent locus controlling its expression (X^or, X^Q) may result in so many membrane abnormalities. To address this issue we have examined the structure and expression of the RH locus in these patients using the recently available information deduced from the cloning of the two *Rh* genes that compose the RH locus.[22-25]

MATERIALS AND METHODS

Patients. Blood samples from Rh-deficient patients were collected on heparin (10 U/mL) and shipped to Paris. Three samples of Rh_{null} of the regulator type were investigated: A.L., provided by L. Lebeck (Loma Linda, CA); Y.T., provided by C. Hyland (Brisbane, Australia); and T.B., provided by Dr M.J. Stelling (Geneva, Switzerland). One sample of Rh_{null} of the amorph type (D.A.A.) was from Dr C. Perez-Perez[26] (Linares, Spain) and one sample of Rh_{mod} (S.S.) from P. Hartnett (Miami, FL). Blood samples from individuals with common Rh phenotypes were from the National Blood Transfusion Service in Paris.

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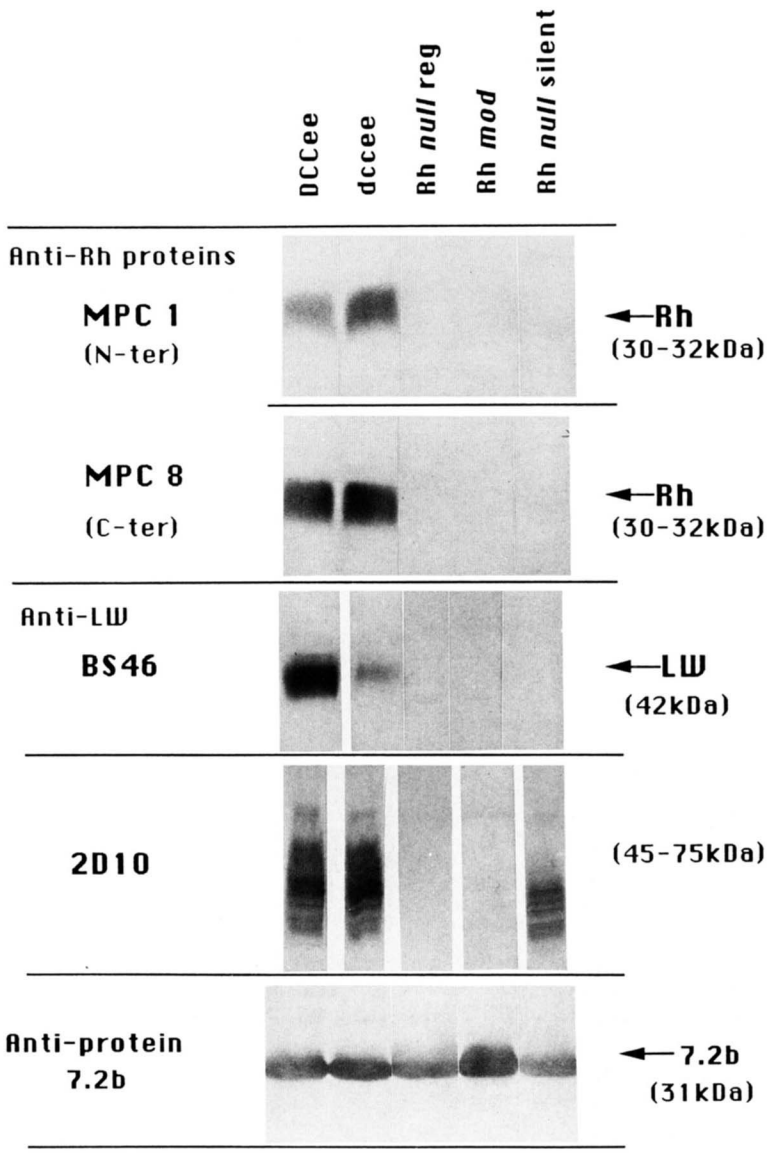

