J Mol Evol (1994) 38:169–176

Journal of Molecular Evolution

© Springer-Verlag New York Inc. 1994

# Molecular Characterization of the Rh-like Locus and Gene Transcripts from the Rhesus Monkey (*Macaca mulatta*)

**Isabelle Mouro,[1] Caroline Le Van Kim,[1] Baya Cherif-Zahar,[1] Isabelle Salvignol,[2] Antoine Blancher,[2] Jean Pierre Cartron,[1] Yves Colin[1]**

[1]Institut National de la Santé et de la Recherche Médicale, Unité 76 Institut National de Transfusion Sanguine, Paris, France
[2]Centre de Transfusion Sanguine, Hopital Purpan, Toulouse, France

Received: 16 February 1993/Accepted: 5 April 1993

**Abstract.** The human Rh blood group locus consists of two structurally related genes (*D* and *CcEe*) in Rh-positive haplotypes but a single gene (*CcEe*) in Rh-negative haplotypes. The genome of rhesus monkeys (*Macaca mulatta*), while not expressing any of the human Rh D, C, c, E, or e specificities, carries a Rh-like locus strongly related to the human Rh locus. Southern blot analysis suggested the presence of only one *Rh-like* gene with an additional truncated fragment corresponding to the 5′ region. RNA preparations from *M. mulatta* bone marrow cells contained Rh-like species of 1.7 kb. Two allelic Rh-like transcripts were amplified by PCR and sequenced. The predicted translation product of the first transcript was a 417-amino-acid protein closely similar to the human Rh counterpart. The predicted product of the second transcript consisted of a 361-amino-acid polypeptide truncated in the $NH_2$ terminal region and differing from the former by a few substitutions. The macaque Rh-like protein sequences differed from those of human D and Cc/Ee polypeptides by 22–25%, whereas the degree of identity between the human proteins was 91.5%. Implications of these results in the analysis of the evolutionary pathway of the Rh locus are discussed.

*Correspondence to:* Y. Colin, INSERM U76, INTS, 6 rue Alexandre Cabanel, 75015 Paris, France

**Key words:** Rhesus blood group system — *Macaca mulatta* — PCR amplification — Southern blot analysis — Rh-like cDNAs — Gene duplication

## Introduction

Rhesus (Rh) together with ABO are the main blood group systems in man that have proven to be important in transfusion and clinical medicine. Grossly, individuals are subdivided into "Rh-positive" and "Rh-negative" according to the presence or absence of the D antigen on red cells. The Rh system, however, is much more complex, and as many of 47 antigens, including those of the Cc and Ee series, have been described (Issitt 1989). Recent studies have shown that the Rh antigens are carried by at least three analogous integral membrane proteins of 30–32 kDa that are not glycosylated or phosphorylated. (For review see Agre and Cartron 1991.) The primary structure of these proteins was deduced from the nucleotide sequence of their cloned mRNAs (Chérif-Zahar et al. 1990; Avent et al. 1990; Le Van Kim et al. 1992), showing that the D and non-D polypeptides exhibit 82% sequence homology (Le Van Kim et al. 1992). Whether the Rh antigens are the products of one gene (Wiener 1944) or several closely linked genes (Fisher 1944) was clarified only recently. Indeed, the Rh locus is composed of two structurally related genes (*D* and

*CcEe)* in Rh-positive haplotypes but of a single gene (*CcEe*) in Rh-negative haplotypes (Colin et al. 1991). Accordingly, the Rh locus most likely evolved by duplication of an ancestral gene.

It is known that, among the nonhuman primates, apes express Rh-like antigens (Socha and Ruffié 1983) while Old World monkeys (baboons, macaques, etc.) do not. Human anti-c antibodies agglutinate gibbon, chimpanzee, orangutan, and gorilla red cells whereas human anti-D react only with chimpanzee, orangutan, and gorilla cells (Wiener et al. 1964; Shaw 1986; Socha and Ruffié 1990). Analogs of nonhuman Rh proteins have also been described recently in monkey, cow, cat, and rat as 32-kDa erythrocyte polypeptides (Saboori et al. 1989). All these studies established that other species than man and apes may carry Rh-like proteins that have not been yet characterized. In this paper, we report on the molecular genetic analysis of the Rh-like locus of rhesus monkeys (*Macaca mulatta*) which was historically associated with the discovery of the Rh system (Landsteiner and Wiener 1940).

## 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, USA). Random priming labeling kits were from Boehringer Mannheim (Germany) and pUC sequencing kits were from Pharmacia (Uppsala, Sweden).

*DNA Probes.* The complete human Rh cDNA (RhIXb clone) and PCR-amplified exon-specific fragments deduced from the *RhCcEe* gene structure analysis (exon1: transcribed sequence from nucleotide [nt.] 1 to nt. 223; exon5: nt. 711 to nt. 875; exon7: nt. 1013 to nt. 1146; exon10: nt. 1305 to nt. 1539; Cherif-Zahar et al. 1993) were prepared as described previously (Colin et al. 1991) and labeled by the random priming method.

