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Rh_{mod} Syndrome: A Family Study of the Translation-Initiator Mutation in the Rh50 Glycoprotein Gene

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Summary

Rh_{mod} syndrome is a rare genetic disorder thought to result from mutations at a "modifier" but not at the suppressor underlying the regulator type of Rh_{null} disease. We studied this disorder in a Jewish family with a consanguineous background and analyzed *RH* and *RHAG*, the two loci that control Rh-antigen expression and Rh-complex assembly. Despite the presence of a *d* (D-negative) haplotype, no other gross alteration was found at *RH*, and cDNA sequencing showed a normal structure for D, Ce, and ce Rh transcripts in family members. However, analysis of *RHAG* transcript, which encodes Rh50 glycoprotein, identified a single G→T transversion in the initiation codon, causing a missense amino acid change (ATG[Met]→ATT[Ile]). This point mutation also occurred in the genomic region spanning exon 1 of *RHAG*, and its genotypic status in the mother and two children was confirmed by analysis of single-strand conformation polymorphism. Although blood typing showed a very weak expression of Rh antigens, immunoblotting barely detected the Rh proteins in the Rh_{mod} membrane. In vitro transcription–coupled translation assays showed that the initiator mutants of Rh_{mod}—but not those of the wild type—could be translated from ATG codons downstream. Our findings point to incomplete penetrance of the Rh_{mod} mutation, in the form of "leaky" translation, leading to some posttranslational defects affecting the structure, interaction, and processing of Rh50 glycoprotein.

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Introduction

Rh (rhesus) deficiency syndrome is manifest as Rh_{null} or Rh_{mod}, a rare genetic disorder of the red blood cells (RBCs) that is characterized by an altered expression of Rh antigens as phenotypic markers. Whereas Rh_{null} cells lack all Rh antigens (Vos et al. 1961), Rh_{mod} cells display a markedly reduced antigen expression (Chown et al. 1972). Clinically, Rh-deficient individuals exhibit a mild to moderate chronic hemolytic anemia accompanied by a varying degree of spherostomatocytosis (Nash and Shojania 1987). In addition, Rh-deficient RBCs show biochemical and physiological changes in the plasma membrane, including an increased cation permeability, elevated ATPase activity, altered lipid organization, and absent or diminished expression of multiple Rh-related integral membrane proteins (reviewed in Agre and Cartron 1991*b*).

Classic genetic studies have established that Rh-deficiency syndrome is transmitted in an autosomal recessive fashion most often associated with consanguinity. Depending on the origin of underlying mutations, Rh_{null} is divided into the amorph and regulator types: the former arises by mutations inactivating the antigen locus *RH* itself, whereas the latter results from mutations at a separate suppressor modulating Rh-antigen expression (Race and Sanger 1975). These genetic postulates are now confirmed by the identification, in unrelated Rh_{null} cases, of different mutations in *RH* versus *RHAG*, which encode Rh30 polypeptides and Rh50 glycoprotein, respectively (Cherif-Zahar et al. 1996, 1998; Huang 1998; Huang et al. 1998*a,* 1998*b;* Hyland et al. 1998; Kawano et al. 1998). Such molecular analyses provide direct evidence proving that both the *RH* locus and the *RHAG* locus are essential for the expression and function of the Rh structures as a multisubunit complex in the RBC plasma membrane.

Although most Rh_{null} cases have been attributed to a defective "regulator," Rh_{mod} was first thought to result from some mutations that might have involved a second, different "modifier" gene (Chown et al. 1972; McGuire Mallory et al. 1976). This hypothesis was based mainly on the observation that Rh_{mod} cases exhibit a variable

antigen expression acted on by their *RH*-genotype compositions. However, the phenotypic diversity may also reflect differences in the degree of penetrance of mutations in the same suppressor that causes the regulator type of Rh_{null} disease. The characterization of the genomic organization of *RHAG* enabled us to devise a mutation-screening procedure to test this hypothesis (Huang 1998; Huang et al. 1998*b*). We investigated a Jewish family of Russian origin in which the Rh_{mod} phenotype is transmitted on a consanguineous background. We describe here a rare mutation in the initiator ATG of *RHAG* and show in vitro that downstream ATGs of the mutated Rh50 can be utilized for alternative translation initiation. Our findings correlate the "leaky" expression of Rh_{mod} with incomplete penetrance of a mutation in the *RHAG* suppressor but not with a mutation in the proposed "modifier" gene.

Subjects, Material, and Methods

Blood Samples and Hemagglutination Test

Blood samples under study were obtained from four members of the Rh_{mod} family: SM (the homozygote of a consanguineous pedigree), her husband, and her two obligate-heterozygous children. SM's husband was from an unrelated family known not to be consanguineous. RBC hemagglutination in test tubes was performed according to standard methods. Blood samples used as controls were drawn from D-positive (D+) and D-negative (D−) human blood donors with the *DCe/DCe* and *dce/dce* genotypes, respectively.

Genomic Southern Blot Analysis

Genomic DNA was isolated from leukocyte pellets. The Rh50 probe was a full-length cDNA (nucleotides −13 to 1256) isolated from a placenta cDNA library (Huang 1998). The Rh30 cDNA probes are region specific and span, respectively, the 5′ (exons 1–3, nucleotides 1 to 480), middle (exons 4–7, nucleotides 515 to 1073), and 3′ portions (exons 8–10 plus the 3′ UTR, nucleotides 1074 to 1456) (Huang 1996). Genomic DNA was digested with restriction enzyme *Sph*I, and blots were hybridized with ^{32}P-labeled cDNA probes.

