Glanzmann Thrombasthenia

Cooperation between Sequence Variants in *Cis* during Splice Site Selection

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Abstract

Glanzmann thrombasthenia (GT), an autosomal recessive bleeding disorder, results from abnormalities in the platelet fibrinogen receptor, GPIIb-IIIa (integrin $\alpha_{IIb}\beta_3$). A patient with GT was identified as homozygous for a G→A mutation 6 bp upstream of the GPIIIa exon 9 splice donor site. Patient platelet GPIIIa transcripts lacked exon 9 despite normal DNA sequence in all of the *cis*-acting sequences known to regulate splice site selection. In vitro analysis of transcripts generated from mini-gene constructs demonstrated that exon skipping occurred only when the G→A mutation was *cis* to a polymorphism 116 bp upstream, providing precedence that two sequence variations in the same exon which do not alter consensus splice sites and do not generate missense or nonsense mutations, can affect splice site selection. The mutant transcript resulted from utilization of a cryptic splice acceptor site and returned the open reading frame. These data support the hypothesis that pre-mRNA secondary structure and allelic sequence variants can influence splicing and provide new insight into the regulated control of RNA processing. In addition, haplotype analysis suggested that the patient has two identical copies of chromosome 17. Markers studied on three other chromosomes suggested this finding was not due to consanguinity. The restricted phenotype in this patient may provide information regarding the expression of potentially imprinted genes on chromosome 17. (*J. Clin. Invest.* 1996. 98:1745–1754.) Key words: RNA splicing • exon skipping • platelet membrane glycoprotein complex IIb-IIIa • molecular genetics • Glanzmann thrombasthenia

Introduction

Glanzmann thrombasthenia (GT)[1] is an autosomal recessive bleeding disorder due to quantitative or qualitative abnormalities in the platelet fibrinogen receptor (1, 2). The disease was first described in 1918 by Glanzmann, a Swiss pediatrician (3). In 1974 Nurden and Caen demonstrated the absence of platelet membrane glycoproteins IIb-IIIa (GPIIb-IIIa) in three patients with this disorder (4). Subsequent studies characterized, isolated, and demonstrated that GPIIb-IIIa functioned as the fibrinogen receptor in platelets (5–12), thus providing the biochemical explanation for the bleeding phenotype seen in GT. With the isolation of cDNA and genomic clones for GPIIb and GPIIIa (13–19), a number of mutations in the genes have been identified which result in GT. The first molecular defect in thrombasthenia was described in 1990 (20) and since then > 20 different mutations have been characterized (for review see reference 21). These studies have provided a wealth of information about platelet physiology, as well as biochemical, immunologic, and molecular biologic information about GPIIb-IIIa and other molecules which comprise the integrin family of adhesive molecules (22, 23).

GT is categorized into three types according to the amount of immunologically detectable GPIIb-IIIa: type I, < 5%; type II, 10–20%; and variant thrombasthenia with nearly normal levels of GPIIb-IIIa (1). Four mutant GPIIIa alleles have been described in five kindreds with variant thrombasthenia (24–28): three of these mutations affect two amino acids (Asp119 and Arg214) and demonstrate a necessary region for fibrinogen binding (24–26, 28). A third mutation alters the cytoplasmic Ser752 and impairs the signaling required for GPIIb-IIIa function (27, 29). However, the patients with variant thrombasthenia represent only 8% of all thrombasthenics (1), and the majority of mutations described to date have been in type I patients. Most of these are caused by small mutations (30–40). Of the 10 point mutations published in full form, five have resulted in mRNA splicing abnormalities (30–33, 38). These splicing abnormalities usually generate unstable mRNAs yielding no translated protein products, or in a few cases, truncated and dysfunctional protein products. Several point mutations have resulted in mRNA splicing defects in which entire exons have been deleted (30, 31, 33). Although alternate exon splicing has been thoroughly investigated (see reviews 41, 42), many mechanistic details are still poorly understood. Most mutations which affect splicing are located within consensus splice donor, acceptor, and branch sites and cause exon skipping as opposed to cryptic splice site utilization (43, 44). Nakai et al. scanned 209 mammalian genetic disease splicing mutations and observed that > 90% create or destroy consensus sequences and that most occurred at the 5′ splice site region; none were upstream of the −1 position (45).

We previously reported the preliminary characterization of a patient with type I GT who had an unusual mutation resulting in abnormal mRNA splicing manifested by the skipping of exon 9 in the GPIIIa gene (46). We now report the full characterization of this genetic defect and the inheritance of the mutant allele. Our data indicate that a mutation 6 bp upstream of the splice donor site of exon 9 acts in concert with an addi-

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1. *Abbreviations used in this paper:* GT, Glanzmann thrombasthenia; OAT, ornithine δ-aminotransferase; ORF, open reading frame; RT, reverse transcription; SSCP, single strand conformation polymorphism.

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tional exonic sequence variation to cause exon skipping with the subsequent utilization of a cryptic splice site, and that the phenotype in this patient was apparently due to disomy of chromosome 17. We discuss the possible reasons for these splicing abnormalities and the genetic implications of chromosome 17 disomy.