Fig 1. Immunostaining of RBC membrane proteins from Rh-deficient erythrocytes. One hundred to 150 μg of total membrane proteins from A.L. (Rh_{null} reg, U-negative), S.S. (Rh_{mod}, U-negative), and D.A.A. (Rh_{null} silent, U-positive) separated by SDS-PAGE on a 12% polyacrylamide separating gel were transfered to nitrocellulose sheets. Each was incubated with rabbit anti-Rh protein antibodies (MPC1 and MPC8, 1:4,000 dilution), with a murine MoAb anti-LW^{ab} (BS46; 10 μg/mL), with the murine MoAb 2D10 (10 μg/mL), and with the rabbit antiprotein 7.2b antiserum, respectively. Antibodies bound were detected by the alkaline phosphatase conjugate substrate method. Arrows on the left side indicate the migration position of the Rh, LW, 2D10, and 7.2b polypeptides.

Membrane protein analysis. For immunostaining analysis, sodium dodecyl sulfate (SDS)-lysates from RBC membrane preparations prepared as described[27] were separated by discontinuous SDS polyacrylamide gel electrophoresis (PAGE),[28] transferred to nitrocellulose sheets,[29] and incubated as described[26,30] with rabbit polyclonal antibodies raised against synthetic Rh peptides (MPC1 and MPC8 reacting with the NH2- and COOH-terminal regions of the Rh proteins, respectively) or with the murine MoAbs BS46 (gift of Dr H.H. Sonneborn, Dreieich, Germany) and 2D10 (gift of Dr A.E.G. von dem Borne, Amsterdam, The Netherlands) that react with blood group LW[31] or Rh-related[18,19] membrane GPs, respectively. A rabbit polyclonal antibody against the membrane protein 7.2b (gift of Dr R. Johnson, Detroit, MI) was also used as control. Bound antibodies were detected with alkaline phosphatase-labeled goat antirabbit IgG or rabbit antimouse diluted (1:800) followed by revelation with the alkaline-phosphatase conjugate substrate kit (Biorad Laboratories, Rockville Centre, NY).

Genomic DNA analysis. Human leukocyte DNA extracted from peripheral leucocytes was digested with restriction enzymes (Appligene, Strasbourg, France; 10 U/mg DNA), resolved by electrophoresis in 0.8% agarose gel, and transferred onto nitrocellulose membrane (BA85; Schleicher & Schüll, Dassel, Germany) as described.[32] DNA probes used for hybridizations were: (1) the complete RhIXb cDNA encoding the Cc/Ee proteins,[22] (2) exon-specific sequences obtained by PCR from exon 1, exon 4, and exon 9 + 10 of the *CcEe* gene (Cherif-Zahar et al, unpublished data). Hybridization with DNA probes (10^6 cpm/mL) was performed for 16 hours at 65°C in 5 × SSPE (1 × SSPE = 0.15 mol/L NaCl, 0.01 mol/L NaH_2PO_4, 0.001 mol/L EDTA, pH 7.4), 5 × Denhardt (1 × Denhardt = 0.02% wt/vol each of Ficoll, polyvinylpyrolidone, and bovine serum albumin [BSA]), 0.1% SDS, and 100 mg/mL sonicated salmon-sperm DNA. Final washes were performed at 65°C for 15 minutes in 0.1 × SSPE, 0.1% SDS.

Reverse transcription coupled with PCR amplification. Total RNA was extracted by the acid–phenol-guanidinium method[33] from 5 mL of peripheral blood (PB) and 0.5 μg was incubated for 40 minutes at 42°C in a reaction mixture (50 μL) containing 100 mmol/L Tris (pH 8.3), 140 mmol/L KCl, 10 mmol/L $MgCl_2$, 30 mmol/L β-mercaptoethanol, 1 mmol/L of each deoxynucleoside triphosphate (dNTP), 40 U of ribonuclease inhibitor (RNasine),

