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Research Article

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Linkage of Combined Factors V and VIII Deficiency to Chromosome 18q by Homozygosity Mapping

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Abstract

Combined Factors V and VIII deficiency is an autosomal recessive bleeding disorder identified in at least 58 families comprising a number of different ethnic groups. Affected patients present with a moderate bleeding tendency and have Factor V and Factor VIII levels in the range of 5–30% of normal. The highest frequency of the mutant gene is found in Jews of Sephardic and Middle Eastern origin living in Israel with an estimated disease frequency of 1: 100,000. We sought to identify the gene responsible for combined Factors V and VIII deficiency using a positional cloning approach. Of 14 affected individuals from 8 unrelated Jewish families, 12 were the offspring of first-cousin marriages. After a genome-wide search using 241 highly polymorphic short tandem repeat (STR) markers, 13 of the 14 affected patients were homozygous for two closely linked 18q markers. Patients and all available family members were genotyped for 11 additional STRs spanning ~ 11 cM on the long arm of chromosome 18. Multipoint linkage analysis yielded a maximal log of the odds (LOD) score of 13.22. Haplotype analysis identified a number of recombinant individuals and established a minimum candidate interval of 2.5 cM for the gene responsible for combined Factors V and VIII deficiency. The product of this locus is likely to operate at a common step in the biosynthetic pathway for these two functionally and structurally homologous coagulation proteins. Identification of this gene should provide new insight into the biology of Factor V and Factor VIII production. (*J. Clin. Invest.* 1997. 99:596–601.) Key words: gene • bleeding disorder • coagulation Factors • Jewish • hemophilia

Introduction

Combined Factors V and VIII deficiency was first described by Oeri et al. in 1954 (1). Affected patients present with a moderate bleeding tendency; most commonly noted are excessive bleeding after surgical procedures, childbirth, and menorrhagia. Frequent bleeding manifestations include epistaxis, gingival bleeding and easy bruising. Factor V and Factor VIII levels in affected individuals are typically in the range of 5–30% of normal. Since the original report, at least 89 patients belonging to 58 families have been identified (2–5). 24 of these families are of Mediterranean origin including nine Italian and nine Israeli families. The remaining 34 families are from North America, Europe, and Japan. The highest frequency of the disorder is found in Jews of Sephardic and Middle Eastern origin living in Israel. At least 15 affected patients belonging to this population have been identified yielding an estimated disease frequency of 1:100,000 (2). Combined Factors V and VIII deficiency is autosomal recessive in inheritance and consanguinity was present (most notably first-cousin matings) in 21 of 34 sibships for whom adequate information was available (3).

Co-inheritance of both Factor V deficiency (parahemophilia) (6) and Factor VIII deficiency (hemophilia A) (7) has been reported in four families (8–11) and is easily distinguished from the combined deficiency disorder. In their original report, Oeri et al. postulated that combined Factors V and VIII deficiency resulted from a defect in a gene which encodes a common precursor of both clotting factors (1). This hypothesis was excluded by the subsequent identification of distinct genes for Factor V (chromosome 1) (12) and Factor VIII (located on the X chromosome) (13). Consistent with the known proteolytic degradation of both Factor V and Factor VIII by activated protein C, Marlar and Griffin in 1980 identified a deficiency of protein C inhibitor in four patients with combined Factors V and VIII deficiency (14). However, subsequent analysis of these patients by the same workers and others excluded this explanation (15–18). Recently, Zivelin et al. used analysis of linkage to intragenic polymorphisms for protein C, protein S, Factor V, and prothrombin to exclude these loci as candidates for the combined Factors V and VIII deficiency gene (19). At present, the nature of the defect in combined Factors V and VIII deficiency remains to be established.

In 1987, Lander and Botstein devised an efficient strategy for mapping human genes that cause recessive diseases (20). This approach, known as homozygosity mapping, searches for regions that are consistently homozygous in multiple affected children from different families. This strategy has recently been successfully applied to a number of diseases (21–25). We now report successful localization of the gene for combined Factors V and VIII deficiency to the long arm of chromosome 18 using a homozygosity mapping approach.