Methods

Subjects. Subject RS is a 24-yr-old African American female with a lifelong bleeding history. Her bleeding is primarily from her nasal and gingival mucosa and with her menses. She required platelet transfusions at age 10 for epistaxis. Her clinical features are typical for Glanzmann thrombasthenia and careful history and physical examination revealed no unusual or additional clinical findings. Her platelets do not aggregate in response to physiologic stimuli, but do aggregate to ristocetin. Platelets from the patient had trace amounts of GPIIb, but lacked detectable GPIIIa by Western immunoblotting and have been previously shown not to bind soluble fibrinogen (47). No history of consanguinity could be elicited despite intense questioning. The father is unavailable for study. The mother of the patient was asymptomatic and had normal platelet aggregation. Blood from normal individuals was used to prepare genomic DNA for control experiments. All of these studies have been conducted according to the principles expressed in the Declaration of Helsinki.

Reagents. Taq polymerase was from Perkin Elmer Cetus Corp. (Norwalk, CT), isotopes were from Amersham Corp. (Arlington Heights, IL), ds Cycle Sequencing System from Gibco BRL (Gaithersburg, MD).

Quantitation of vitronectin receptors. Monoclonal antibody LM609 (the generous gift of Dr. David Cheresh, Scripps Research Institute, La Jolla, CA) specific for $\alpha_v\beta_3$ was used to quantitate the platelet vitronectin receptor as previously described (48).

Single strand conformation polymorphism (SSCP) analysis. Preparation of genomic DNA and screening for mutations was performed by SSCP analysis as previously described (31). Radiolabeled PCR products were denatured in 95% formamide and electrophoresed through 6% acrylamide, 10% glycerol, and 1 × TBE (89 mM Tris-borate pH = 8.3, 2 mM EDTA) nondenaturing gels. The gels were run for 16 h at 10 W at room temperature, dried, and exposed to film for 48 h.

Oligonucleotide synthesis. Oligonucleotides for SSCP were as previously described (31). Primers for RNA analysis were designed from GPIIIa cDNA sequence (16) and are listed in Table I. Nucleotide sequence numbering is according to Zimrin et al., (18); exon numbering, according to Villa-Garcia et al., (19) and Zimrin et al. (18).

RNA preparation. Total platelet RNA was prepared and reverse transcribed into cDNA with random primers as previously described (49). Using oligonucleotides designed from exonic sequence, the platelet cDNA was amplified and analyzed on 2% agarose gels. Abnormal fragments were gel purified and the nucleotide sequence determined as described below.

Nucleotide sequence determination. Sequence information was obtained from eluted DNA from SSCP gels (31), directly from amplified genomic DNA, or from reverse transcribed platelet mRNA. 2 μl of the PCR products were used in a sequencing reaction which included a [γ-^{33}]P-labeled primer and 2.5 units Taq polymerase (ds Cycle Sequencing System; Gibco BRL). 20–30 cycles of amplification were performed as previously described (31). Extension products were separated on 6% acrylamide/7 M urea gels and assayed by autoradiography.

Southern blot analysis. Genomic DNA preparation for Southern blot analysis, as well as the blotting procedure were performed as previously described (20). A 2.0-kb cDNA probe 5′ to the internal EcoRI site in the GPIIIa cDNA (50) and a 345-bp probe containing exons 1–3 of the fibrillin cDNA (51) were used for genomic DNA hybridizations. Equivalency of loading between lanes was determined by using the ImageQuaNT version 4.1 software on a phosphorimager, (445SI-486; Molecular Dynamics, Sunnyvale, CA).

Reverse transcriptional PCR (RT-PCR) products derived from platelet RNA were separated on a 2% agarose gel, transferred to nylon filters, and hybridized with an internal oligonucleotide as described (52). Relative abundance of RNA transcripts was determined by carefully aligning the autoradiogram with the filter, cutting out that portion of the filter corresponding to a particular band, and determining the signal strength with a scintillation counter.

Minigene transcript analysis of mutant and wild-type alleles. Approximately 2 kb of genomic DNA was PCR amplified from the patient and her mother using PCR primers RSY.S and RSY.A contained in exons 8 and 10, respectively. These fragments were cloned in frame into the ornithine δ-aminotransferase (OAT) cDNA contained in pGEM-4Z (Promega Corp., Madison, WI) as described (52). The EcoRI fragment from these plasmids were isolated and cloned into the pcDNAI/Amp expression plasmid (Invitrogen, San Diego, CA), and mapped for the proper orientation. The nucleotide sequence of the entire exon 9 and the flanking 50 bp was determined, and demonstrated that we had isolated the wild-type maternal allele (pcDNA.E1), the mutant maternal allele (pcDNA.E2), and the single patient allele (pcDNA.RS).