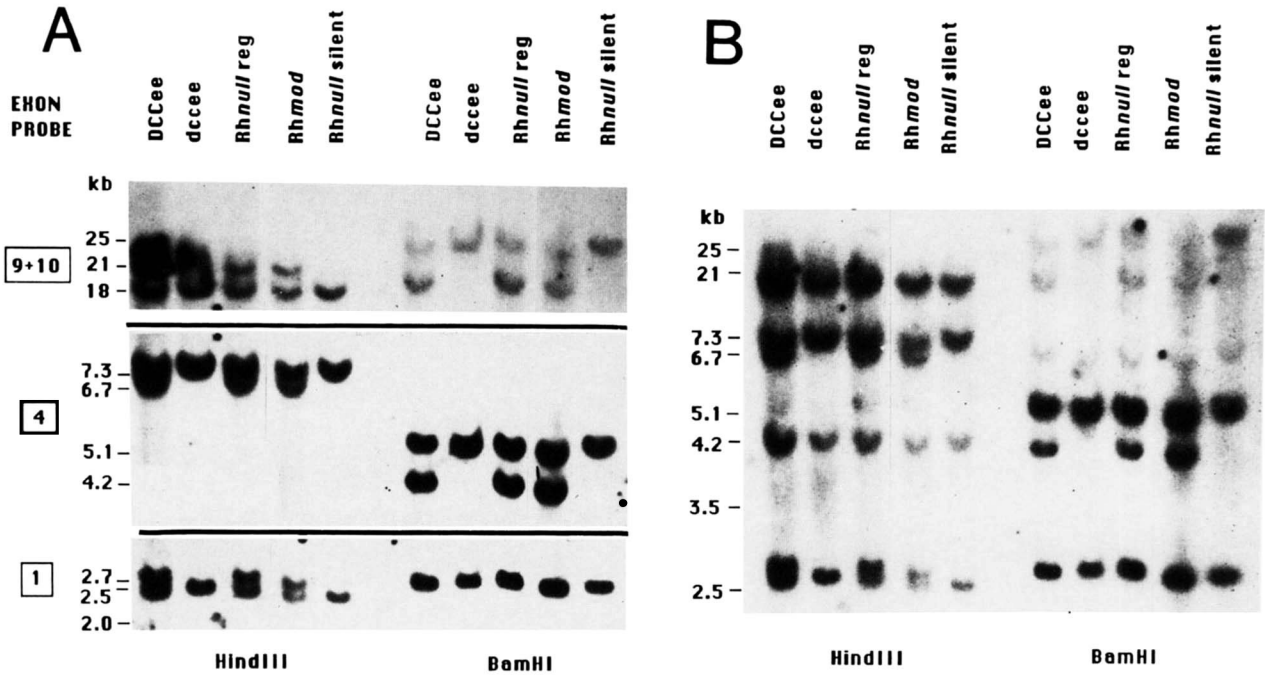

Fig 2. Southern blot analysis of the RH locus in the Rh-deficient patients. DNA from RhD-positive and RhD-negative donors with the indicated phenotype and from Rh-deficient individuals were digested by *Hin*dIII or *Bam*HI restriction enzyme and hybridized on Southern blot with the exon-specific Rh probes (A) as indicated or (B) with the full-length Rh cDNA probe.

and 10 U of avian myeloblastosis virus reverse transcriptase (Promega Biotec, Madison, WI). The cDNA products were then subjected to PCR in 50 mmol/L KCl, 10 mmol/L Tris (pH 8.3), 0.01% gelatin, 0.2 mmol/L of the four dNTPs, 50 pmol of each primer, and 2.5 U of *Thermus acquaticus* DNA polymerase (Perkin-Elmer, Norwalk, CT). All primers were chosen from the RhIXb cDNA sequence. PCR amplifications (30 cycles) were performed under the following conditions: 1 minute of denaturation at 94°C, 1.5 minutes of primer annealing at 55°C, and 2 minutes of extension at 72°C. Amplification products were purified using agarose gels electrophoresis, phosphorylated with polynucleotide kinase, and subcloned in pUC18 vector.