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Methods

Subjects. Blood samples were obtained, in compliance with institutional review board policies, from 54 individuals in 9 Jewish families of Sephardic and Middle Eastern origin affected with combined Factors V and VIII deficiency, including 14 affected individuals (see Fig. 1). 12 affected patients were the progeny of first-cousin marriages (families 2–7, and 9), one was the offspring of a fourth-cousin marriage (family 1), and one the offspring of an unrelated marriage (family 2, individual 4). Multiple affected individuals were observed in four of the families. Total genomic DNA was prepared as previously described (26).

Genotype analysis. Primer pairs for the detection of 241 polymorphic markers were obtained from Research Genetics (Huntsville, AL) or the Biopolymers Core at the University of Michigan. These markers consisted of highly polymorphic short tandem repeats (STR), mostly tetranucleotide repeats, developed by the Cooperative Human Linkage Center (CHLC), Genethon, The Marshfield Clinic, and the Eccles Institute for Human Genetics (27–30). The average heterozygosity of the markers selected for this study is 0.75. PCR reactions were performed in 96-well microtiter plates in a total reaction volume of 10 μl. The forward strand primer (10 ng per PCR reaction) was end-labeled in the presence of γ-^{32}P-ATP (Amersham Corp., Arlington Heights, IL) and polynucleotide kinase (New England Biolabs Inc., Beverly, MA). To the end-labeled primer was added the deoxynucleotides (final concentration of 200 μM for each), 10× PCR buffer, 10 ng of the reverse primer, *Taq* DNA polymerase, and 20 ng of genomic DNA. PCR was performed in an MJ Research PTC-100 96V thermocycler. The optimal annealing temperature for each primer pair was empirically determined by amplification of normal DNA samples. PCR was performed for 30 cycles with one minute of denaturation at 94°C, 1 min of annealing at the appropriate temperature, and one minute of elongation at 72°C. A final 5-min elongation ensured complete extension of the PCR products. After cycling was complete, the PCR reactions were stopped by the addition of 10 μl of standard dideoxy sequencing stop solution (95% formamide, 1 mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol). PCR reactions were stored at −20°C until analysis. Completed PCR reactions were denatured 5 min at 94°C and the amplification products were separated on 8% denaturing polyacrylamide gels. Gels were exposed to x-ray film for 2–48 h. The gel films were analyzed by determining the number of affected individuals homozygous at each marker. Allele sizes were determined by comparison to MspI digested pBR322 which was included as a size standard on the gels. All markers were scored independently by two observers, the scores compared, and any discrepancies resolved by re-examination of the gels.

Linkage analysis. Genetic analysis was performed using MAPMAKER/HOMOZ, Version 1.0 (31), a new algorithm for rapid linkage calculations designed especially for homozygosity mapping. Autosomal recessive inheritance with complete penetrance and a disease allele frequency of 0.003 (based on a disease frequency of 1:100,000) were assumed. Order and distances between markers were determined using MULTIMAP, Version 1.1 (32). Any distance determined to be less than 0.5 cM was set at 0.5 cM for the MAPMAKER/HOMOZ analysis. For linked markers, allele frequencies were determined by analysis of either 15 Centre d'Etudes du Polymorphisme Humain (CEPH) families (representing 108 independent chromosomes) or 17 unaffected chromosomes available in the 9 combined Factors V and VIII deficiency families reported here. Approximately the same allele distribution was observed in the two populations.

Results

A genome-wide search for regions of homozygosity-by-descent with 241 STR markers was conducted with DNA from 14 individuals (including 12 offspring of first-cousin marriages) affected with combined Factors V and VIII deficiency from eight unrelated Jewish families (Fig. 1). DNA was not available on the deceased affected individual of an additional family (family 8, Fig. 1), but an affected haplotype could be deduced (see Fig. 2). Five of the families are of Tunisian origin, two of Iraqi, one of Iranian, and one of Egyptian origin. Two markers on chromosome 18q (D18S64 and D18S849) demonstrated homozygosity in 13 of 14 affected individuals (see Fig. 2). These two markers map adjacent to each other, with CRIMAP (33) analysis placing D18S64 2.1 cM telomeric to D18S849. Affected patients were homozygous for one of two different alleles for D18S64 and homozygous for one of four (possibly five if family 8 is included, see below) different alleles at D18S849. Genotyping of 30 unaffected individuals representing the parents, siblings, or offspring of the 14 affected