Point mutations were introduced into pcDNA.E1 and pcDNA.RS using the Altered Sites II in vitro mutagenesis kit (Promega Corp.). Briefly, the EcoRI fragments of pcDNA.E1 and pcDNA.RS were cloned into pALTER-1 and single-strand DNA prepared. The desired mutation was generated using a synthetic oligonucleotide and

Table I. GPIIIa Oligonucleotide Primers

Name	Sequence (5′ to 3′)	Location:description
3H.S	cagttcaattcttgtcttcttgt	intron 8 for SSCP of exon 9
3H.A	gctccaggacaaaggccct	intron 9 for SSCP of exon 9
3a24	TATCCCTCTTTGGGGCTGATGACTG	exon 7 for mRNA analysis
3IA1	AAGGGCGATAGTCCTCCTC	exon 10 for mRNA analysis
RA1	ACCTTGGCCTCAATGCTGAAG	exon 10 for mRNA analysis
RA3	CAATGCTGAAG<u>CTCACCCCATA</u>	spans exons 10 and 8
3aex8	CCAGCTCATTGTTGATGCTT	exon 8 probe for Southern
RSY.S	ACCG***GAACCAATTC***TGTCCATGGATTCCAGCA	exon 8 sense for minigene
RSY.A	GGC***ATGCAT***CAAAGGTGACCTGGACGAT	exon 10 antisense for minigene

Intron sequence in lowercase; exon 8 sequence underlined; artificial sequences for cloning purposes are in italics; XmnI and NsiI restriction sites are in bold.

T4 DNA polymerase, subcloned back into pcDNAI/Amp, and the plasmid was sequenced to confirm the mutation had been properly introduced.

Plasmid DNA was purified using Nucleic Acid purification columns (Qiagen Inc., Chatsworth, CA) and electroporated into K562 cells as described (19). 16 h later total RNA was isolated, reversed transcribed into cDNA, and analyzed by PCR amplification with OAT and GPIIIa specific primers. To confirm the identity of these PCR products, they were cloned into the pCRII plasmid (Invitrogen) and sequenced.

Microsatellite analysis. Oligonucleotides were used to PCR amplify the region of genomic DNA known to contain polymorphic dinucleotide repeats. These primers have been described previously (53–55) and were identified by searching the official Genome Data Base (GDB) (56, 57) source node at Johns Hopkins University in Baltimore on the GDB main computer. PCR conditions were as above except the sense primer was end-labeled with ^{32}P-γATP and the annealing temperatures varied between 55–62°C. Products were separated in 6% acrylamide/7-M urea gels, and assayed by autoradiography.

RNA secondary structure prediction. RNA secondary structure was analyzed by the algorithm of Zuker and Stieger (58) using the computer program RNAFOLD in the PC/GENE software from IntelliGenetics (Mountain View, CA) on a Computer Ergonomics (Columbia, MD) 486 computer. The input sequence contained all of exon 9 (four combinations of sequence variants) and the last and first 10 bp of introns 8 and 9, respectively. A graphic display was generated from this prediction using the program MOLECULE written by Dr. J. Ryan Thompson (Carnegie-Mellon University, Pittsburgh, PA) according to the algorithm of Lapalme et al. (59).

Results

Because the molecular defect in GT may be in either of two genes, we performed preliminary studies to assess GPIIIa expression. Platelets from patient RS and a normal control were studied for their vitronectin receptor ($\alpha_v\beta_3=\alpha_v$/GPIIIa) content (48). The patient's platelets did not bind an antibody to the vitronectin receptor (LM609), suggesting a GPIIIa defect (data not shown). Genomic Southern blotting of patient DNA using a series of restriction enzymes was normal (see below), suggesting normal gene dosage and the lack of a large rearrangement. We used the SSCP technique to analyze all GPIIIa exons from patient RS. Fig. 1 *A* shows such an analysis of exon 9 from a group of normal individuals and patient RS. Despite the presence of additional bands due to the known silent C/A polymorphism at position 20507 in this exon (18), a clearly abnormal pattern of migration was seen for the sample from RS (Fig. 1, lane *13*). The abnormal 209-bp fragment was isolated and sequenced, and a G to A substitution was found 6 bp upstream of the splice donor site (AC<u>G</u>GTGAGgt→AC<u>A</u>GTGAGgt) at position 20624 of the GPIIIa gene (Fig. 1 *B*). There were no other nucleotide differences between the sequence from the patient DNA fragment and that of the normal control. This G to A substitution represents a synonymous change and has not previously been reported. To address whether this sequence change represented a polymorphism, we performed SSCP analysis of exon 9 on DNA samples from 45 normal individuals and 4 additional patients with GT (90 and 8 chromosomes, respectively). In no case did we find the SSCP pattern corresponding to this G to A transition (data not shown). Because the patient was African American, we also studied DNA from 58 African Americans using an HphI restriction digest of the PCR fragments (the HphI recognition sequence is specifically encoded by the G variant) and again found no alleles containing the A at position 20624. Thus, the A was not present in 206 chromosomes from normal individuals lessening the possibility that the sequence variation at position 20624 represents a polymorphism.