*Southern Blot Analysis.* Human and *M. mulatta* (rhesus monkey) genomic DNA extracted from peripheral leukocytes (Sambrook et al. 1989) was digested by restriction enzymes (10 U/μg DNA), resolved by electrophoresis in 0.8% agarose gel, and transferred as described by Southern to a Zeta probe GT nylon membrane (Biorad, Richmond, VA, USA). Hybridization with the DNA probes ($10^6$ cpm/ml) was performed for 24 h at 65°C in 7% SDS, 0.5 M $NaHPO_4$, 1 mM EDTA. Final washes were carried out at 65°C for 45 min in 5% SDS, 40 mM $NaHPO_4$, 1 mM EDTA and for 30 min in 1% SDS, 40 mM $NaHPO_4$, 1 mM EDTA.

*Northern Blot Analysis.* Total RNAs from *M. mulatta* bone marrow cells and from the human erythroleukemic cells K562 and HEL were prepared by the guanidium-isothiocyanate/cesium chloride method (Sambrook et al. 1989), resolved by electrophoresis on 1% agarose, 1.1% formaldehyde, and transferred onto Zeta probe GT nylon membrane. Hybridization with the cDNA probe was performed under the conditions described for the Southern blots.

*Polymerase Chain Reaction (PCR) Amplification.* The primers used for PCR amplification were synthesized on a Milligen Biosearch 8700 DNA synthesizer and purified on a 20% acrylamide/urea gel. The nucleotide sequence of these primers was deduced from the 5′ and 3′ noncoding region of the previously isolated human Rh cDNA clone (Chérif-Zahar et al. 1990) and was, respectively, $^{5'}$GCA CAG AGA CGG ACA CA$^{3'}$ and $^{5'}$TGC TGT CAT GAG CGT TTC TC$^{3'}$; 1.5 μg of total RNA from *M. mulatta* was converted in the first cDNA strand as described previously (Colin et al. 1989). One-fifth of the cDNA products were enzymatically amplified between the 5′ and 3′ primers described above by the PCR method (Saiki et al. 1988) in the presence of Perfect Match Enhancer (Stratagene, La Jolla, CA, USA). Thirty-five cycles of amplification were performed in a Perkin-Elmer Cetus thermal cycler under the following conditions: denaturation for 1 min at 94°C, primer annealing at 46°C for 1 min, and extension at 72°C for 1 min 30 sec. Amplified cDNA products were purified on agarose gels and then subcloned in Puc 18 vectors.

*DNA Sequencing.* Inserts from recombinant Puc 18 vectors were sequenced on both strands by the dideoxy chain termination method (Sanger et al. 1977) with a Pharmacia T7 sequencing kit.

## Results

### *Southern Blot Analysis of the "Rh-like" Locus from* M. mulatta

As a first approach to determine whether the *M. mulatta* genome contains an Rh-like locus, a comparative Southern blot analysis of the human and monkey genomes was performed. DNAs from one RhD-positive (DCCee) and one RhD-negative (ddccee) donor and as well as from *M. mulatta* were digested by *Eco*RI, *Hin*dIII, and *Bgl*II and hybridized with the human Rh cDNA probe isolated previously (Chérif-Zahar et al. 1990). As shown in Fig. 1A, a strong cross-hybridization was observed between *M. mulatta* DNAs and the human probe, even under high-stringency conditions. Because the human Rh locus is composed of either two related genes (*D* and *CcEe*) or only one gene (*CcEe*) in RhD-positive and RhD-negative haplotypes, respectively, such a polymorphism was investigated by analyzing the hybridization pattern of the genome from 10 unrelated *M. mulatta* monkeys. While D-positive and D-negative hybridization patterns were clearly distinct (Fig. 1A), the presence or the absence of only one band resumes the polymorphism observed in *M. mulatta* DNA with the three restriction enzymes used (Fig. 1A). In order to determine whether this polymorphism might be