Reverse Transcriptase–PCR (RT-PCR)

Erythroid total RNA was isolated as described elsewhere (Goossens and Kan 1981) and was extracted with the Trizol reagent (Bethesda Research Laboratories [BRL]). Rh30 and Rh50 transcripts were analyzed by gene-specific RT-PCR, as described elsewhere (Huang 1996, 1998). Primers for cDNA synthesis and PCR amplification are listed in table 1. The Rh30 or Rh50 mRNA was converted into cDNA by means of either a gene-specific 3′ UTR primer or an adaptor-linked oligo$(dT)_{16}$ primer (Frohman et al. 1988) (table 1). The cDNA was then amplified by two pairs of specific primers spanning the entire coding sequence for the Rh30 or Rh50 protein.

Rapid Amplification of cDNA 5′ Ends (5′ RACE) and Subcloning

To determine whether the transcription start site is affected, the 5′ end of Rh50 cDNAs was mapped by means of the 5′ RACE System Version 2.0 kit (BRL). Approximately 2 μg of total RNA was reverse-transcribed by primer Ex-3a (table 1). The single-stranded cDNA was dC-tailed and then was amplified by adaptor-linked poly(dG) and primer Ex-2a. The cDNA products from the control and SM were purified and ligated to the pCR2.1 TA vector (Invitrogen), and positive clones were identified by X-Gal selection and PCR amplification.

Amplification and Analysis of Genomic Sequences

To verify the mutation identified by cDNA analysis, the *RHAG* sequences were amplified by means of total genomic DNA used as a template. On the basis of the structural organization of *RHAG* (Huang 1998), gene-specific primers were designed to amplify the 5′ promoter, the exon 1–spanning region, and the exon/intron junctions. The amplified products were purified and either were sequenced or were analyzed by single-strand conformation polymorphism (SSCP), as described elsewhere (Huang et al. 1998*b*).

Immunoblot Analysis of RBC Membrane Proteins

Isolation of RBC membrane proteins and their immunoblot analysis were performed as described elsewhere (Towbin et al. 1979; Huang et al. 1998*a*). The two available monoclonal antibodies (mAbs) were used: 2D10, for Rh50 (Mallinson et al. 1990), and LOR-15C9, for Rh30(D) (Apoil et al. 1997). For band visualization, peroxidase-conjugated anti–mouse Ig and anti–human Ig were used as the second antibodies for 2D10 and LOR-15C9, respectively.

DNA Sequencing

Unless described otherwise, the amplified cDNA and genomic products were sequenced directly after separation by native 5% PAGE. For subcloned inserts of 5′ RACE and in vitro–translation constructs, multiple clones were sequenced on a 373A automated DNA sequencer using fluorescent-dye tags as chain terminators (Applied Biosystems).

Table 1

Synthetic Oligonucleotides for Analysis of the Rh50 Glycoprotein Gene

Category[a]	Sequence	Nucleotide Position[b]	Location
I. cDNA PCR:			
3′-UTRa	5′-AATGGGAAAGGAAGCTGGAGAGCA-3′	1321 to 1298	3′-UTR
Ex-1s	5′-AGTGTGCCTCTGTCCTTTGCCACA-3′	−27 to −4	5′-UTR
Ex-4s	5′-GAAGAGTCCGCATACTACTCAGAC-3′	601 to 624	Exon 4
Ex-5a	5′-CTGTTTGTCTCCAGGTTCAGCAAT-3′	708 to 685	Exon 5
Ex-10a	5′-CCATGTCCATGGAACTGATTGTCA-3′	1256 to 1233	Exon 10
$(dT)_{16}$ anchor	5′-GTCATGACTCGAGTCGACATCGA$(T)_{16}$-3′		
II. 5′ RACE:			
Ex-3a	5′-ATTGTCATGATCAGCATTTGGGTG-3′	437 to 414	Exon 3
Ex-2a	5′-CCAAAGCAGCAACGATGAGGTTG-3′	268 to 246	Exon 2
III. SSCP:			
5′P-1s	5′-CATGCAGTCAAATTCCACACACAG-3′	−97 to −74	5′ Region
Ex-1a	5′-CTCAAAGAATATGCCCATGTCTG-3′	147 to 125	Exon 1
IV. Genomic PCR:			
5′P-2s	5′-TCCAGCTTTGCTTGACTGATTGCC-3′	−679 to −656	5′ Region
In-1a	5′-CAAAGAGCTCAAGGGTTTATAGTA-3′	95 to 72	Intron 1
V. Construction of in vitro–translation plasmids:[c]			
Ex-1s	5′-AGTGTGCCTCTGTCCTTTCCCACA-3′	−27 to −4	5′-UTR
Ex2-Sa	5′-TTATTTCTTCAGGAAGGTCATGAG-3′	222 to 202	Exon 2
Ex3-La	5′-CTATTATTCACTAACCAGGTATTC-3′	483 to 466	Exon 3

NOTE.—For brevity, Rh30 primers have not been listed (see Huang 1996), since no abnormality was found at *RH*.

[a] Sense (denoted by an "s" suffix) and antisense (denoted by an "a" suffix) primers to 5′ or 3′ UTR; exons (as "Ex"), 5′ regions (denoted as "P"), and introns (denoted as "In") are indicated.

[b] Positions of exon primers are counted from the first base of initiation codon ATG, whereas the intron primer is designated on the basis of its distance from the adjacent exon.

[c] Introduced termination codons are underlined. Ex2-Sa was used for the construction of WT-S and Rh_{mod}-S pairs, and Ex3-La was used for the construction of WT-L and Rh_{mod}-L pairs.