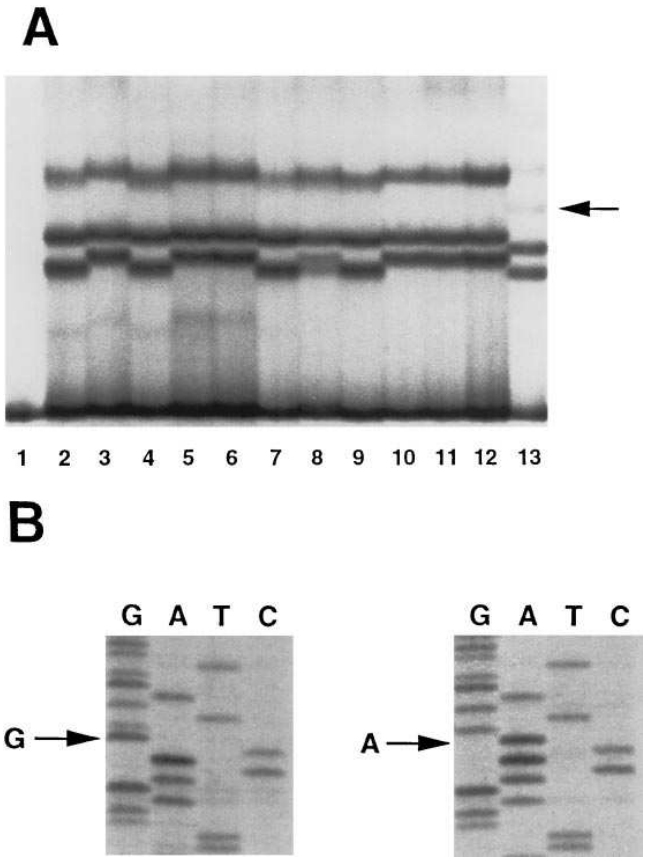


Figure 1. SSCP analysis of exon 9 of the GPIIIa gene. (*A*) PCR amplified genomic DNA from 11 different normal individuals (lanes *1–12*) and patient RS (lane *13*) using primers 3H.S and 3H.A. The variable bands seen in the normal individuals have been previously sequenced (31) and are due to the known silent polymorphism in this exon (18). (*B*) Nucleotide sequence of exon 9 SSCP products. (*Left*) sequence of a fragment derived from a normal individual; (*right*) sequence of the fragment indicated by the arrow in Fig. 1 *A*.

Direct sequencing of genomic DNA revealed the patient was homozygous for the G to A substitution, suggesting this may be responsible for the nonexpression of GPIIIa. However, because this nucleotide substitution does not change an amino acid, we sought to determine whether it alters pre-mRNA splicing. The major PCR product identified when patient platelet RNA was amplified with sense and antisense primers complementary to regions of exons 7 and 10, respectively, was smaller than normal (Fig. 2 *A*, lane *3*). There were also two less abundant products of approximately normal size observed in the patient sample which were only consistently seen upon overloading of PCR products (see below). Isolation and sequencing of the major 442-bp fragment from the patient sample revealed the absence of exon 9 but the insertion of 5 bp between the sequences corresponding to exons 8 and 10. (Fig. 2 *B*). These 5 bp match precisely the 5 bp preceding the exon 10 spice acceptor site and are preceded in genomic DNA by an

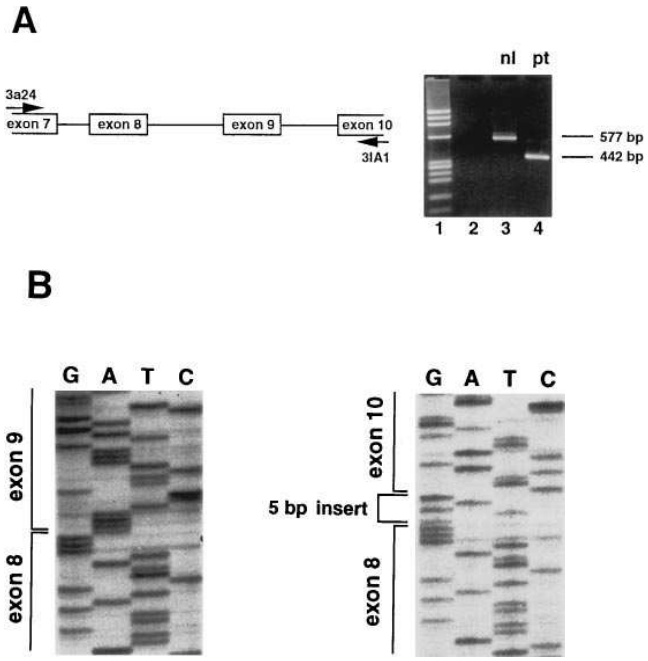


Figure 2. Platelet GPIIIa RNA analysis. (*A*) The cartoon on the left indicates the relative positions of the PCR primers in exons 7 and 10 of the GPIIIa gene, and does not reflect a specific sized PCR product. To the right is shown an ethidium-stained agarose gel containing products from RT-PCR reactions using primers 3a24 and 3IA1 with no input template (lane *2*), and platelet cDNA from a normal individual (lane *3*), and patient RS (lane *4*). Size markers are ϕx174 DNA digested with HaeIII (lane *1*). (*B*) Nucleotide sequence of the fragments shown in *A*. (*Left*) GPIIIa mRNA from normal platelets; (*right*) platelet GPIIIa mRNA from RS.

AG dinucleotide. This suggested that the majority of the transcripts manifest the skipping of exon 9 and the utilization of a cryptic splice acceptor site 5 bp upstream of exon 10. Note that the isolated skipping of exon 9 would result in a frameshift and consequent premature termination codon, and mutant transcript instability would be predicted (52). However, some proportion of such transcripts utilize a cryptic splice site, restoring the open reading frame (ORF) and hence stability of those molecules. We have determined the sequence of the two faint upper bands seen in Fig. 2 *A*, lane *4*. The lower of these two is normal in size, contains exon 9, the G to A substitution, and otherwise has normal sequence (data not shown). The slightly larger than normal fragment has been difficult to separate from the normal fragment, but the sequence we have obtained indicates it is a heteroduplex comprised of both the 577 and 442 bp cDNA species. Fig. 3 summarizes the abnormal splicing from the mutant allele and indicates the position of the cryptic splice site.