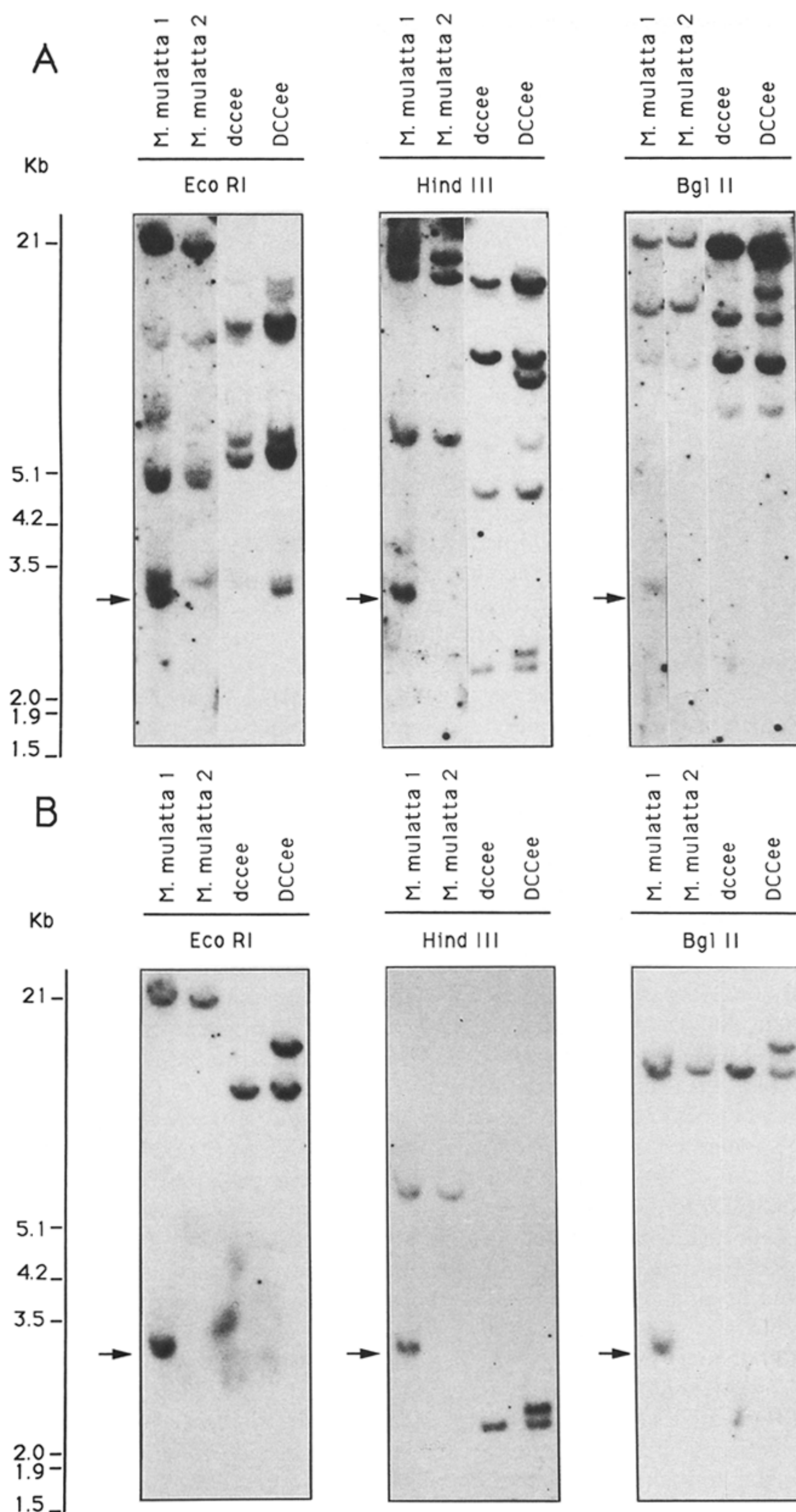


**Fig. 1.** Southern blot analysis of the *M. mulatta* Rh-like locus and comparison with the human Rh locus. DNA from *M. mulatta* monkeys and from RhD-positive (DCCee) and RhD-negative (dccee) human donors was digested with the restriction enzymes *Hin*dIII, *Eco*RI, and *Bgl*II and hybridized on Southern blots with the human Rh cDNA (**A**) or exon 1-specific (**B**) probes. Out of 10 monkey DNA samples studied, nine exhibited the restriction pattern of *M. mulatta* 1 and one had the pattern of *M. mulatta* 2. A longer exposure was performed for the monkey samples than for the human samples.

associated with the presence of one or two *Rh-related* genes in different *M. mulatta* genomes, the same blots were rehybridized with exon-specific probes derived from the human *CcEe* gene structure. As expected, the exon1-specific probe detected two bands in the human D-positive DNA (*D* and *CcEe* gene fragments), but only one (*CcEe* gene fragment) in the D-negative sample (Colin et al. 1991). Two hybridization bands were also detected in nine of the *M. mulatta* DNAs, but only one in the 10th sample (Fig. 1B). All other *CcEe* gene exonic probes (exons5, 7, and 10) revealed only one band

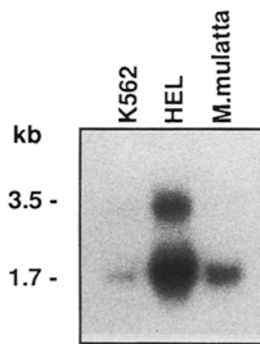


**Fig. 2.** Identification of *M. mulatta* Rh-like transcript by Northern blot analysis: 5 μg of total RNAs from *M. mulatta* bone marrow and human erythroleukemic cells (K562 and HEL) was resolved on a denaturing agarose gel, transferred to Zeta Probe GT membrane, and hybridized with the $^{32}$P-labeled human Rh cDNA probe. The weak signal at 3.5 kb seen with the human erythroleukemic cells was not detected in *M. mulatta* RNAs preparation, even on longer exposure.

in all macaque DNA samples tested so far. Intensities of these nonpolymorphic bands were rather constant for a given probe. By contrast, these three exonic probes revealed clearly two bands in human D-positive control DNA and only one in human D-negative sample. All these results indicate that *M. mulatta* genome contains a single Rh-like gene while an additional Rh-related 5′-gene fragment was detected in nine out of 10 individuals.