In Vitro Transcription–Coupled Translation Analysis

To evaluate the effect of the initiator mutation, two shortened forms of *RHAG* cDNA from SM and her husband (the control) were constructed and tested by in vitro transcription–coupled translation. This was necessary because a size difference of seven amino acids cannot be detected if the full-length forms are used in protein translation. The subcloned cDNAs were used as amplicons for PCR with gene-specific primers containing introduced stop codons (table 1). The products were purified by PAGE and were subcloned into the pRc/CMV vector with a T7 promoter (Invitrogen). The orientation, open reading frame, and change in ATG initiation codon of the wild-type and Rh_{mod} constructs were verified by sequencing. The plasmids were linearized with *Bst*XI, and transcription-coupled translation assays were performed according to the supplier's specifications (Promega). ^{35}S-methionine (NEN Life Science Products) was used as a label, and the translated products were analyzed by SDS-PAGE followed by autoradiography.

Results

Family Data and RBC Hemagglutination

SM and her brother SS (deceased) were first described as Rh_{null} siblings of a consanguineous Jewish pedigree of Russian origin. Their parents were typed as D− but produced three D+ children. By 1984, SM had a well-compensated hemolytic anemia, whereas SS had a normal hematological count with numerous spherocytes and stomatocytes after splenectomy.

The results of RBC direct tests were as follows: SM, D−C−E−c−e−; her husband, D+C+E−c+e+; her son, D+C+E−c−e+; and her daughter, D+C+E−c+e+. Further tests on SM, with eight anti-e, four anti-Rh17, and one anti-Rh29, were all negative; however, when her cells were treated with papain and were tested directly, they reacted weakly with one polyclonal anti-e and reacted moderately with four monoclonal anti-e (MS-17, -21, -62, and -69), all anti-Rh17, and anti-Rh29. When tested either by saline-RT and spin (SRS) or by indirect antiglobulin test (IAT), the untreated cells were nonreactive to three anti-D (LOR-15C9, DB176A, and DM75-2). Although nonreactive by SRS, the untreated cells were agglutinated strongly in IAT, by the fourth anti-D (ND04001). These results show that SM is a genuine case of Rh_{mod} disease, rather than a case of Rh_{null} disease.

Southern Blot Analysis of RH *and* RHAG

Figure 1 shows the Southern blots for the Rh_{mod} family. In the *RH* polymorphic region (Huang et al. 1996), a

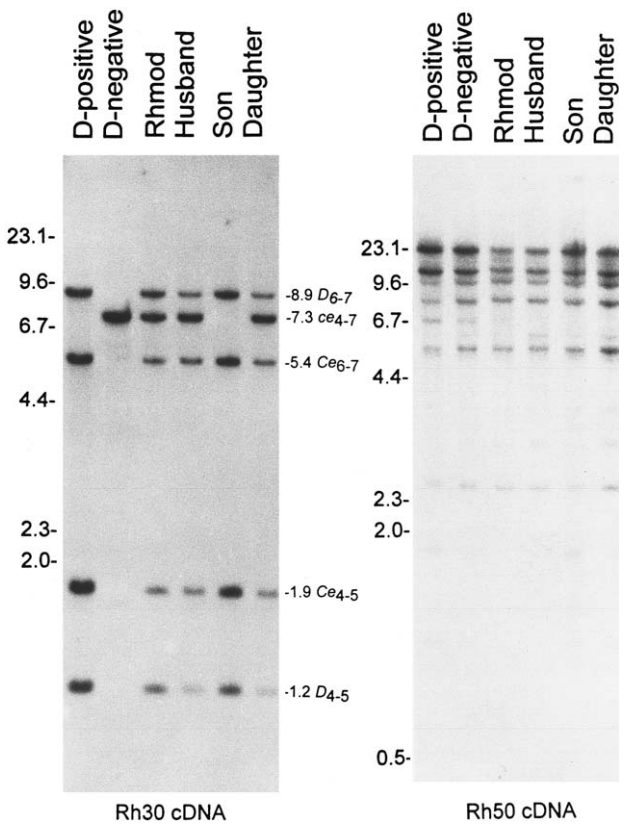

Figure 1 Southern blot analysis of *RH* and *RHAG* loci in the Rh_{mod} family. Genomic DNAs were digested with restriction enzyme *Sph*I, and the blots were hybridized with the Rh30 (*left panel*) and Rh50 (*right panel*) cDNA probes. The gene origin and exon content of various *Sph*I bands assigned to the polymorphic region of *RH* (Huang et al. 1996) are denoted. Note the reduction of intensity in *RHD*-specific 8.9- and 1.2-kb bands and the presence of a *ce*-specific band in all members except the son. The blot probed with full-length Rh50 cDNA showed no significant difference between controls and Rh_{mod} family members. Size markers (in kb) of *Hin*dIII-cleaved lambda-phage DNA are shown to the left of the gel panels.

reduced dosage in the *D*-specific 8.9- and 1.2-kb bands, indicative of a *d* (D−) haplotype on one chromosome, was evident in SM, her husband, and her daughter (fig. 1, *left*). The three also showed a reduced intensity in the *Ce*-specific 5.4- and 1.9-kb bands but had a *ce*-specific 7.3-kb band. The banding pattern in the son was apparently identical to that in D+ individuals of the *DCe/DCe* genotype. The data indicated the transmission of two haplotypes *DCe* and *dce* in the family. Analysis of *RHAG* showed no gross alteration, despite the possible occurrence of some polymorphisms in noncoding regions (fig. 1, *right*).