We considered several additional issues with respect to the molecular genetics of this mutation. First, is the G to A substitution in exon 9 necessary to cause the abnormal splicing phenotype? Second, is the G to A substitution in exon 9 sufficient to cause the abnormal splicing phenotype? Third, how was the abnormal allele inherited? To address the first question we determined the nucleotide sequence corresponding to all of the *cis*-acting elements known to influence splice site selection. These include the splice donor, branch point, and splice acceptor for introns 8 and 9 (Fig. 3). No sequence alteration was identified in these regions, and we conclude that the G to A substitution in exon 9 is associated with, and perhaps necessary for the abnormal splicing.

Since the mother is heterozygous for the G→A substitution (see below), if the G→A substitution causes exon skipping we would predict that some portion of her platelet GPIIIa transcripts would demonstrate exon 9 skipping. To determine whether exon 9 skipping did not occur at low levels in normal individuals and to study maternal transcripts, we analyzed platelet RNA from the mother, the patient, and a series of normal individuals (Fig. 4 *A*). Using primers flanking exon 9, we observed a faint band in the mother's sample (Fig. 4 *A*, lane *2*) which comigrated with the fragment manifesting exon skipping from RS. The abnormal fragment was not seen in platelet RNA from normal individuals (Fig. 4 *A*, lanes *1*, *4–9*). Although we did not rigorously attempt to perform a quantitative RT-PCR, these data nevertheless suggested that a small percent of the maternal platelet mRNA contained GPIIIa transcripts lacking exon 9. We confirmed this by utilizing an antisense PCR primer which would only anneal to cDNA derived from mRNA that had skipped exon 9 and utilized the cryptic splice site in intron 9 during splicing. Fig. 4 *B* demonstrates that platelet total RNA from the patient (Fig. 4 *B*, lane *3*) and the mother (Fig. 4 *B*, lane *2*), but not a normal control

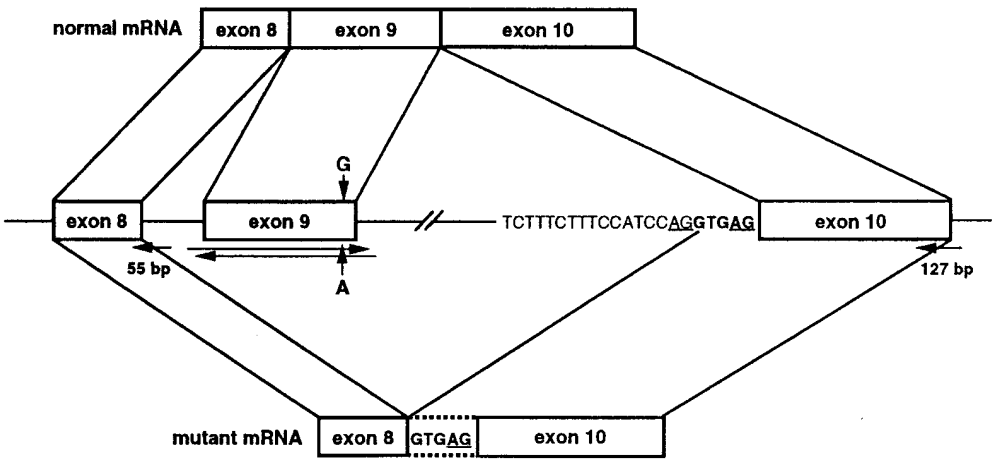


Figure 3. Model for the splicing abnormality in the major GPIIIa transcript. The wild-type G and mutant A at position 20624 are indicated with vertical arrows. Note the cryptic splice site and polypyrimidine tract which immediately precede the normal exon 10 splice acceptor site. Several features of the mutant transcript are illustrated: the skipping of exon 9 and the additional 5 bp between exons 8 and 10. Regions for which nucleotide sequence was determined are indicated by horizontal arrows, with the numbers of nucleotides sequenced indicated below.