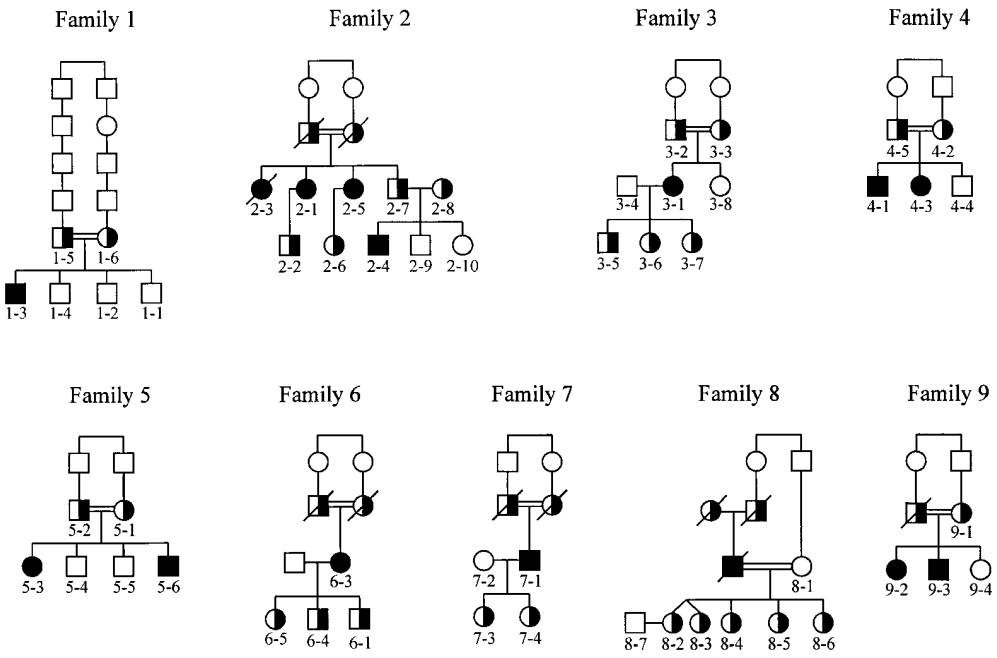

Figure 1. Pedigrees of combined Factors V and VIII deficiency families. Pedigrees of the nine families of Sephardic and Middle Eastern Jewish origin used in the linkage analysis are shown. DNA samples were obtained on those individuals identified by number (family number-individual number). Double lines indicate consanguineous matings.

	Tunisian	Tunisian		Tunisian	Tunisian	Tunisian		Iraqi				Iraqi		Egyptian	Iranian
	1-3	5-3	5-6	6-3	8	9-2	9-3	2-1	2-3	2-5	2-4	4-1	4-3	7-1	3-1
centromere															
AFM191XCP	193|181	187	187	195|193	195	185|181	185|181	195	195	195	197|195	197	197	195	185
D18S1152	114|118	106	106	106	108	102|108	102|108	106		106	108|106	108	108	106	114
D18S858	199|205	205	205	205|193	199	196|205	196|205	205	205	205	199|205	199	199	208	205
D18S41	199|203	203	203	203|193	203	197|199	197|199	199	199	199	199	199	199	207	199
D18S849	302	288	288	288|282	286	288	288	302	302	302	302	302	302	298	270
D18S1144	173	159	159	159|165	177	159	159	177	177	177	177	177	177	163	163
D18S1129	91	107	107	107	109	107	107	97	97	97	97	97	97	95	97
D18S1103	94	94	94	94	94	94	94	106	106	106	106	106	106	106	106
D18S1155	198	198	198	198	198	198	198	208	208	208	208	208	208	208	208
D18S1109	136	136	136	136	136	136	136		124	124	118|124	118	118	124	120|118
D18S64	200	200	200	200	200	200	200	192	192	192	192	192	192	192	206|192
GATAP32103	180	180	180	180	180	180	180|192	188|184	184	184	188|184	188	188	184	180
D18S862	141	141	141	141	141	141	141		141|135	141|135	144|135	144	144	141	126|129
telomere															