### *Identification of Nucleotide Sequence of* M. mulatta *"Rh-like" Transcripts*

Northern blot analysis performed under high-stringency conditions with the human Rh cDNA probe revealed the presence of Rh-related transcript(s) in bone marrow cells of *M. mulatta* (Fig. 2). The mRNA species exhibited the same size as the major 1.7-kb human Rh transcripts identified in K562 and HEL cells (Cherif-Zahar et al. 1990). However, the minor mRNA species of 3.5 kb found in human cells and corresponding to the use of a second polyadenylation signal in the RhD gene (Le Van Kim et al. 1992) was not detected in monkey cells.

Taking into account the similarities between the human and monkey Rh transcripts, oligonucleotides deduced from the 5′ and 3′ untranslated sequences common to D and CcEe human cDNAs (see Materials and Methods) were used as primers in PCR amplifications of *M. mulatta* first-strand cDNAs. Two PCR experiments were carried out independently to eliminate possible mutations due to the *Taq* polymerase activity. A PCR product of 1.3 kb was obtained, which corresponded to the size of the human Rh cDNAs amplified in the same conditions. Sequence analysis of several recombinant clones revealed the presence of two types of *M. mulatta* Rh-like cDNAs called MAC-A and MAC-B. Transcripts MAC-A exhibit the same open reading frame of 1,251 bases as the human Rh cDNAs (Fig. 3). Transcripts MAC-B differ from the former by six nucleotide substitutions (positions 212, 320, 575, 747, 784, 1028) and by the deletion of one nucleotide in position 85 resulting in a premature stop codon at nucleotide 113. Since an AUG codon surrounded by consensus translation initiation sequences (Kozak 1986) is found in position 169, this mRNA might be translated in a truncated polypeptide which differs from the Rh-like protein described above by the absence of 56 $NH_2$-terminal amino acids and by five amino acid substitutions (positions 71, 107, 192, 262, 343). The coding nucleotide sequences of the MAC-A and MAC-B transcripts (Fig. 3) diverged by 10–11% compared either with the human RhD or RhCcEe cDNAs, while the divergence between the two human cDNAs is 3.5% (Le Van Kim et al. 1992).

### *Amino Acid Analysis and Structural Comparison of Human and Monkey Rh Polypeptides*

The predicted translation products of MAC-A and MAC-B cDNAs are proteins of 417 and 361 amino acids ($M_r$ 45,500 and 39,700, respectively), which exhibited 22–25% divergence compared either with the human D or RhCc/Ee proteins (Fig. 4). The strong similarities observed between sequences of macaque and human Rh-polypeptides are in accordance with the resemblance between two-dimensional iodopeptide maps of human and macaque Rh-related polypeptides (Saboori et al. 1989). Hydropathy plot calculations (Engelman et al. 1986) suggest that the *M. mulatta* Rh-like proteins have a membrane organization similar to the human Rh proteins with multispanning hydrophobic transmembrane domains (Cherif-Zahar et al. 1990; Avent et al. 1990; Le Van Kim et al. 1992). The discrepancy between the apparent molecular mass of the isolated proteins (32 kDa) and the calculated molecular mass of the predicted products of the Rh-like