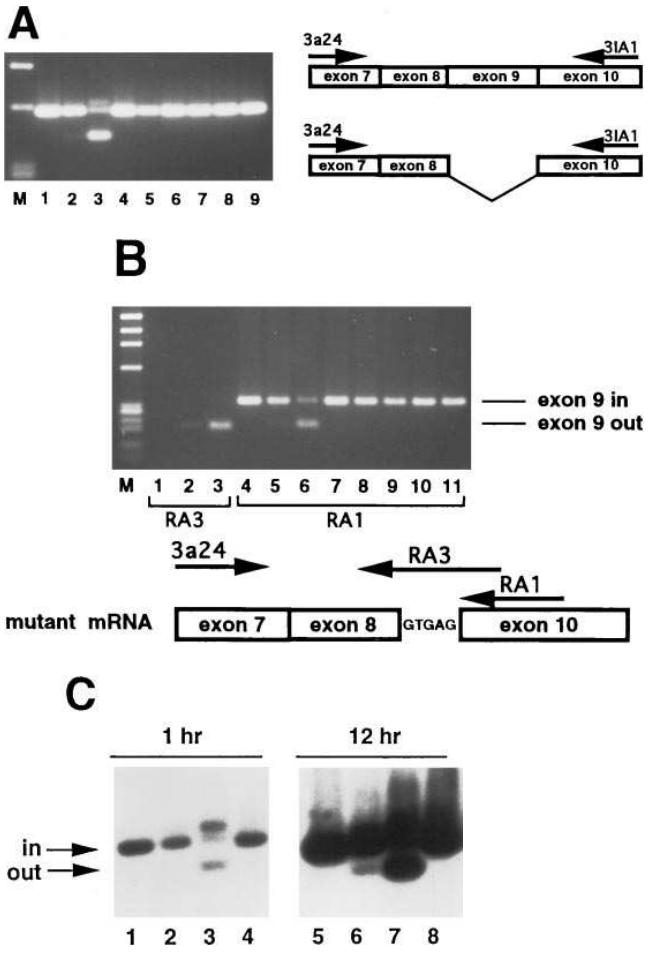


Figure 4. Analysis of GPIIIa exon 9 splicing. (*A*) Primers 3a24 and 3IA1 were used to amplify cDNA from normal (lanes *1*, *4–9*), maternal (lane *2*), and patient (lane *3*) platelets. Size markers (M) are ϕx174 DNA digested with HaeIII. Position of primers on each transcript is displayed to the right. The 5-bp insertion in the smaller transcript is not shown. (*B*) Primers 3a24 and RA3 were used to amplify cDNA from normal (lane *1*), maternal (lane *2*), and patient (lane *3*) platelets. Primer RA3 is specific for mRNA lacking exon 9 that has utilized the cryptic splice site. Different antisense primers RA3 and RA1 were used for products shown on this gel, and are indicated below. Primers 3a24 and RA1 were used to amplify cDNA from normal (lanes *4*, *7–11*), maternal (lane *5*), and patient (lane *6*) platelets. Primer RA1 will amplify mRNA independent of the presence of exon 9. Size markers (*M*) are ϕx174 DNA digested with HaeIII. Note that antisense primers RA1 and RA3 generate smaller products than seen in *A*. (*C*) Southern analysis of PCR products indicating relative abundance of GPIIIa transcripts with or without exon 9. Normal (lanes *1* and *4*), maternal (lane *2*), and patient (lane *3*) platelet RNA was reverse transcribed, PCR amplified with primers 3a24 and 3IA1, separated in 1.5% agarose, transferred to nylon membranes, and hybridized with ^{32}P-labeled primer 3aex8. The same filter is shown twice, with the length of exposure to film at −80°C indicated above each exposure. *In* refers to transcripts containing exon 9; *out* to transcripts lacking exon 9.

(Fig. 4 *B*, lane *1*), contain such transcripts. Shifting the antisense primer 10 bp in the 3′ direction, such that it is entirely complementary to exon 10 sequence, allows the amplification of the "exon in" species in normal individuals (Fig. 4 *B*, lanes *4*, *7–11*) and both the "exon in" and "exon out" species in the mother (Fig. 4 *B*, lane *5*) and patient (Fig. 4 *B*, lane *6*). We used Southern blot analysis to estimate the relative abundance of the two species of GPIIIa mRNA in the mother's platelets and to confirm the identity of the smaller cDNA fragment (Fig. 4 *C*). Prolonged exposures revealed the exon out form in the maternal amplified cDNA (Fig. 4 *C*, lane *6*) but not in normal controls (Fig. 4 *C*, lanes *5* and *8*). Within the limits of this RT-PCR amplification, we estimate that the exon 9 deleted form represents ∼ 3% of the maternal platelet GPIIIa transcripts. These comprehensive RNA analyses indicated that (*a*) the maternal and patient 442-bp amplified fragment (Fig. 2 *A* and Fig. 4 *A*) was indeed the result of exon 9 skipping, and (*b*) this abnormal splicing did not appear to occur in normal individuals. These data also suggest the G→A substitution was necessary for exon 9 skipping.

To prove whether the G to A transition is necessary and sufficient to dictate abnormal splicing, we analyzed the splicing of this portion of the GPIIIa gene transfected into K562 cells. Fig. 5 *A* schematically displays the minigene constructs used in these experiments. As shown in Fig. 5 *B*, the patient's allele and the mother's mutant allele cause exon 9 skipping (Fig. 5 *B*, lanes *2* and *4*), whereas the mother's wild-type allele splices normally (Fig. 5 *B*, lane *3*). Note that in the context of these artificial minigene constructs this abnormal splicing process is not maximally efficient and a substantial portion of the processed transcripts contain exon 9. The key finding, however, is that exon 9 is deleted only in the presence of the G to A substitution (Fig. 5, lanes *2* and *4*). To confirm the identity of the PCR products in lanes *2* and *3* we subcloned and sequenced these fragments (data not shown). The larger fragments in lanes *2* and *3* contained sequence for OAT and GPIIIa exon 8–10. The smaller fragment in lane *2* contained OAT and GPIIIa exon 8 and 10 sequence, with the previously identified 5 bp of intronic sequence between exons 8 and 10.