Figure 2. Haplotype analysis of affected individuals for markers on chromosome 18q. Affected individuals are indicated by family number-individual number as in Fig. 1. Families are grouped by ancestry. Families 1, 5, 6, 8, and 9 are Tunisian, families 2 and 4 are Iraqi, family 7 is Egyptian, and family 3 is Iranian. The markers, shown at left, are arranged from centromere (*top*) to telomere (*bottom*). Those markers for which each affected individual was homozygous are indicated by light gray shading. The 3 markers for which all affecteds were homozygous are indicated by dark gray shading. Five individuals (5-3, 5-6, 4-1, 4-3, and 7-1) were homozygous for all markers in the region. Though no DNA was available from the deceased affected individual in family 8, the observation of only one paternal haplotype among four unaffected offspring (Figs. 1 and 3) suggests that this affected individual was also homozygous for all markers in the region. Based on the observed recombinants, the minimum candidate interval places the combined Factors V and VIII deficiency gene between markers D18S1144 and D18S1109.

individuals in the nine families revealed very few homozygotes for either of these markers: only 2 of 30 individuals at D18S849 (Fig. 3) and 6 of 30 at D18S64 (not shown). A total of 12 different alleles were observed for D18S849 among these nine families, while five different alleles were noted among the nine families for D18S64. Taken together, these data are suggestive of linkage of the combined Factors V and VIII deficiency locus to these markers. Recently, Kruglyak et al. have developed a new algorithm for rapid linkage calculations designed specifically for homozygosity mapping, MAPMAKER/HOMOZ (31). Multipoint linkage analysis over a region extending from 5 cM centromeric to D18S849 to 13.7 cM telomeric to D18S64 using MAPMAKER/HOMOZ yielded a peak LOD score of 11.1 at D18S849.

An additional 27 short tandem repeat markers mapping approximately to the region of interest were identified based on the CHLC and Genethon genetic maps (27, 28). DNA samples from all affected individuals were typed for the additional markers. 14 of 27 markers identified eight or more homozygotes among the 14 affected individuals. All available members of the 9 families were genotyped for these 14 markers. Three of the markers were uninformative in our patient panel and thus were excluded from further analysis. Before calculating multipoint LOD scores for the remaining 11 markers, it was necessary to determine the order and distance between them relative to D18S849 and D18S64 in the candidate region. For this analysis, DNA samples from 15 large CEPH families (representing 108 independent chromosomes) were genotyped with the full marker panel. The best order of the markers and estimated distances between them in cM, as determined using MULTIMAP (32), was: cen-AFM191XCP-1.5-D18S1152-0.3-D18S858-0.3-D18S41-1.0-D18S849-0.4-D18S1144-0.3-D18S1129-0.4-D18S1103-0.4-D18S1155-1.4-D18S1109-0.3-D18S64-2.4-GATAP32103-1.6-D18S862-tel. MAPMAKER/HOMOZ analysis using all 13 markers in the region yielded a maximum LOD score of 13.22 at D18S1129 (Fig. 4). MAPMAKER/HOMOZ analysis using allele frequencies estimated from the 17 unaffected chromosomes for markers D18S1144, D18S1129, D18S1103, D18S1144, and D18S1109 yielded a maximum LOD score of 13.36 at D18S1129.

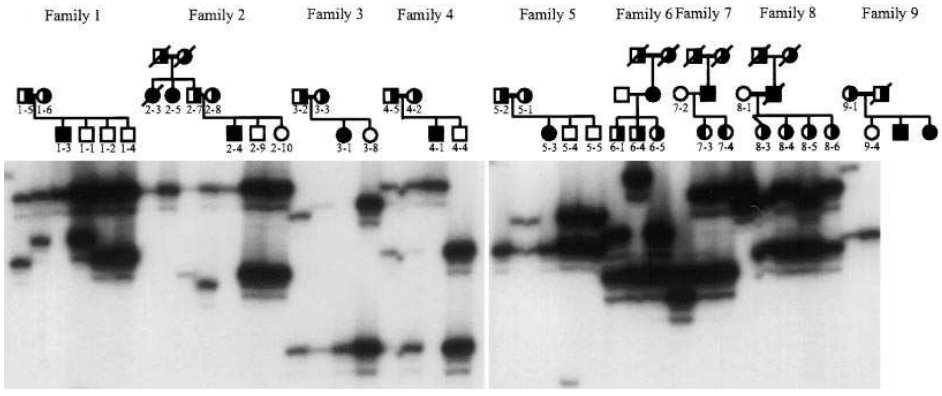

Figure 3. Genotype analysis of individuals at D18S849. Shown below the abbreviated pedigree of each family is the autoradiogram after PCR and gel electrophoresis for marker D18S849. 12 different alleles were observed among the families. As seen, only two unaffected individuals (5-2 and 9-4) were homozygous at this marker. 13 of 14 affected individuals were homozygous for one of four different alleles. Only affected individual 6-3 (not shown) was heterozygous at this marker (See Fig. 2).