RT-PCR Analysis of RhD and RhCE Transcripts

To exclude mutation at *RH,* the structure of Rh transcripts was characterized by RT-PCR and sequencing. In SM, her husband, and her daughter, three types of cDNA were identified, which were found to encode the D, Ce, and ce Rh polypeptides, respectively. In the son, only D and Ce transcripts were identified. The entire sequence of three cDNAs from SM was obtained but showed no difference from that of normal subjects. The combined data of genomic and cDNA analyses allowed for the exclusion of mutation at the *RH* locus, as well as for the inference of gene linkage and segregation in all family members (fig. 2).

RT-PCR Analysis of RHAG *Transcripts*

Next we analyzed the Rh50 transcript expressed in SM's erythroid cells. No shortened cDNA species indicative of aberrant RNA splicing were detected (gels not shown). Sequencing of the entire cDNA identified a single G→T transversion in the +3 position of the ATG codon for translation initiation (ATG[Met^1]→ATT[Ile]). Since there is a potential ATG triplet (nucleotides −96 to −94) located farther upstream (Huang 1998), 5′ RACE was performed to map the 5′ end of Rh50 transcripts. Sequencing of multiple clones revealed that no inserts had a sequence beyond −75, demonstrating that the upstream ATG does not occur in mature mRNAs. In SM and her husband, the same −27A was determined as the major transcription start site, consistent with the assignment reported by others (Iwamoto et al. 1998). These results indicate that the initiator mutation does not affect *RHAG* transcription or splicing.

Genomic Analysis of the 5′ Region of RHAG *in the* Rh_{mod} *Family*

To confirm the identified mutation and to determine its genotype status in family members, the 5′ region of *RHAG,* encompassing the promoter, exon 1, and a part of intron 1, was amplified and sequenced. As shown, a single peak—for nucleotide 3T in SM and for nucleotide 3G in her husband—was present in the parents, whereas the same position displayed a composite of T/G in their children (fig. 3*A*). This indicated both homozygosity of the mutation in SM and heterozygosity of the mutation in the children. The genotype status was further demonstrated by SSCP analysis of the genomic fragments containing exon 1 (fig. 3*B*). In addition, no difference was found in the promoter region and exon/intron junctions (data not shown), compared with the wild-type *RHAG* sequences (Huang 1998).

Immunoblot Analysis of Rh30 and Rh50 Proteins

To determine the expression of RhD and Rh50, gels loaded with equal amounts of RBC membranes were blotted and subjected to mAb probings. On the anti-RhD immunoblot, bands of expected molecular weight—that is, 30–32 kD—were seen in D+ and in three family members but not in either SM or D− sub-

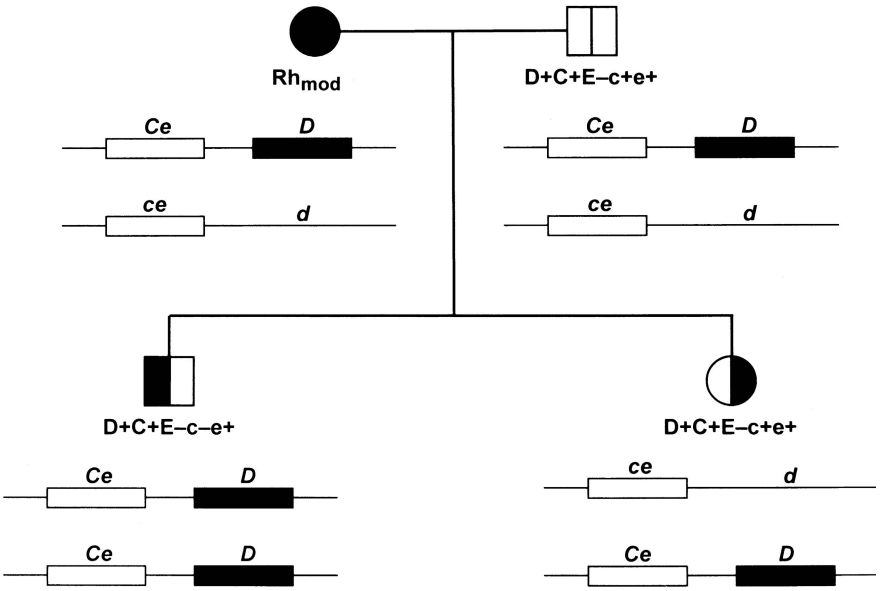

Figure 2 Inheritance and linkage of *RH* in the Rh$_{mod}$ family. Genotypes of four members were determined on the basis of the combined data from *Sph*I RFLPs (fig. 1) and sequences of the corresponding transcripts. Rh-antigen phenotypes are shown below each family member: a plus sign (+) denotes that the individual is positive; a minus sign (−) denotes that the individual is negative. The *Ce* or *ce* gene (*unblackened boxes*) is placed upstream of the *D* gene (*blackened boxes*) (Carritt et al. 1997). "*d*" denotes a deletion of the *D* gene (*horizontal line*). The Rh$_{mod}$ proband (i.e., SM) and her husband are genotypically identical at *RH*, in that they both carry the *DCe* and *dce* haplotypes. The random assortment of the two haplotypes resulted in *DCe/DCe* and *DCe/dce* children.

jects (fig. 4*A*). On the immunoblot visualized by anti-Rh50 at a dilution of 1:100, a broad diffuse band in the range of 36–50 kD was seen in all individuals except SM (fig. 4*B*). These data suggested an apparent absence of RhD and Rh50 in the Rh$_{mod}$ membrane. However, faint bands were visible when the blot overloaded with Rh$_{mod}$ membrane ghosts was probed with undiluted 2D10 (gel not shown). This observation is consistent with the results of blood typing, demonstrating a severe deficiency but not a total abolishment of Rh-protein expression.

