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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References

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 by showing that it is an example for a gene cluster; RH 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. 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. 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, 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

### Table 1. Antithrombin levels with antigen and activity methods

<table>
<thead>
<tr>
<th></th>
<th>Antigen (Lia test), %</th>
<th>Thrombin-based activity test, %</th>
<th>Factor Xa-based activity test, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proband (our results)</td>
<td>93</td>
<td>60-64</td>
<td>110</td>
</tr>
<tr>
<td>Proband (Canadian results)</td>
<td>93</td>
<td>72-74</td>
<td>53-56</td>
</tr>
<tr>
<td>Sister (our results)</td>
<td>96</td>
<td>62</td>
<td>92</td>
</tr>
<tr>
<td>Brother (our results)</td>
<td>92</td>
<td>55</td>
<td>89</td>
</tr>
</tbody>
</table>

NA indicates not applicable.

Our present investigation shows that the 3 patients have normal antigen levels measured with Lia antigen test. 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\(^1\) 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 Coamate LR kit (Haemochrom Diagnostica) with S-2772 and the automatic method for Thrombolyzer (Benhek 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).
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.


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. SM1 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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