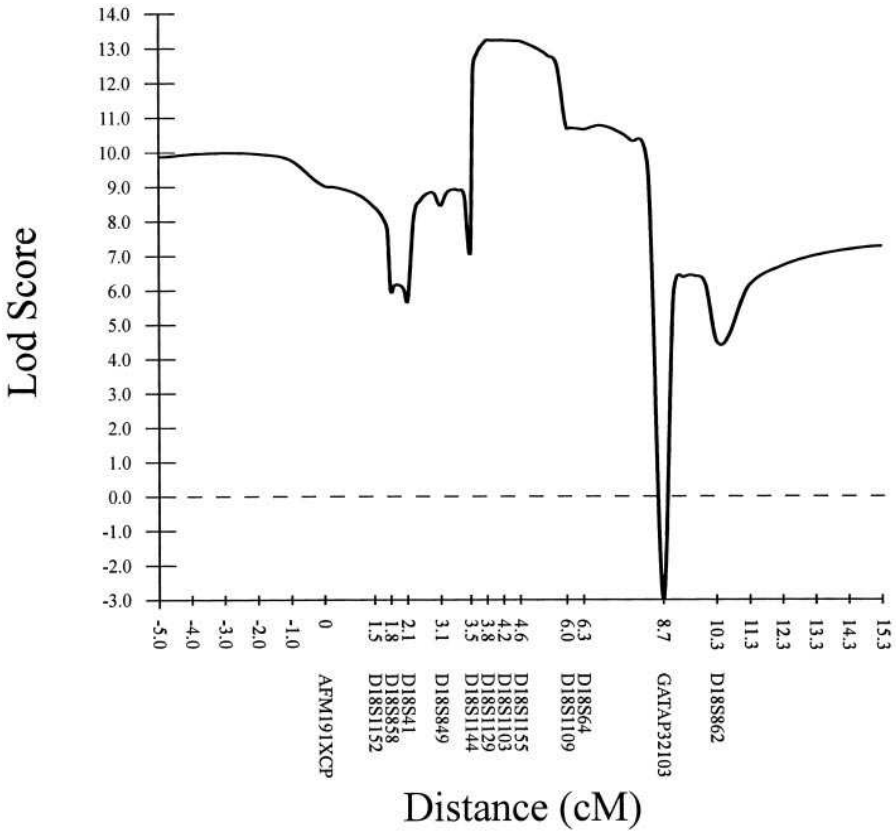

Figure 4. Multipoint linkage analysis of combined Factors V and VIII deficiency. Multipoint linkage analysis was performed using the 13 markers shown on the abscissa with MAPMAKER/HOMOZ. The best distances and order between the markers was determined using MULTIMAP. The markers are shown centromere (*left*) to telomere (*right*). The dotted horizontal line within the plot indicates a LOD score of 0. For marker GATAP32103, the extension to −3.00 represents a point where the LOD score reached $-\infty$. MAPMAKER/HOMOZ analysis using all 13 markers in the region yielded a maximum LOD score of 13.22 at D18S1129.

We next performed haplotype analysis of the 14 affected individuals or their available parents, siblings, or offspring. Shown in Fig. 2 are the haplotypes of the affected individuals from each family. Five affecteds were determined to be homozygous for every marker in the 10.3-cM interval. Though no DNA was available from the deceased affected individual in family 8, the observation of only one paternal haplotype among four unaffected offspring (Fig. 2) suggests that this affected individual was also homozygous for all markers in the region. A number of recombinants were detected which helped to limit the candidate interval. Recombination during ancestral meioses is suggested by the haplotypes observed in affected individuals in families 1, 2, 3, 6, and 9. Recombination during parental meioses is also suggested in families 2 and 9. In family 2, comparison of individual 2-1 to siblings 2-3 and 2-5 indicates a recombination between markers GATAP32103 and D18S64. In addition, a double recombination event between markers D18S1144 and D18S1129 and between GATAP32103 and D18S862 is suggested in an unaffected individual in family 9 (9-4) who is identical to her affected siblings centromeric to D18S1129 and telomeric to GATAP32103. Based on the observed recombinants, the candidate interval is defined by markers D18S1109 (for which affected individuals 2-4 and 3-1 are heterozygous) and DS18S1144 (for which affected individual 6-3 is heterozygous) (Fig. 2). By MULTIMAP (32) analysis this is an $\sim$ 2.5 cM interval.