Effect of the Initiator Mutation on In Vitro Translation

The mutational effect was analyzed by in vitro transcription–coupled translation of two pairs of truncated constructs, made from the wild-type cDNA and from SM's cDNA, which differed only in the third position of the initiation codon—with the wild-type cDNA having G and SM's cDNA having T. The *RHAG* sequence was placed under the control of the T7 promoter for RNA transcription; it starts from nucleotide −27A and terminates at the indicated codon position in the two pairs of constructs (fig. 5*A*).

As shown, in vitro–translation products derived from the wild-type and Rh$_{mod}$ constructs were readily detectable (fig. 5*B*). The paired longer constructs, WT-L and Rh$_{mod}$-L, showed no apparent difference (fig. 5*B, left*), since their expected sizes (161 amino acids vs. either 154 or 146 amino acids) were still too close to be differentiated. Nevertheless, the comparable intensity of the band seen in Rh$_{mod}$ showed an efficient translation in the absence of the cognate initiation signal. Moreover, the difference in size and banding pattern became evident when the paired shorter constructs, WT-S and Rh$_{mod}$-S, were used for translation (fig. 5*B, right*). WT-S gave rise to a single band, expected of a 74-amino-acid product, whereas Rh$_{mod}$-S yielded two different polypeptides, of smaller size. The two smaller ones were most likely initiated from Met^{8} and Met^{16} (fig. 5*C*), containing 67 and 59 amino acids, respectively. This is because any translated products starting from Met^{44} would be <3.5 kD. Thus, in the presence of a wild-type initiation signal, the usage of internal ATGs for alternative translation was avoided; by contrast, the absence of the cognate initiator resulted in a preferential selection of the immediately adjacent ATGs. The latter case likely reflects effects of both the position and context of the cryptic translation initiation–signal sequences (fig. 5*C*). Taken together, the results correlated the Rh$_{mod}$ phenotype with the occurrence of alternative translation leading to some post-translational defects (fig. 6).

Discussion

We have described molecular studies of a Jewish family in which Rh$_{mod}$ occurs as an autosomal recessive char-