DNA sequencing. Inserts from recombinant pUC18 vectors were sequenced on both strands using the dideoxy chain termination method.[34]

RESULTS AND DISCUSSION

Protein defects. In preliminary studies membrane preparations from the regulator (reg), the amorph (silent), and the Rh_{mod} types of Rh-deficient erythrocytes that are unreactive (or very weak reactive for Rh_{mod}) with all anti-Rh antibodies of different specificities (D, C, c, E, e, etc) were separated by SDS-PAGE and examined by immunostaining with antibodies raised against synthetic peptides and directed against the Rh proteins (30 to 32 Kd), the LW GP (42 Kd), and Rh-related GPs (45 to 75 Kd). Neither the MPC1 nor the MPC8 antibodies that recognize the NH2-terminal (residues 34 to 46) and the COOH-terminal (residues 409 to 417) of the Rh proteins,[26,30] respectively, from RhD-positive (DCCee) and RhD-negative (ddccee) samples, reacted with the membrane proteins of Rh-deficient cells (Fig 1). Because the Cc/Ee and D proteins have conserved NH2- and COOH-terminal domains,[25] it is concluded that the Rh-deficient cells lack both the Cc/Ee and the D proteins. This conclusion is consistent with the finding that the polyclonal antipeptide antibodies react with RBCs from homozygous D – – individuals who carry the D but not the Cc and Ee proteins.[30] The present immunoblotting analysis of Rh-deficient cells, together with those reported by Suyama and Goldstein,[35] support the conclusions of previous studies that have suggested the lack of Rh proteins in Rh_{null} cells using the indirect method of labeling.[13,14] Although Rh-deficient erythrocytes and RBCs from patients with hereditary stomatocytosis show similar morphologic abnormalities, both conditions are distinct at the molecular level[36] because the antiprotein 7.2b antiserum that selectively recognized a 31-Kd protein that was deficient in patients with hereditary stomatocytosis[37] reacted normally with all Rh-deficient erythrocytes (Fig 1).

Rh-deficient cells also lack the LW and Rh-related GPs.[16,26,38] The BS46 (anti-LW) antibody reacted only with RBC membrane proteins from common Rh phenotypes and not with those from Rh-deficient individuals (Fig 1). The murine MoAb 2D10 also recognizes membrane GPs of 45 to 75 Kd that are absent from most Rh_{null} or Rh_{mod} cells lacking the blood group U, but are weakly expressed on some Rh_{null} cells that are U-positive.[18,19] Accordingly, 2D10 did not react with the U-negative A.L. and S.S. samples (Fig 1) but did react weakly with the U-positive D.A.A. sample.[26] These multiple protein abnormalities have not been explained on a molecular basis. A currently favored hypothesis is that Rh polypeptides are required for the correct transport or cell-surface expression of certain GPs or, alternatively but not exclusively, that a GP missing in Rh_{null} cells is necessary for the cell-surface expression of Rh pro-

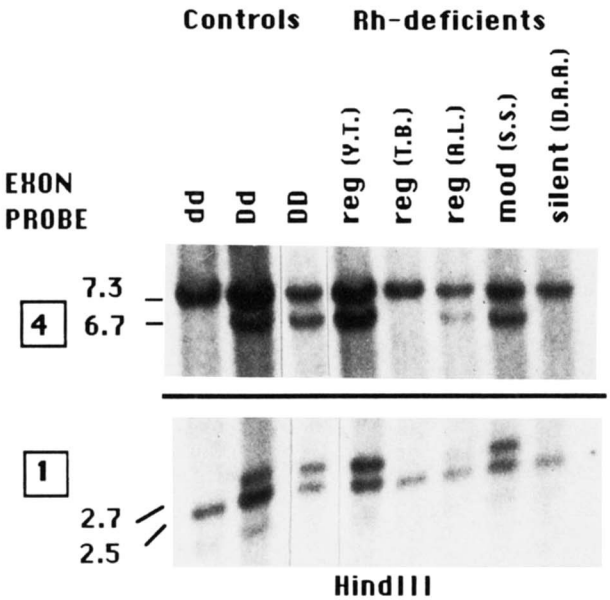

Fig 3. Determination of the zygosity for the RhD gene by Southern blot analysis. Genomic DNA from donors with the indicated genotype for the *D* gene and from the Rh-deficient patients Y.T., T.B., A.L. (Rh_{null} reg); S.S. (Rh_{mod}); and D.A.A. (Rh_{null} silent) were digested with the *Hin*dIII restriction enzyme and hybridized with the exon 4 and exon 1 probes. Gene dosage effects were estimated by determination of the relative intensity of 6.7/7.3-kb fragments (exon 4 probe) and from 2.7/2.5-kb fragments (exon 1 probe) corresponding to the *D* and *CcEe* genes, respectively, after densitometry analysis of the autoradiogram.