**Fig. 3.** Nucleotide sequence of the *M. mulatta* Rh-like cDNA, comparison with the human Rh cDNAs, and predicted amino acid sequence of the Rh-like protein. The amino acids which differ from those observed in the human proteins are *underlined.* Nucleotide positions which differ from both human RhD and RhCcEe cDNAs are *underlined. Symbols* ○ and ^ indicate positions which differ from only D or CcEe cDNAs, respectively. Polymorphic position between the two Rh-like allelic cDNAs and deduced encoded polypeptides are printed in *italics.*

```
                                                      gcacagagacggacacagg      -1

Met Ser Ser Lys Tyr Pro Arg Ser Val Arg Cys Cys Leu Pro Leu Trp Ala Leu      18
ATG AGC TCT AAG TAC CCG CGG TCT GTC CGG TGC TGC CTG CCC CTC TGG GCC CTA      54

Thr Leu Glu Ala Ala Leu Ile Leu Leu Phe Phe Phe Phe Thr Tyr Tyr Asp Ala      36
ACA CTG GAA GCA GCT CTC ATT CTC CTC TTC TTT TTT TTT ACC TAC TAC GAC GCT     108

Ser Leu Glu Asp Gln Lys Gly Leu Val Ala Ser Tyr Gln Val Cys Gln Asp Leu      54
TCC TTA GAG GAT CAA AAG GGG CTC GTG GCG TCC TAT CAA GTC TGC CAA GAT CTG     162
                                                      o

Thr Val Met Ala Val Leu Gly Leu Gly Phe Phe Thr Ser Asn Leu Arg Arg Asn      72
ACC GTG ATG GCG GTC CTT GGC TTG GGC TTC TTC ACC TCG AAT TTG CGG AGA AAC     216
                  ^  o                             ^  o

Ser Trp Ser Ser Val Ala Phe Asn Leu Phe Leu Leu Ala Leu Gly Val Gln Trp      90
AGC TGG AGC AGT GTG GCC TTC AAC CTC TTC CTG CTG GCC CTT GGT GTG CAG TGG     270

Ala Ile Leu Leu Asp Gly Phe Leu Ser Gln Phe Ser Pro Gly Lys Val Ala Ile     108
GCA ATC CTG CTG GAC GGC TTC CTG AGC CAG TTC TCT CCT GGG AAG GTG GCC ATC     324
                                                 o

Lys Leu Phe Ser Ile Arg Leu Ala Thr Arg Ser Thr Met Ser Met Leu Ile Ser     126
AAA CTG TTC AGT ATT CGG CTG GCC ACC AGG AGC ACT ATG TCG ATG CTG ATC TCA     378
                                                 o

Met Asn Ala Val Leu Gly Lys Val Asn Leu Val Gln Leu Val Val Met Glu Leu     144
ATG AAT GCT GTC CTG GGG AAG GTC AAC TTG GTG CAG TTG GTG GTG ATG GAG CTG     432
 ^   ^

Val Glu Leu Thr Val Phe Gly Thr Met Arg Ile Val Ile Asn Asn Ile Phe Lys     162
GTG GAG CTG ACA GTC TTT GGC ACC ATG AGG ATA GTC ATC AAT AAT ATC TTC AAA     486
                             o

Ile Asp Tyr Gly Met Asn Met Met His Ile His Val Phe Ala Ala Tyr Phe Gly     180
ATA GAC TAC GGC ATG AAC ATG ATG CAC ATC CAC GTG TTC GCA GCC TAT TTT GGG     540
                         ^   ^       ^

Leu Thr Val Ala Trp Cys Leu Pro Lys Pro Leu Pro Lys Gly Thr Glu Asp Lys     198
CTG ACT GTG GCC TGG TGC CTG CCA AAG CCT CTA CCC AAG GGA ACA GAG GAT AAA     594
    o                                            o                     ^

Tyr Gln Thr Thr Thr Ser Pro Ser Leu Phe Ala Met Leu Gly Thr Leu Phe Leu     216
TAT CAG ACA ACA ACG AGC CCC AGT TTG TTT GCC ATG CTG GGC ACC CTC TTC TTG     648
         ^

Trp Met Phe Trp Pro Thr Phe Asn Ser Ala Leu Leu Leu Asn Pro Ile Glu Arg     234
TGG ATG TTC TGG CCA ACT TTC AAC TCT GCT CTG CTG CTA AAT CCA ATC GAA AGG     702
      o                 ^           ^                           ^

Lys Asn Ala Val Phe Ser Thr Tyr Tyr Ala Leu Ala Val Ser Ala Val Thr Ala     252
AAG AAC GCC GTG TTC AGC ACC TAC TAT GCT CTA GCA GTC AGC GCG GTT ACA GCC     756
            ^                           o             ^

Ile Ser Val Ser Ser Leu Ala His Pro Gly Gly Lys Ile Asn Met Thr Tyr Met     270
ATC TCA GTG TCA TCC TTG GCT CAC CCC GGA GGG AAG ATC AAC ATG ACT TAT ATG     810
                                         ^               o

His Asn Ala Ala Leu Ala Gly Gly Val Ala Leu Ser Ala Ser Cys His Val Ile     288
CAC AAT GCA GCG TTG GCA GGA GGT GTG GCT CTG AGT GCC TCA TGT CAC GTG ATC     864

His Ser Pro Trp Ile Ala Met Val Leu Gly Leu Val Ala Gly Leu Ile Ser Ile     306
CAT TCT CCT TGG ATT GCC ATG GTG CTA GGT CTT GTG GCT GGG CTG ATC TCC ATC     918
                                                                     o

Gly Gly Ala Lys Cys Leu Pro Val Cys Phe Asn Arg Val Leu Gly Ile His Glu     324
GGG GGA GCC AAG TGC CTG CCG GTG TGT TTT AAC CGA GTG CTG GGG ATT CAC GAG     972
                 o           o                                   o

Ser His Ser Val His Tyr Thr Phe Gly Leu Pro Ala Leu Leu Gly Glu Ile Thr     342
AGC CAC AGC GTG CAC TAC ACC TTC GGC TTG CCG GCT CTG CTT GGA GAG ATC ACC    1026
 ^       ^        oo  ^                                                 o

Tyr Ile Val Leu Met Ala Leu Arg Val Val Trp Ala Ser Ser Asn Met Ile Gly     360
TAC ATT GTG CTG ATG GCG CTT CGT GTC GTC TGG GCC AGC AGT AAC ATG ATC GGC    1080
                             o     ^     o o ^^

Phe Gln Val Leu Leu Ser Thr Gly Thr Leu Ser Leu Ala Met Ala Met Ser Ile     378
TTC CAG GTC CTT CTC AGC ACT GGG ACA CTC AGC TTG GCC ATG GCG ATG AGT ATC    1134

Thr Ser Gly Leu Leu Thr Gly Leu Leu Leu Asn Leu Lys Ile Trp Lys Gly Pro     396
ACA TCT GGT CTC CTG ACA GGT TTG CTT CTA AAT CTC AAA ATA TGG AAA GGA CCT    1188
                                              o

His Val Ala Lys Tyr Phe Asp Asp Gln Ala Phe Trp Glu Phe Pro His Leu Ala     414
CAT GTG GCT AAA TAT TTT GAT GAC CAA GCC TTC TGG GAG TTT CCT CAT TTG GCT    1242
     o

Val Gly Phe                                                                 417
GTT GGA TTT taagaaaagcatccaagaaaaacaaggcctgttcaaaaacaagacaacttcctttcac    1310

tgttgcctgcatttgtacgtgagaaacgctcatgacagca                                  1354
```