We next analyzed splicing of the patient and wild-type maternal alleles in which the −6 position from the splice donor site of intron 9 had been switched; i.e., to a wild-type G in the patient allele and a mutant A in the maternal allele. The mutation in patient exon 9 is necessary for exon skipping, since replacing it with the normal G corrects the splicing abnormality (Fig. 5 *B*, lane *7*). However, introducing the mutant A into the maternal wild-type allele was not sufficient to cause exon skipping (Fig. 5 *B*, lane *5*). Because the G to A substitution in exon 9 could not be solely responsible for the splicing defect, and because the mother was heterozygous for the C/A polymorphism at the 5′ end of exon 9 (nucleotide 20507), while the patient was homozygous A at this position, we hypothesized that the G to A mutation at the 3′ end of exon 9 (position 20624) would result in abnormal splicing only when allelic with the 5′ polymorphic A. This hypothesis is summarized in Fig. 5 *C*. To exclude the possible contribution to splicing of any other sequence variations in the maternal mutant allele, we introduced both sequence substitutions into the maternal wild-type allele. We observed that the 3′ A caused abnormal splicing only in the context of the 5′ A (compare Fig. 5 *B*, lane *6* with lane *5*).

We were puzzled by the fact that the patient was homozygous for the G to A substitution, since this appeared to be an exceedingly rare sequence variant and there was no history of consanguinity. One possibility consistent with this finding was haploinsufficiency due to a large gene deletion. The quantitative Southern blot data shown in Fig. 6 discount this explanation. Fig. 6 *A* indicates that equivalent amounts of normal, ma-

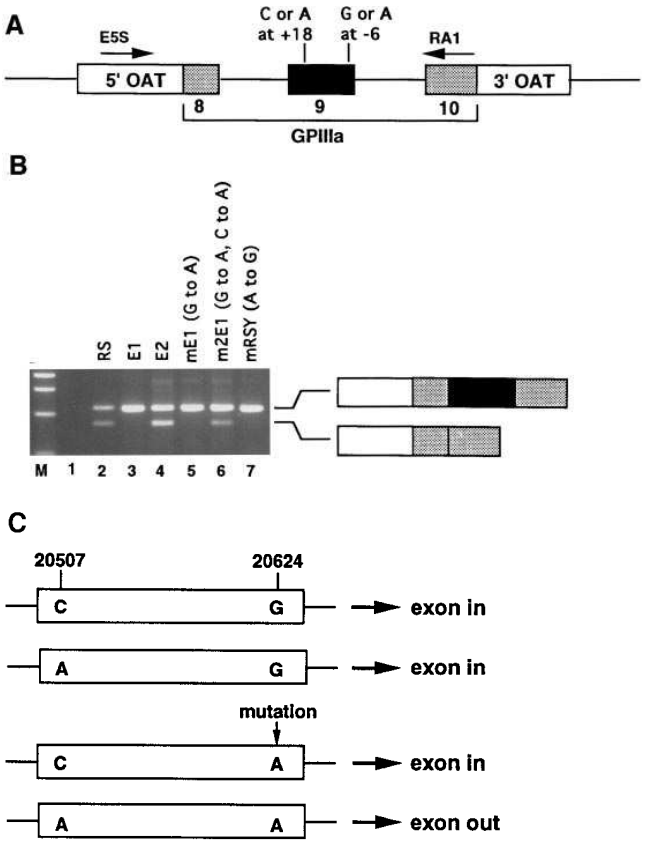


Figure 5. In vitro analysis of transcripts generated from the mutant allele. (*A*) 2 kb of contiguous GPIIIa gene from exons 8 to 10 from the patient and her mother were engineered in frame into the OAT cDNA in the pcDNAI expression plasmid. Plasmids were transfected into K562 cells and the resulting transcripts analyzed 16 h later by RT-PCR using primers E5S (5′-GAGACTGCCTGTAAACTAGC-3′) and RA1, specific for OAT (60) and GPIIIa, respectively. In some constructs, position −6 to the splice donor site was mutated to G or A; similarly, position +18 from the splice acceptor site was mutated from a C to an A. Note that exon 9 is 140 bp. (*B*) Templates for PCR reactions were: no template cDNA (lane *1*), cDNA from K562 cells transfected with constructs containing the patient's mutant allele (lane *2*, *RS*), the maternal wild-type allele (lane *3*, *E1*), the maternal mutant allele (lane *4*, *E2*), the maternal wild-type allele in which the G at position 20624 had been mutated to an A (lane *5*, *mE1*), the maternal wild-type allele in which the C at position 20507 had been mutated to an A and the G at position 20624 had been mutated to an A (lane *6*, *m2E1*), and the patient's mutant allele in which the A at position 20624 had been mutated to a G (lane *7*, *mRSY*). PCR products were amplified with E5S and RA1. Diagram to right of the gel demonstrates exon composition of transcripts. Size markers (*M*) are ϕx174 DNA digested with HaeIII. In controls not shown, cDNA from K562 cells that were not transfected with an OAT-GPIIIa minigene construct yielded no PCR products with primers E5S and RA1, but did amplify with control GPIIIa sense and antisense primers demonstrating the template cDNA was able to be amplified. (*C*) Hypothesis for exon 9 skipping in patient RS. The nucleotide positions of the 5′ polymorphism and the 3′ mutation are indicated above. As referenced in the text, the C at position 20507 creates a TaqI site; the G at 20624, an HphI site.