teins. This explanation suggests that the Rh antigen structure is a large membrane complex containing the Rh polypeptides, Rh-related GPs, and possibly other unrelated GPs.[4]

Southern blot analysis of the RH locus in Rh_{null} and Rh_{mod} phenotypes. Genomic DNA from the Rh_{null} reg Y.T., the Rh_{null} silent D.A.A., and the Rh_{mod} S.S. were digested with *Hin*dIII and *Bam*HI restriction enzymes and subjected to Southern blot analysis with Rh-specific probes. DNAs from RhD-positive (DCCee) and RhD-negative (ddccee) donors were used as a control. Figure 2A shows the hybridization patterns obtained with probes for exon-1, exon-4, and exon-9+10 of the *CcEe* gene (Cherif-Zahar et al, unpublished data) that correspond to residues (1-48, 162-211, and 408-416, respectively) of the mature *CcEe*-encoded proteins. Two hybridizing bands were detected with each exon-specific probe after *Hin*dIII and *Bam*HI digestion of the DCCee sample (in the *Hin*dIII profile, ie, exon-1: 2.7 kb and 2.5 kb; exon-4: 7.3 kb and 6.7 kb; exon-9+10: 21 kb and 18 kb). However, only one band was detected (*Hin*dIII profile, exon-1: 2.5 kb; exon-4: 7.3 kb; exon-9+10: 18 kb) with the ccddee sample, as expected from recent studies showing that the RH locus is composed of two homologous genes (*D* and *CcEe*) in RhD-positive individuals, but of only one (*CcEe*) in RhD-negative individuals.[24]

The hybridization patterns of Y.T. (Rh_{null} reg) and S.S. (Rh_{mod}) did not differ from those obtained with the RhD-positive control, and those from D.A.A. (Rh_{null} silent) were identical with those of the control RhD-negative phenotype (Fig 2A). No additional or missing band could be detected with the Rh-deficient samples suggesting a large sequence insertion and/or deletion at the RH locus of these individuals. This inference was confirmed by hybridization with the full length RhIXb cDNA probe (Fig 2B). It is not surprising that no defect of the *Rh* genes occurs in Rh deficiency caused by a postulated recessive suppressor gene independent of the RH locus. Studies of families have shown that these individual family members carry the normal *Rh* genes but fail to express Rh antigens.[5] Our results provide the first direct proof that confirms this hypothesis.

Hybridization with exon-specific probes was also performed to determine whether the Rh-deficient individuals carrying the *D* gene were homozygous or heterozygous for this gene. Gene dosage effects were obvious when *Hin*dIII digests of DNA from individuals of known *DD, Dd,* and *dd* genotypes, which all carry two copies of the *CcEe* gene, were estimated by densitometric analysis of autoradiograms (Fig 3). As expected from the presence of two copies of the *D* and *CcEe* genes in *DD* genome, the *D*-specific and *CcEe*-specific fragments[24] were detected with the same intensity as with the exon 4 probe (6.7 kb and 7.3 kb, respectively) and the exon 1 probe (2.7 kb and 2.5 kb, respectively). On the other hand, a 1:2 gene dosage effect was observed between the *D* and *CcEe* fragments in the heterozygous *Dd* genome. Only the *CcEe* gene was detected in the Rh-negative (*dd*) DNA. Examination of the hybridization pattern of the Rh-deficient samples (Fig 3) showed that S.S. (Rh_{mod}) and Y.T. (Rh_{null} reg) were homozygous for the *D* gene, whereas A.L. (Rh_{null} reg) was heterozygous for the *D* gene. T.B. (Rh_{null} reg) and D.A.A. (Rh_{null} silent) carried only the *CcEe* gene as Rh-negative donors. Although the Rh genotypes of the Rh-deficient individuals in this study were not known, these findings agree with studies of families that suggest that Rh haplotypes carrying or not carrying the *D* gene are segregating in Rh*null* reg and Rh*mod* pedigrees.[39,40] These data also show that the regulatory genes that determine the Rh_{null} reg and Rh_{mod} phenotypes are effective both on chromosomes that carry either one or two Rh genes (*CcEe* or *CcEe* and *D*). Moreover, our findings show for the first time that zygosity for the *D* gene (*DD* or *Dd*) can be easily determined without studying families by using exon-specific probes on a DNA sample. Together with the possibility of detecting the *D* gene,[25] this may have applications, for instance, in the prediction of the D genotype of a father in couples where there is an RhD-negative woman at risk of fetal alloimmunization.[41]