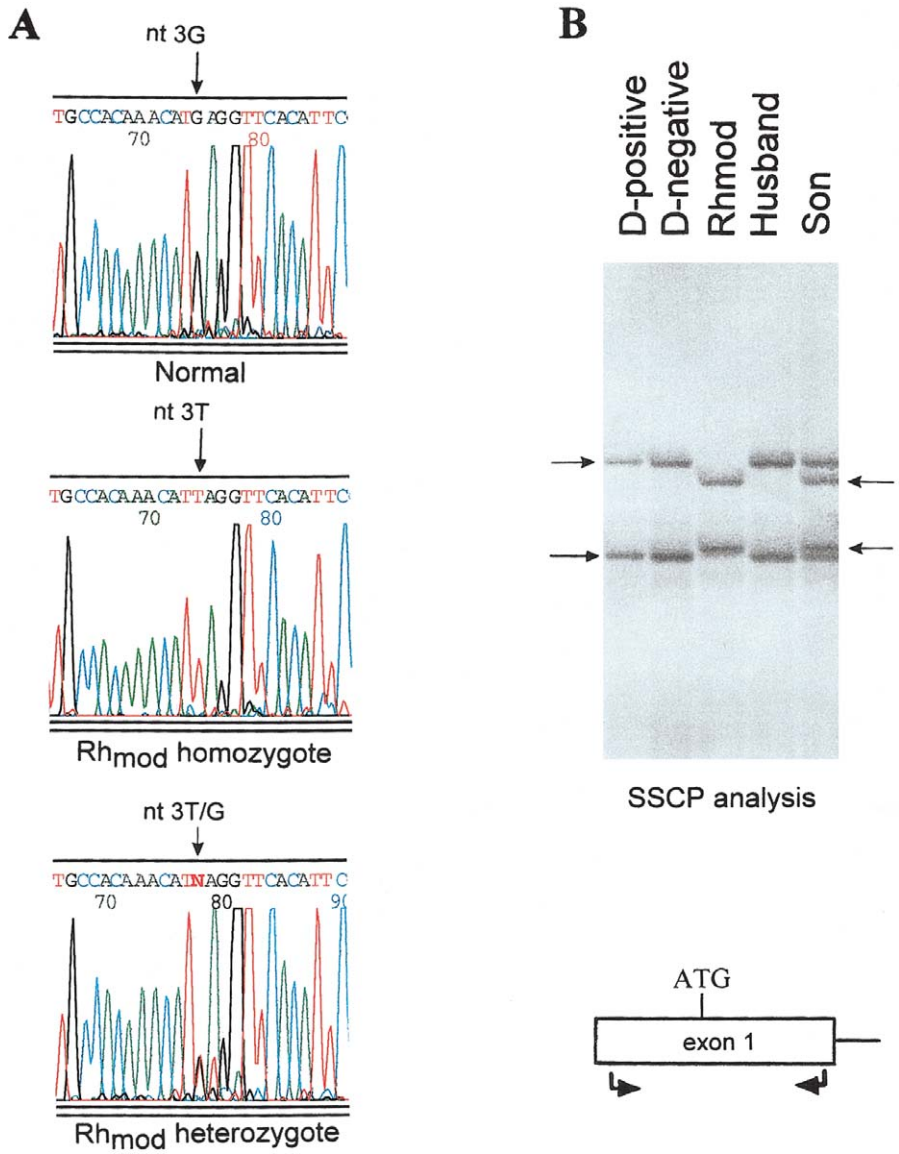

Figure 3 Amplification and analysis of the initiator mutation in the Rh_{mod} family. *A*, DNA sequence profiles spanning the genomic region relevant to translation initiation. The genomic sequences encompassing exon 1 were amplified by primers 5P-1 and In-1a (see table 1) and then were directly sequenced. The nucleotide in the +3 position of initiation codon ATG is indicated by a downward-pointing arrow. The husband shows a normal sequence, whereas the Rh_{mod} proband (i.e., SM) and her children are homozygous and heterozygous for the G→T transversion, respectively. *B*, SSCP analysis. The genomic products used for SSCP analysis were amplified by primers 5P-2 and Ex-1a. They were denatured by heating, were separated on an 8% polyacrylamide gel, and then were stained with silver nitrate. The normal and abnormal bands are denoted by arrows to the left and right of the gel panels, respectively. The results readily demonstrate the homozygosity and heterozygosity for the point mutation.

acter on a consanguineous background. To determine the nature and type of the underlying defect, we investigated *RH* and *RHAG*, whose products are essential both for the assembly of Rh structures and for the integrity of RBC membranes (Anstee and Tanner 1993; Cartron and Agre 1995). Analysis of Rh transcripts provided results consistent with the pattern of antigen expression, excluding mutation in the *RH*-antigen locus itself. Screening for mutation of *RHAG* led to the identification of a single G→T transversion in the ATG codon for translation initiation of Rh50 in the Rh_{mod} erythroid cells. To our knowledge, this is the first example of polytopic transmembrane (TM) proteins known to be defective in translation initiation in association with a genetic disorder. Other initiator mutations have been shown to target the genes encoding erythrocyte intra-

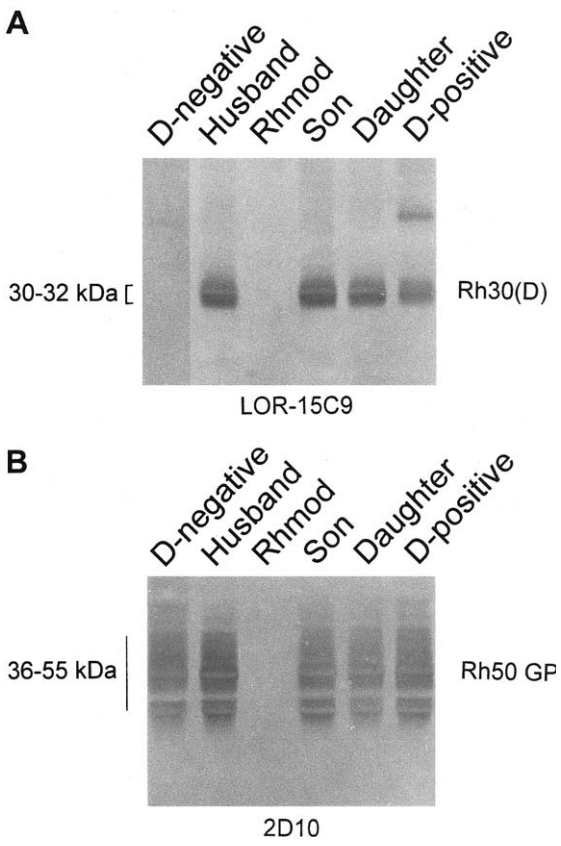

Figure 4 Immunoblot analysis of Rh30 polypeptides and Rh50 glycoprotein in the RBC membrane. Equal amounts of RBC-membrane ghosts from controls and Rh_{mod} family members were loaded and separated by PAGE under nonreducing conditions. *A,* Immunoblots probed with the monoclonal antibody 2D10 against Rh50. A broad diffuse band in the range of 36–50 kD is detectable in normal and Rh_{mod} heterozygotes but is barely seen in the Rh_{mod} homozygote (i.e., SM). *B,* Immunoblots probed with LOR-15C9, a monoclonal antibody specific for the RhD polypeptide only. A band of 30–32 kD is seen in all D+ individuals but not in either the D− control or the Rh_{mod} homozygote. Thus, the RhD protein was severely deficient, although the RhD gene was structurally normal and actively transcribed in Rh_{mod}.

cellular proteins—that is, globins in thalassemias (Baysal and Carver 1995), as well as protein 4.1 and β-spectrin in hereditary spherocytosis (Venizia et al. 1992; Basseres et al. 1998).

Although their amino acid sequence is only 36% identical, Rh30 and Rh50 share a quite similar 12-TM topology, serving as the antigen carrier and coexpressor, respectively (Avent et al. 1990; Cherif-Zahar et al. 1990; Le Van Kim et al. 1992; Ridgwell et al. 1992; Arce et al. 1993). They are thought to form a structural core, providing contact sites to promote the assembly of a multisubunit complex that may include several other membrane glycoproteins—for example, LW, CD47, and GPB (Agre and Cartron 1991*a*). Such a hypothesis had been highlighted by the rare occurrence of Rh_{null} disease and has now been substantiated by the findings made in more-recent biochemical and molecular-genetic studies.