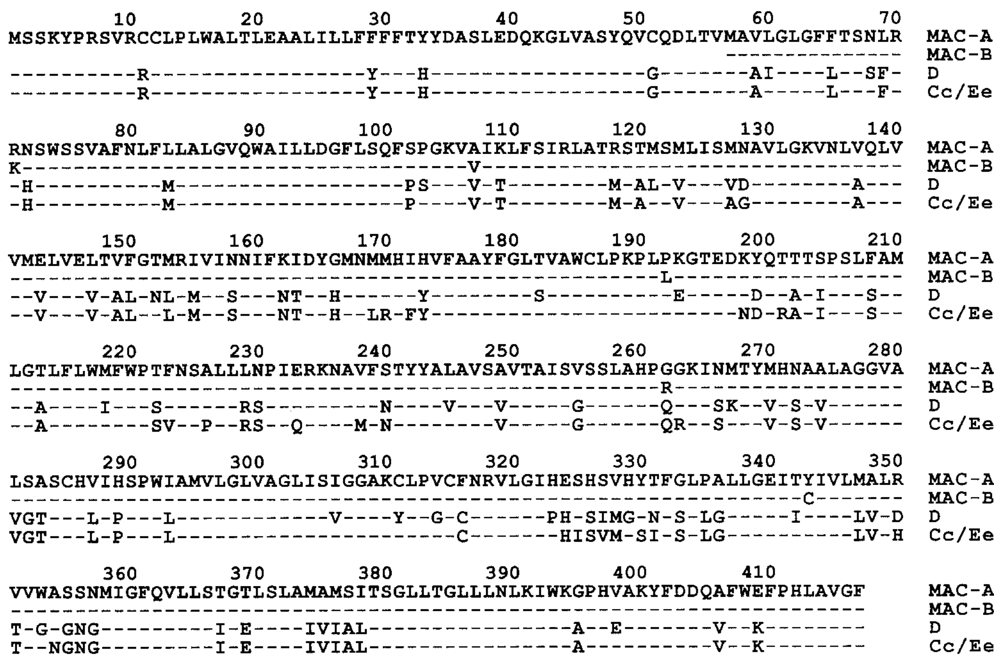


**Fig. 4.** Amino acid sequence comparison of *M. mulatta* Rh-like proteins and human Rh proteins. MAC-A and MAC-B are the deduced proteins encoded by the two Rh-like allelic cDNAs. (See text.) D and Cc/Ee proteins are deduced from previous cDNA analysis (Le Van Kim et al. 1992; Cherif-Zahar et al. 1990). Identical positions are noted as *dashes*.

cDNAs (45.5 kDa and 39.7 kDa) is more likely the result of the abnormal behavior of these highly hydrophobic molecules in $NaDodSO_4$ gels, as previously observed for the human Rh polypeptides (Agre and Cartron 1991). Out of the seven cysteine residues of the 417-amino-acid Rh-like monkey protein (MAC-A, Fig. 4), five are conserved in the Cc/Ee proteins and four in the D polypeptide. The exofacial cysteine 285, which may be involved in the antigenic properties of the human Rh proteins (Cherif-Zahar et al. 1990; Le Van Kim et al. 1992; Green 1966) is conserved between all proteins. Of the two cysteine residues (Cys-11 and Cys-51) present in this *M. mulatta* protein but absent in the human polypeptides, one (Cys-51) has an exofacial position. Cysteine 316 is present in D and Cc/Ee proteins but is absent from the macaque proteins. The predicted MAC-B protein would carry only four cysteines (positions 186, 285, 311, 315) due to the truncated 56 N-terminal amino acids.

## Discussion

Our results have established that the genome of rhesus monkeys (*M. mulatta*), while not expressing any of the epitopes recognized by the human Rh alloantibodies of the D, C, c, E, or e specificities (for review see Socha and Ruffié 1983), carries an Rh-like locus strongly related to the human Rh locus. In human, the Rh locus is polymorphic since RhD-positive haplotypes carry two *Rh-related* genes (*D* and *CcEe*) and RhD-negative haplotypes only one (*CcEe*) (Colin et al. 1991). This polymorphism was not observed in rhesus monkeys, which all possess a single copy of *Rh-like* gene per haploid genome. However, in DNA samples of nine out of 10 animals, the human exon1 probe detected an Rh-related 5′-fragment which most likely corresponds to a truncated *Rh* gene. Two allelic transcripts (MAC-A and MAC-B, Fig. 4) of the *Rh-like* gene have been isolated. The deduced amino acid sequence indicated that the monkey Rh-like proteins exhibit 75–78% similarity to the human Rh proteins.