Southern blot analysis of the RH locus in the Rh_{null} silent family. Genomic DNA isolated from Epstein-Barr virus (EBV)-immortalized lymphoblastoid cell lines derived from each family member of the Rh_{null} silent D.A.A.[26] was digested with *Hin*dIII and probed with the RhIXb cDNA on Southern blot (Fig 4). No obvious alteration of the restriction profiles from either D.A.A. (genotype denoted − − −/− − − on Fig 4, indicating two copies of the silent *Rh* allele) or her parents and children could be detected. Comparison of the Rh genotypes and the restriction profiles (best shown with the 7.3-kb and 6.7-kb hybridization bands) is consis-

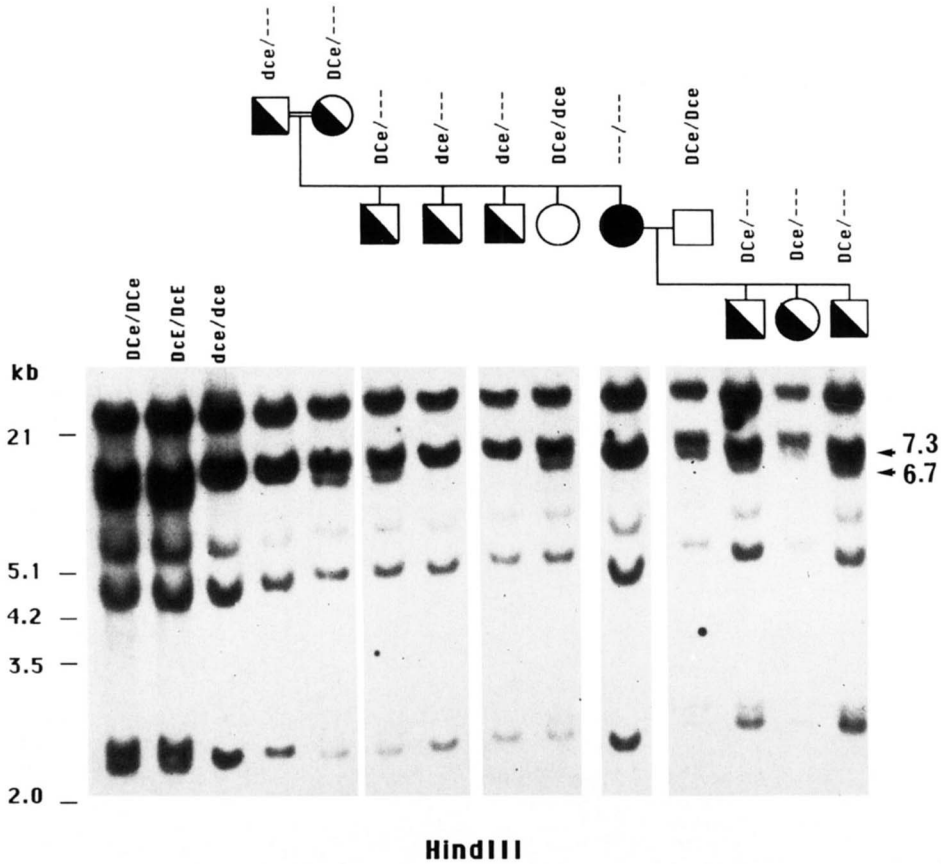

Fig 4. Pedigree of family ALA showing the inheritance of a silent *Rh* gene and *Hin*dIII restriction patterns of genomic DNA. Solid symbols refer to homozygous (the preposita D.A.A.) or heterozygous individuals for the silent *Rh* gene. Open symbols refer to wild-type *Rh* genes. (— — —) Refers to the silent Rh chromosome segregating in the family. DNA from each family member was digested by *Hin*dIII enzyme and hybridized with the Rh cDNA probe on Southern blot. Arrows on the right side indicate the 7.3-kb and 6.7-kb hybridization bands corresponding to the *CcEe* and *D* genes, respectively.

