Table 1. Antithrombin levels with antigen and activity methods

	Antigen (Lia test), %	Thrombin-based activity test, %	Factor Xa–based activity test, %
Proband (our results)	93	60-64	110
Proband (Canadian results)	NA	72-74	53-56
Sister (our results)	86	62	92
Brother (our results)	92	55	89

NA indicates not applicable.

Our present investigation shows that the 3 patients have normal antigen levels measured with Lia antigen test.[2] The factor Xa inhibition–activity assay also gave normal values, but decreased antithrombin activity values (about 60%) were found with a thrombin inhibition–based activity test using bovine thrombin (Table 1). Both activity methods were based on chromogenic substrates and were carried out in the presence of excess heparin. Our results differ from the original Canadian report[1] especially concerning the factor Xa inhibition–based test.

Chromogenic peptide substrate assays have been used for many years to measure antithrombin activity. These assays are based on either thrombin inhibition[3] or factor Xa inhibition.[4] For thrombin-based tests bovine thrombin should be preferred because human thrombin also reacts with heparin cofactor II and may lead to overestimation of antithrombin.[5,6] In our present study we used bovine thrombin (Sigma Aldrich, St Louis, MO) and substrate S-2238 (Haemochrom Diagnostica, Molndal, Sweden) for the thrombin inhibition assay, which was modified for the Cobas Mira analyzer (Roche Diagnostics, Basle, Switzerland). For factor Xa inhibition we used the Coamatic LR kit (Haemochrom Diagnostica) with S-2772 and the automatic method for Thrombolyzer (Benhk Elektronik, Norderstedt, Germany). The Canadian study used human thrombin in the thrombin inhibition assay, which can explain the slight difference compared with our results. Other factors that can contribute to differences in test results are plasma amount, incubation time, and heparin concentration in the assays. These factors however can hardly account for a discrepancy between 55% (Canadian study) and 110% (present study) as in the case of the factor Xa inhibition method (Table 1).

In the present study with the factor Xa–based method normal antithrombin activity was found in the patient with type II deficiency; her siblings also had low levels with the thrombin-based test. We would therefore like to stress that there is a possibility of not detecting all type II deficiencies with a factor Xa inhibition–based test, which is the most widespread routine method. In our investigation the factor Xa–based method overestimated antithrombin activity levels. According to our knowledge this has not previously been described and can cast some doubts about the validity of the use of this test as the test of choice when screening for antithrombin deficiency. Therefore we believe that both the bovine thrombin– and factor Xa inhibition–based tests, together with an antigen method, should be performed in patients with suspected antithrombin deficiency type II.

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To the editor:

RHCE represents the ancestral *RH* position, while *RHD* is the duplicated gene

In 2000, we elucidated the structure of the *RH* locus[1] by showing that it is an example for a gene cluster; *RHD* and *RHCE* face each other by their 3′ tail ends, and a third gene, *SMP1*, was found to be interspersed between the 2 rhesus genes. Two 9 000 base pair (bp) DNA segments, dubbed "rhesus boxes," of identical orientation fringed the *RHD* gene (Figure 1, top).

Based on this structure of the *RH* locus, the *RHD* gene deletion was parsimoniously explained by an unequal crossing-over event.[1] Furthermore, the inverse orientation of the *RH* genes may facilitate gene conversion among both rhesus genes, which would explain the high frequency of *RHD-CE-D* or *RHCE-D-CE* hybrid alleles.[2] However, it remained unknown which rhesus gene, if any, represented the ancestral positioning. The close proximity of the *RHCE* and *SMP1* in humans was startling too.

The duplication of the rhesus gene is known to have occurred during primate evolution,[3] giving rise to the *RHD* and *RHCE* genes in humans. Hence nonprimate mammals, like mice, may reveal the ancient state of the *RH* locus. In this context an 89 065 bp genomic DNA segment that was recently deposited in public databases (GenBank entry AL611963), which encompassed the mouse *RH* locus (Figure 1, bottom), was most disclosing. In order to compare the topology in mouse to the human *RH* locus we assembled a 315 242 bp DNA segment that included the human *RH* locus.

The assembly of this human genomic DNA was complicated by the fact that the current GenBank entry AL139426 contained sequences representative of *RHD*, *SMP1*, both rhesus boxes, and parts of *RHCE* but did not represent their correct topology. To overcome this limitation we compared the sequence of AL139426 to the sequences of *RHD* (X63097) and *RHCE* (M34015) cDNA, of *RHD* (AB035192) and *RHCE* (AB035191) intron 3, of *RHD* (AB035185) and *RHCE* (AB035184) intron 9, and of the upstream (AJ252311) and downstream (AJ252312) rhesus boxes. We determined multiple misassemblies occurring in long regions between almost identical paralogous sequences (join of *RHD* exon 3 to