The Rh-like proteins presumably inserted in the red cell membrane would account for the reactivity of the *M. mulatta* red blood cells with two monoclonal antibodies (anti-Rh29) that recognize nonpolymorphic epitopes at the red cell surface of human and nonhuman primates (Socha and Ruffié 1990). Furthermore, these polypeptides may correspond to the Rh analog protein of 32 kDa isolated from rhesus monkey erythrocyte membranes (Saboori et al. 1989). Among the 95- and 90-amino-acid substitutions found between the *M. mulatta* and the human D and Cc/Ee proteins, respectively, only 11 and nine are within the first 100 residues and only three and four within the last 40 amino acids, indicating a high degree of conservation of the $NH_2$- and COOH-terminal regions of the Rh-related proteins (Fig. 4 and Le Van Kim et al. 1992). Whether these regions may define the common iodopeptides

found on two-dimensional maps of the 32-kDa proteins isolated from human, rhesus monkey, cow, cat, and rat erythrocytes and may represent conserved, functionally important domains of the Rh-related polypeptides (Saboori et al. 1989) could be determined when the Rh-like molecules from these different species are cloned.

Since their discovery, a controversy has arisen because of the confusion between the human alloantibody responsible for hemolytic disease of the newborn (Levine and Stetson 1939; Levine et al. 1941a,b) and the heteroantibody (named "anti-Rhesus") raised in rabbits and guinea pigs injected with red cells from rhesus monkeys (Landsteiner and Wiener 1940). These antibodies were recognized later as distinct antigens and the name "Rh" was retained for the clinically important alloantibody, whereas the heteroantibody was renamed "LW" in honor of the discoverers, Landsteiner and Wiener (Levine et al. 1961, 1963). However, our present results and those discussed above demonstrate that the *M. mulatta* genome contains a functional Rh-like locus and that macaque erythrocytes carry membrane proteins strongly related to the human Rh polypeptides. Moreover, recent findings based on the reactivity of nonhuman primate erythrocytes with a large panel of Rh and Rh-related monoclonal antibodies indicate that macaque red cells give positive reactions not only with anti-LW antibodies, but also with anti-Rh29 (nonpolymorphic epitope of Rh proteins) and with a few anti-D antibodies (Socha and Ruffié 1990). Therefore, among the heteroantibodies obtained after immunization of rabbits and guinea pigs with rhesus monkey erythrocytes, some could display Rh specificity, as claimed by Landsteiner and Weiner (1940) and by Moureau (1941), and others LW specificity (Levine et al. 1941a,b).

Since eukaryotic cells transfected with the recombinant human cDNAs failed to react with any of the D, C, c, E, and e antibodies (Hermand et al. 1993 and our unpublished data), sequence comparison of Rh-like proteins from different apes and monkeys with Rh polypeptides from donors of different Rh phenotypes will hopefully provide an alternative approach to delineate these epitopes. Red-blood-cell-typing data indicate that anthropoid apes (gibbons, gorilla, orangutans, chimpanzees) carry the c blood group antigen and that gorilla and chimpanzees in addition carry a red blood cell antigen strongly related to the human D antigen named $Rh_0^{Go}$ (Moor Jankowski and Wiener 1972) and $R^c$ (Socha and Moor Jankowski 1980), respectively. However, none of the higher primates expresses E or e antigens, a finding confirmed recently with monoclonal antibodies, except for some chimpanzees (Socha and Ruffié 1990). On the other hand, Cercopithecoidae (macaques, baboons, etc.) and lower primates are unreactive with c and most D blood group antibodies (Socha and Ruffié 1983, 1990). It was thus suggested that an ancestral *Rh* gene might have evolved to encode for the blood factor "c" and later to factor "D," probably by a duplication and mutational events (Wiener et al. 1964). Our present results support this hypothesis. The presence of an additional truncated 5′-fragment in some macaque haplotypes indicates that the Rh locus of the common ancestor of anthropoid apes and Old World monkeys was already composed of two *Rh-related* genes, one of which was partially or totally deleted in contemporary rhesus monkeys. In man, only some haplotypes conserved the two ancestral copies. However, it is also possible that the common ancestor of anthropoid apes and Old World monkeys had only one *Rh-related* gene and that independent duplication events took place in the ancestors of rhesus monkeys and these of man.

Antibodies of Rh specificities have been extensively used to identify Rh-related blood group systems in nonhuman primates such as the RCEF system in chimpanzees (Wiener and Socha 1974; Socha and Moor-Jankowski 1980; Socha and Ruffié 1990). However, the contribution of these serological studies to the understanding of the primate phylogeny was limited because they required the conservation of well-defined antigenic structures. It is anticipated that the availability of molecular Rh and Rh-like probes will help to delineate more precisely the evolutionary pathway of the Rh locus and will provide important clues to the conserved domains of these proteins that may be assigned to a potential function.

*Acknowledgments.* This work was supported in part by the Institut National de la Santé et de la Recherche Médicale and the Caisse Nationale d'Assurance Maladie des Travailleurs Salariés. The support from NATO (grant 88/0556) is also acknowledged.