tent with the inheritance of the silent Rh allele (---) with a chromosome carrying only the *CcEe* gene at the RH locus (hybridization band of 7.3 kb), as found in the RhD-negative condition (ddccee sample). In this experiment, the silent chromosome could not be distinguished from a normal RhD-negative chromosome. Obviously, a large deletion at the RH locus cannot account for this particular example of the amorphic type of Rh_{null}, but it is not to be concluded that this may not occur in other individuals with a similar phenotype.

Analysis of Rh transcripts in the Rh_{null} amorph phenotype. To determine whether the lack of expression of the Rh polypeptides in the RBCs of D.A.A. might result from a mutation in the coding sequence of the *CcEe* gene, overlapping regions of the reticulocyte Rh cDNA of this patient and of an Rh-positive control were amplified in vitro (not shown). Amplification products of expected size obtained from the D.A.A. samples were cloned in a pUC18 vector, and several clones were sequenced to detect possible errors caused by mutations that could have occurred during amplification. No difference was seen between the nucleotide sequence of the Rh transcript from D.A.A. and the previously characterized transcript of the *CcEe* gene from the individual with common Rh phenotypes.[22,25] Therefore, it is concluded that the absence of Rh antigens at the RBC surface of the Rh_{null} amorph phenotype is neither the result of a gene rearrangement at the RH locus nor the result of point mutations(s) in the Rh coding region.

This conclusion raised the hypothesis that the transcriptional activity of the Rh_{null} gene or the message stability may have been affected. To estimate the relative level of the Rh transcript in Rh_{null} and normal cells, Northern blot analysis was performed on RNA preparations from circulating reticulocytes of D.A.A. and the Rh-negative donor. Although the Rh mRNA species were easily detected in the control K562 and HEL human erythroleukemic cell lines,[22] the level of hybridization of the Rh probe in reticulocyte RNA preparations was too low to ascertain whether the observed reduction of signal in the Rh_{null} sample, compared with normal reticulocytes, signified an altered *Rh* gene transcription (not shown). However, it is assumed that mutation(s) in regulatory elements of the *Rh* gene (*CcEe* in this patient) may have caused a drastic reduction of mRNAs encoding the Rh polypeptides. Under this hypothesis, it cannot be ruled out that the Rh_{null} silent gene could direct the synthesis of a very low amount of Rh polypeptides, and this might account for the characterization of a so called homologous Rh-like polypeptide in Rh_{null} erythrocytes recently reported by Connor et al.[42] However, we and others have been unable to detect such a polypeptide, either by immunostaining or by radioactive surface labeling[13,14,26,30,35] (and this work). This apparent discrepancy might result from a difference in the sensitivity of the techniques used. Further comparison of the present data cannot be done because neither the regulator nor amorph status of the Rh_{null} sample studied, nor the level of expression and the membraneous/cytosolic nature

of the Rh-like polypeptide, were clearly discussed by Connor et al.[42] Because Rh_{null} cells have a normal aminophospholipid translocase activity,[43,44] but lack or are severely defective in Rh proteins, it is unlikely that Rh polypeptides and the translocase are identical molecules as proposed earlier.[43]

Because the silent and regulator types of Rh-deficiencies occur under different genetic background, distinct mechanisms are expected to explain the very low or nonexpression of Rh proteins in each instance. However, as discussed above, the Rh membrane complex hypothesis may provide a simple explanation for the absence of the Rh polypeptides and other membrane proteins in both instances, assuming that either one of the Rh protein(s) or some as yet unidentified component(s), respectively, might be necessary for the correct transport or cell-surface expression of all these proteins as a stable complex in the membrane.

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