It appears that there is a direct interaction between the Rh30 and Rh50 proteins, involving their N-terminal domains (Eyers et al. 1994). Furthermore, LW, CD47, and GPB are dispensable with regard to the assembly of Rh structures, since their null status did not affect either Rh-antigen expression or RBC shape and physiology (Hermand et al. 1996; Lindberg et al. 1996; Huang 1997). Most notably, both amorph and regulator mutations causing Rh_{null} disease have been identified (Cherif-Zahar et al. 1996, 1998; Huang 1998; Huang et al. 1998*a,* 1998*b;* Hyland et al. 1998; Kawano et al. 1998). The mutational spectrum not only confirms an essential role for *RH* and *RHAG* but also indicates the presence of additional interaction sites in the C-terminal domains of the two proteins. The reported defects include two missense changes, but the remaining changes are either small exonic deletions or splice-site mutations resulting in frameshift and premature termination. The abnormal translation initiator shown here specifies a new molecular basis, thus giving novel insight into the phenotypic diversity of Rh-deficiency syndrome.

As shown, the G→T mutation did not alter *RHAG* expression at the level of transcription or splicing but led to alternative translation initiation. This initiation was apparently confined to the two ATGs immediately adjacent to the wild-type one, despite the fact that multiple ATGs are present farther downstream (Ridgwell et al. 1992). Alignment of the sequences flanking the first four in-frame ATGs of *RHAG* (fig. 5*C*) reveals that they each share a significant homology with the Kozak (1987) consensus. The cognate ATG(Met^1) initiation site contains nine identical nucleotides and has been shown to be most optimal, since its corresponding constructs did not give rise to aberrant products. The other three ATGs have seven or eight nucleotides identical to the consensus sequence, with the ATG(Met^8) and ATG(Met^{16}) cryptic signals being preferentially selected for translation initiation in Rh_{mod}. This selection is unlikely, because of an alteration in binding of the 40S ribosomal subunits to the cap site of Rh50 mRNA, since the Rh_{mod} transcripts were shown to have the same 5′ end as was seen in the wild type. Rather, our data suggest an adjustment in the mode for the ribosomes to scan mRNA and to find an AUG suitable to initiate translation, for the strength of AUG is set mainly by its sequence context and relative position with respect to the cap site (Iida and Masuda 1996).

The "leaky" translation, although observed in vitro, provides a direct link to the reduced antigen expression in Rh_{mod}, suggesting that some posttranslational defects may have affected the structure, interaction, and pro-