## References

Agre P, Cartron JP (1991) Molecular biology of Rh antigens. Blood 78:551–563

Avent ND, Ridgwell K, Tanner MJA, Tanner DJ (1990) cDNA cloning of a 30 kDa erythrocyte membrane protein associated with Rh (Rhesus)-blood-group-antigen expression. Biochem J 271:821–825

Chérif-Zahar B, Bloy C, Le Van Kim C, Blanchard D, Bailly P, Hermand P, Salmon C, Cartron JP, Colin Y (1990) Molecular cloning and protein structure of a human blood group Rh polypeptide. Proc Natl Acad Sci USA 87:6243–6247

Chérif-Zahar B, Mattéi MG, Le Van Kim C, Bailly P, Cartron JP, Colin Y (1991) Localization of the human Rh blood group gene structure to chromosome 1p34.3–1p36.1 region by in situ hybridization. Hum Genet 86:398–400

Chérif-Zahar B, Le Van Kim C, Rouillac C, Raynal V, Cartron JPC, Colin Y (1993) Organization of the gene encoding the human blood group RhCcEe antigens and characterization of the promoter region. Genomics (in press)

Colin Y, Le Van Kim C, Tsapis A, Clerget M, d'Auriol L, London J, Galibert F, Cartron JP (1989) Human erythrocyte glycophorin C. J Biol Chem 264:3773–3780

Colin Y, Chérif-Zahar B, Le Van Kim C, Raynal V, Van Huffel V, Cartron JP (1991) Genetic basis of the RhD-positive and RhD-negative blood group polymorphism. Blood 78:2747–2752

Engelman DM, Steitz TA, Goldman A (1986) Identifying nonpolar transbilayer helices in amino acid sequences of membrane proteins. Ann Rev Biophys Biophys Chem 15:321–353

Fisher RA, cited in Race RR (1944) An "incomplete" antibody in human serum. Nature 153:771–772

Green FA (1966) Sulfhydryl reagents and Rh activity of erythrocyte stroma. Nature 211:852

Hermand P, Mouro I, Huet M, Bloy C, Suyama K, Goldstein J, Cartron JP, Bailly P (1993) Immunochemical characterization of Rh proteins with antibodies raised against synthetic peptides. Blood (in press)

Issitt PD (1989) The Rh blood group system, 1988: eight new antigens in nine years and some observations on the biochemistry and genetics of the system. Transf Med Rev 3:1–12

Kozak M (1986) Bifunctional messenger RNAs in eukaryotes. Cell 47:481–483

Landsteiner K, Wiener AS (1940) An agglutinable factor in human blood recognized by immune sera for Rhesus blood. Proc Soc Exp Biol Med 43:223

Levine P, Celano M, Fenichel R, Pollack W, Singher H (1961) A 'D-like' antigen in Rhesus monkey, human Rh positive and human Rh negative red blood cells. J Immunol 87:747–752

Levine P, Cellano MJ, Wallace J, Sanger R (1963) A human "D-like" antibody. Nature 198:596–597

Levine P, Stetson RE (1939) An unusual case of intragroup agglutination. JAMA 113:126–127

Levine P, Burnham L, Katzin EM, Vogel P (1941a) The role of iso-immunization in the pathogenesis of erythroblastosis fetalis. Am J Obstet Gynecol 42:925–937

Levine P, Katzin EM, Burnham L (1941b) Isoimmunization in pregnancy, its possible bearing on the etiology of erythroblastosis fetalis. JAMA 116:825–827

Le Van Kim C, Mouro I, Chérif-Zahar B, Raynal V, Cherrier C, Cartron JP, Colin Y (1992) Molecular cloning and primary structure of the human blood group RhD polypeptide. Proc Natl Acad Sci USA 89:10925–10929

Moor Jankowski J, Wiener AS (1972) Red cell antigens of primates. In: Pathology of Simian primates part I. Karger, Basel, pp 270–317

Moureau P (1941) Recherches sur un nouvel hémo-agglutinogène du sang humain. Acta biol Belgica 1:123

Petz LD, Garratty G (1980) Acquired immune hemolytic anemia. New York, Churchill Livingstone

Race RR, Sanger R (1975) Blood groups in man, 6th ed. Oxford, Blackwell, pp 178–260

Saboori A, Denker BM, Agre P (1989) Isolation of proteins related to the Rh polypeptides from non-human erythrocytes. J Clin Invest 83:187–191

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487–491

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA 74: 5463–5467

Shaw MA (1986) Monoclonal antibodies anti-LWab and anti-D reagents recognize a number of different epitopes. J Immunogenet 13:377–386

Socha WW, Ruffié J (1983) Blood groups of primates: theory, practice and evolutionary meaning. Alan R Liss Inc, New York, pp 75–90

Socha WW, Ruffié J (1990) Monoclonal antibodies directed against human red cell antigens in tests with the red cells on nonhuman primates. Rev Fr Transfus Hémobiol 33:39–48

Socha WW, Moor Jankowski J (1980) Chimpanzee R-C-E-F blood group system: a counterpart of the Rh-Hr blood groups. Folia Primatol (Basel) 33:172–188

Wiener AS (1944) The Rh series of allelic genes. Science 100: 595–597

Wiener AS, Moor Jankowski J, Gordon EB (1964) Blood groups of apes and monkeys. IV. The Rh-Hr blood types of anthropoid apes. Am J Hum Genet 16:246–253