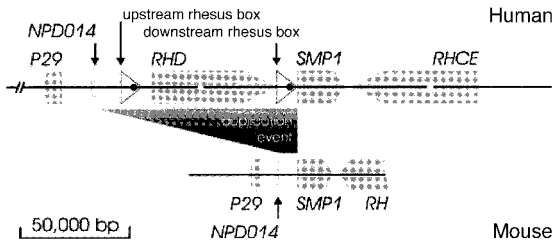

Figure 1. *RH* locus in human and mouse. The human *RH* locus (top) is compared to the mouse *RH* locus (bottom). The orientations and positions of genes are indicated by gray arrows, the rhesus boxes by white triangles. The fragment of the mouse *RH* gene shown covers exons 2 to 10. An open reading frame (ORF) in the rhesus box is depicted as a black circle, the succinate dehydrogenase complex, subunit D precursor pseudogene in *RH* intron 3 as a white circle; no sequences homologous to these ORF were found in the mouse locus. During the duplication event, *RHD* and its fringing rhesus boxes were inserted between *NPD014* and *SMP1*. The depicted DNA segments are GCIP-binding protein *P29* (accession numbers human, NM_015484; mouse, AY033432), *NPD014* (AF247168, AK003799), *SMP1* (NM_014313, AK014282) and *RH(CE)* (M34015, NM_011270), and the ORF in the upstream and downstream rhesus boxes (XP_044740 and XP_057505).

RHCE exon 4, *RHCE* exon 3 to *RHD* exon 4, 5′ upstream rhesus box to 3′ downstream rhesus box, 3′ upstream rhesus box to 5′ upstream rhesus box, and failed assembly of *RHCE* intron 9). We compiled the 315 242 bp human genomic DNA contig (Figure 1, upper panel) including both rhesus genes and a stretch of surrounding DNA comprising more than 100 000 bp using AL031432 (5′ of *RHD*), AL031284 (*RHCE*), AB035185 (*RHD* intron 9), AB035184 (*RHCE* intron 9) and a corrected version of AL139326. This third party annotated human DNA segment was deposited under GenBank accession number BN000065.

The position and orientation of proteins in the human and mouse DNA segments were determined by a homology search against the nonredundant protein database of the GenBank (Tblastx) utilizing the NCBI Blast page. Then, each possible match was manually evaluated after a 2-sequence alignment (Blast 2 sequences).[4] Both genomic DNA segments contained the *RH* gene(s), *SMP1*, and the 2 additional genes, GCIP-interacting protein *P29* and *NPD014*. In addition, the human DNA segment, but not the mouse DNA segment, contained the 2 rhesus boxes carrying one open reading frame each and a succinate dehydrogenase pseudogene located in the introns 3 of *RHD* and *RHCE* (Figure 1). The 3′

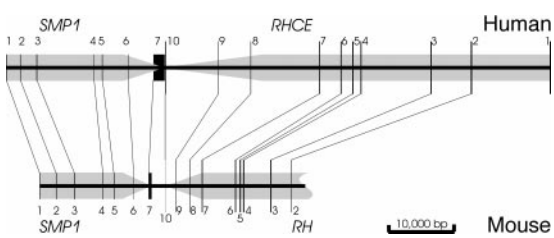

Figure 2. Topology of *SMP1* and *RHCE*. The orientations and positions of the genes are indicated by gray arrows and the localization of the exons by black lines at their approximate scale. The relative position and orientation of *RHCE* and *SMP1* is conserved. Human and mouse *SMP1* have a very similar topology. Both human rhesus genes are much enlarged relative to the mouse *RH* gene because of considerably elongated introns.

Table 1. Conservation of proteins at the *RH* gene locus between mouse and human

	Protein database accession numbers		Length (amino acids)		Amino acid positions	
Protein	Human	Mouse	Human	Mouse	Identical (%)	Conserved (%)
RhD	Q02161	NP_035400	417	418	239 (56)	296 (69)
RhCE	CAA44811	NP_035400	417	418	240 (56)	296 (69)
npd014	AAG44543	BAB23002	242	248	177 (75)	187 (79)
p29	NP_056299	AAK54459	243	242	221 (90)	229 (93)
smp1	AAD17754	BAB29242	157	157	149 (94)	152 (96)

ends of the rhesus boxes carry GC-rich regions that are typical for some strong promoters. The juxtapositioning of this structure right in front of the *SMP1* start codon may modify the expression of smp1 in primates compared to nonprimate species.

Based on the gene positions and orientations, *RHCE* was determined to represent the ancestral state. The close proximity of *SMP1* and *RH* known in humans[1] was also observed in the mouse *RH* locus (Figure 2). In the mouse, there were 8 639 bp between *NPD014* and *SMP1*. This size of a DNA stretch corresponded to the 11 437 bp between *NPD014* and the upstream rhesus box rather than to the 91 136 bp between *NPD014* and *SMP1*. The limited conservation of the noncoding regions did not allow a more detailed analysis of the *RH* duplication site in the moment.

Among the 4 proteins, smp1 was most conserved and Rh was least conserved (Table 1). There are 2 human smp1–analogous proteins, smp1 (accession number AAD17754) located in chromosome 1 and c21orf4 (P56557) located in chromosome 21. These 2 human proteins corresponded to 2 different mouse proteins, BAB29242 and BAB32266, that had 94% and 98% homology to the human genes, respectively.

In conclusion, *RHD* arose by a duplication of *RHCE*. It is likely that the orientation of *RHD* was inverted during this event. We propose that the rhesus boxes were instrumental for the duplication. *SMP1* is a highly conserved gene located in the immediate proximity of *RH* during much of the mammalian evolution. An understanding of the events shaping the rhesus polymorphism and the underlying mechanisms will contribute to improving genotyping strategies for rhesus as well as possibly for a host of other loci with clustered genes in the genome.

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