Discussion

The current genome-wide linkage analysis maps the gene responsible for combined Factors V and VIII deficiency to a 2.5-cM candidate interval on chromosome 18q. MAPMAKER/HOMOZ analysis of 14 affected individuals from 8 Jewish families of Sephardic and Middle Eastern origin yielded a maximum LOD score of 13.22. As yet, no strong linkage disequilibrium is observed between the combined Factors V and VIII deficiency locus and any one haplotype. However, linkage disequilibrium is observed between the disease locus and two possible founder haplotypes. As seen in Fig. 2, the affected haplotype in all Tunisian-Jewish affected individuals shares the same alleles for the six markers including and telomeric to D18S1103 (affected individual 9-3 is heterozygous at GATAP32103). This haplotype is not present on any of 17 unaffected chromosomes. The affected individuals in the remaining four non-Tunisian-Jewish families share a haplotype for markers D18S1103 and D18S1155, two of three markers within the candidate interval. Again, this haplotype, which is different than that seen in the Tunisian-Jewish affecteds, is not found on any of the unaffected chromosomes in these families. These two different founder haplotypes may be indicative of a split between Tunisian Jews and other Jews of Sephardic and Middle Eastern origin (34, 35). The extent of the complete linkage disequilibrium in the Tunisian-Jewish families is at least 6 cM and suggests that the mutation in this branch is more recent than that in the non-Tunisian families who demonstrate complete linkage disequilibrium over a smaller distance (< 1.0 cM) (36).

As recently reviewed by Jorde (37), linkage disequilibrium mapping offers a method of narrowing the candidate interval in populations with well-defined founding dates including some Jewish populations (34, 35). When a disease mutation is first introduced into such a population, it resides on a single chromosome and thus a single haplotype of linked markers. If

any striking disequilibrium is found for any particular marker of a founder haplotype, one can estimate the distance the disease gene is from the marker based on the number of affecteds who carry this particular allele. Recently, the method has been used to predict the locations (within 70 kb in some instances) of several disease genes (38, 39) including familial Mediterranean fever in non-Ashkenazi Jewish founder haplotypes (40). Risch and colleagues applied this technique to identify a single founder haplotype for idiopathic torsion dystonia in Ashkenazi Jews and estimated that the founder mutation first appeared approximately 350 years ago (36). By a similar approach, it may be possible to deduce a single founder haplotype for combined Factors V and VIII deficiency. We hypothesize that the mutation in these two branches may be derived from the same founder who predates the split of these two closely related populations. If this is correct, characterization of new markers between D18S1129 and D18S1155 should eventually identify a common ancestral haplotype which may significantly limit the size of the candidate interval for this disease gene.

Physical mapping studies of the human genome by the MIT Center for Genome Research has identified a 1.6 megabase (Mb) yeast artificial chromosome (YAC) spanning markers D18S1129, D18S1103, D18S1155, and D18S64 (30). D18S1109 has not been placed on the physical map, but genetic mapping studies, placing it between D18S64 and D18S1155, would suggest that it is also on this YAC. By our MULTIMAP analysis, this is approximately a 2.5 cM interval. Three YACs have been identified which are positive for D18S849 and D18S1144 including one which is only 0.33 Mb, consistent with our genetic mapping studies. However, no YACs have been identified containing markers D18S1144 and D18S1129 indicating that the estimated genetic distance of 0.3 cM may be smaller than the physical distance in this region.

No genes known to interact with either Factor V or VIII currently map to the candidate interval. The gene responsible for combined Factors V and VIII deficiency is therefore likely to be a novel locus which functions at a common step in the biosynthetic pathway for these two functionally and structurally homologous coagulation proteins. Identification of this gene should provide new insight into the biology of Factor V and Factor VIII.

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