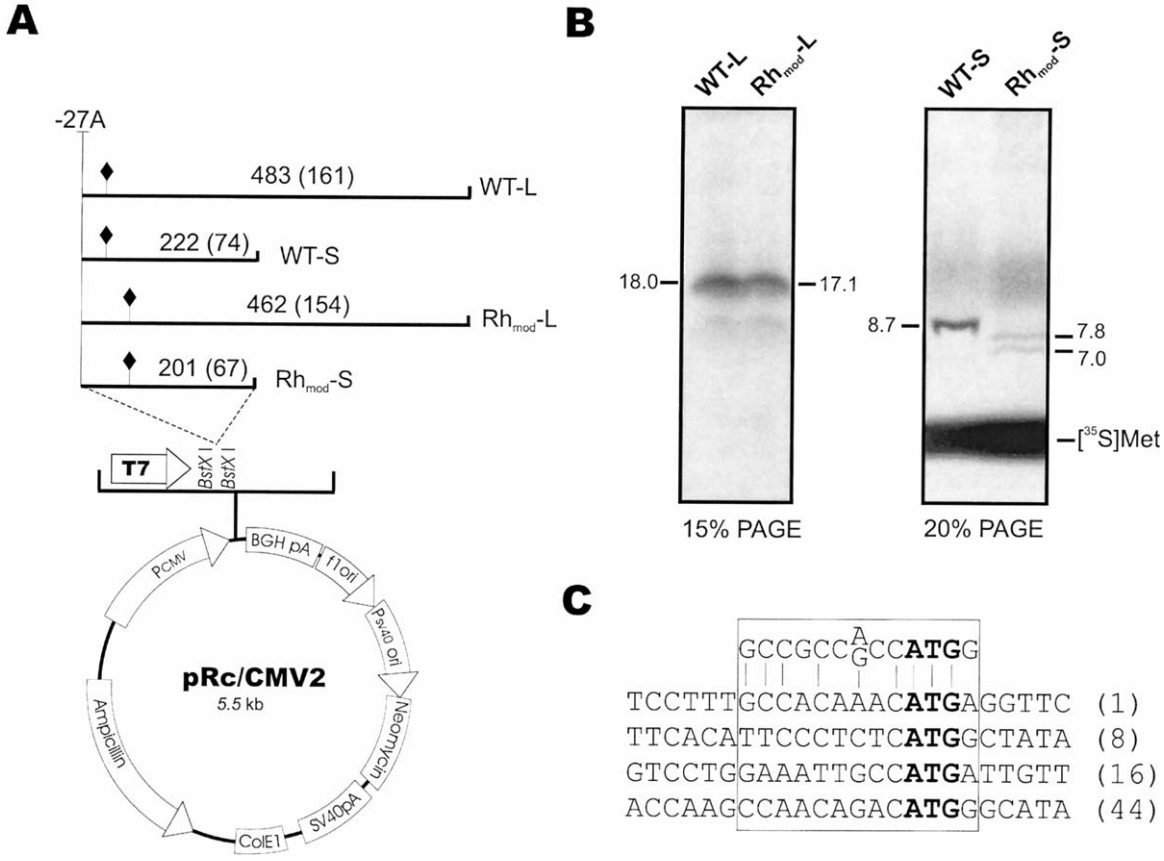

Figure 5 In vitro transcription–coupled translation assays of the wild-type and Rh_{mod} Rh50 constructs. *A,* Diagrams of the two pairs of Rh50 constructs cloned into the pRc/CMV2 vector. The four constructs all start from −27A and are placed downstream of the T7 promoter. The sizes (in bp) of their coding sequences, from ATG (denoted by blackened diamonds [♦]) to the last codon, is given, and the predicted length of their polypeptides is bracketed. "WT-L" and "WT-S" denote long and short wild-type constructs, respectively; "Rh_{mod}-L" and "Rh_{mod}-S" denote long and short mutant constructs, respectively. *B,* Analysis of in vitro–translation products. In each panel, equal amounts of linearized control and Rh_{mod} plasmids were used. The apparent molecular size (in kD) of each band is given. *Left,* 15% SDS-PAGE of [^{35}S]-Met–labeled products translated from the paired long constructs. *Right,* 20% SDS-PAGE of [^{35}S]-Met–labeled products translated from the paired short constructs. Note the presence of a single band in WT-S and of two smaller bands in Rh_{mod}-S. The dark band at the bottom is due to free [^{35}S]-Met. *C,* Alignment of Kozak consensus (*top*) with the first four in-frame ATGs (*boldface*), at positions 1, 8, 16, and 44, and their flanking sequences in *RHAG*. The sequences surrounding ATG codons farther downstream, at positions 60 and 69, are not shown.

cessing of Rh50 glycoprotein (fig. 6). The use of ATG(Met^{8}) for translation initiation is expected to produce a shortened polypeptide lacking seven amino acids, thereby eliminating the cytoplasmic N-terminal segment. Nevertheless, the predicted first-TM1 α-helix (Ridgwell et al. 1992) would be affected only slightly. This is in contrast with the initiation from ATG(Met^{16}), which would basically destroy the TM 1 segment, introducing more-dramatic changes into the mutant protein. With these N-terminal truncations, the proteins might decrease their insertion into the lipid bilayer (Singer 1990), impair their interaction with Rh30 proteins (Eyers et al. 1994), and become either defective in intracellular transport or susceptible to proteolytic degradation (Cheng et al. 1990; Deen et al. 1995). Such possible posttranslational events, either alone or in combination, could repress—but not entirely abrogate—the complex assembly (fig. 6). These considerations may account for the barely present Rh50 glycoprotein and the severely reduced antigen expression in the Rh_{mod} membrane.

Rh_{mod} syndrome is variable in terms of both antigen expression and hematological manifestation (Chown et al. 1972; McCuire Mallory et al. 1976; Nash and Shojania 1987). This phenotypic diversity may attest to the genetic complexity of regulator gene(s) (Tippett 1990). Comparison of the present initiator defect with the missense change (Ser^{79}→Asn) found in Rh_{mod}(VL) (Cherif-Zahar et al. 1996) indicates that differences in the degree of penetrance depend on the location and nature of the respective mutations. Although our results point to "leaky" translation as a form of incomplete penetrance, further studies will be required in order to derive a com-

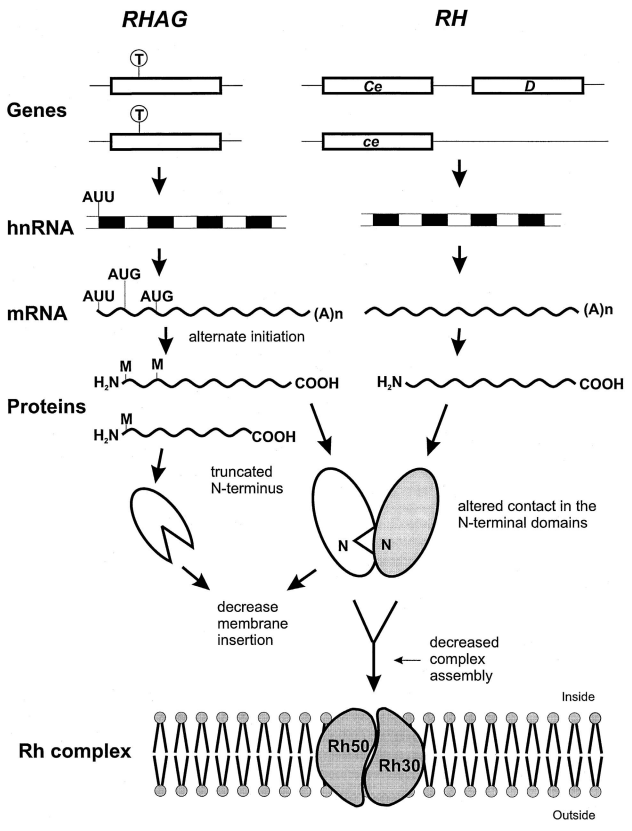

Figure 6 Model for the Rh_{mod} phenotype caused by an incompletely penetrated initiator mutation in the *RHAG* gene. In the Rh_{mod} proband (i.e., SM), the two copies of *RHAG* contain the same G→T transversion in the ATG initiation codon (*circled* "*T*") and are denoted by horizontal unblackened boxes; her *RH* locus harbors structurally normal *D, Ce,* and *ce* genes that are actively expressed. As shown, the translation-initiator mutation alters neither gene transcription nor mRNA splicing; however, it leads to alternative translation initiation, by selective use of the downstream cryptic initiation-signal sequences, thus producing truncated Rh50 proteins with different N-terminal sequences, which are denoted by notches. Such products are aberrant in structure and may directly affect their contact with the Rh30 protein, via their N-terminal domains. They may also be defective in other posttranslational events, such as membrane insertion and cellular transport. These potential changes could largely decrease the formation of a stable Rh complex in the plasma membrane, although the *RH* locus itself harbors no mutation.

plete picture for Rh_{mod} syndrome and to exclude the involvement of a second "modifier" gene in other cases.

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