ternal, and patient genomic DNA were loaded (Fig. 6 *A*, lanes *1–6*). In Fig. 6 *B*, note the equivalent intensity of the ~3.3 and ~5.2 kb TaqI fragments between the control (Fig. 6 *B*, lane *1*) and the patient (Fig. 6 *B*, lane *3*), as well as all BamHI fragments (Fig. 6 *B*, lanes *4–6*), indicating that the patient has two copies of the GPIIIa gene. Essentially equivalent DNA loading of all lanes was confirmed by reprobing this filter with a cDNA probe from the fibrillin gene (Fig. 6 *B*, lanes *7–12*).

Because the patient was homozygous for a total of three GPIIIa polymorphisms, including the TaqI polymorphism at position 20507 of exon 9 (as shown in Fig. 6 *B*), we considered the possibility that she had two identical or nearly identical copies of chromosome 17. To address this issue, we examined five highly informative microsatellite polymorphisms distributed throughout chromosome 17. We found that the patient was homozygous for all five of these markers (Fig. 7 *A*). Note the normal control is heterozygous for D17S849, D17S250, and D17S801 and homozygous for D17S799, and D17S784. The mother is heterozygous for D17S799 and D17S784, and homozygous for D17S250, D17S801, and D17S849. Using the calculated population frequencies for the different marker alleles, there is a 1 in 2,000 chance that an individual would be homozygous for all five markers. The frequency of the GPIIIa polymorphisms are not known, but we have analyzed 40 unrelated individuals to determine the allelic frequencies at position 20507 (data not shown). The frequency of the homozygous "A" state is 0.39. By including this GPIIIa marker, there would be only a 1 in 5,000 chance that an individual would be homozygous for these 6 chromosome 17 polymorphisms, strongly suggesting two copies of the same chromosome 17 in this patient. This idea becomes virtually certain if one considers that none of the 206 normal chromosomes studied had the A at position 20624.

Two identical copies of a chromosome may result when both copies were inherited from one parent (uniparental isodisomy), or as a product of a consanguinous union. To address these possibilities, we analyzed chromosomes 3, 4, and 7 to determine if the patient had the expected frequency of heterozygosity elsewhere in the genome. We studied two polymorphic markers on each chromosome and found that similar to the control, the patient was heterozygous for four of the six markers (Fig. 7 *B*). Because paternal DNA was not available for study, we cannot eliminate the possibility of consanguinity with 100% certainty. However, the studies on chromosomes 3, 4, and 7 are inconsistent with a consanguineous inheritance.

Discussion

We have performed a detailed molecular genetic analysis of the GPIIIa gene in a patient with GT and identified a novel mechanism for producing the thrombasthenic phenotype as well as a novel cause of exon skipping. The most significant findings from our studies are (*a*) that two sequence variations within a single exonic which do not alter consensus splice sequences and which do not generate nonsense or missense mutations, can produce an RNA splicing abnormality. The profound effect on splicing of such nucleotide changes supports a hypothesis whereby exon secondary structure effects mRNA splicing; (*b*) we believe this to be the first example of chromosome 17 disomy in any human disease; (*c*) Assuming this patient inherited two copies of maternal chromosome 17, the lack of any additional clinical phenotype beyond that of typical GT has implications regarding the possibility of imprinted genes on chromosome 17.

The spectrum of mutations identified to date in type I thrombasthenia includes nonsense mutations, frameshift mu-

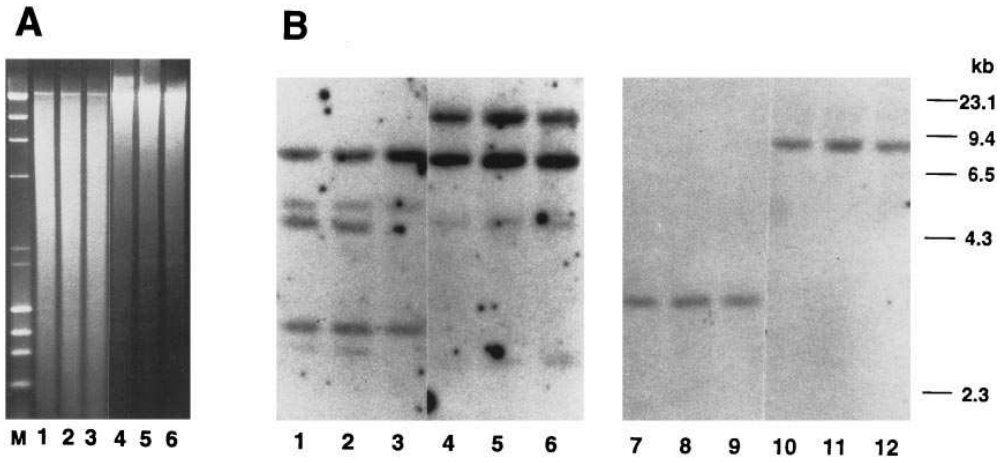
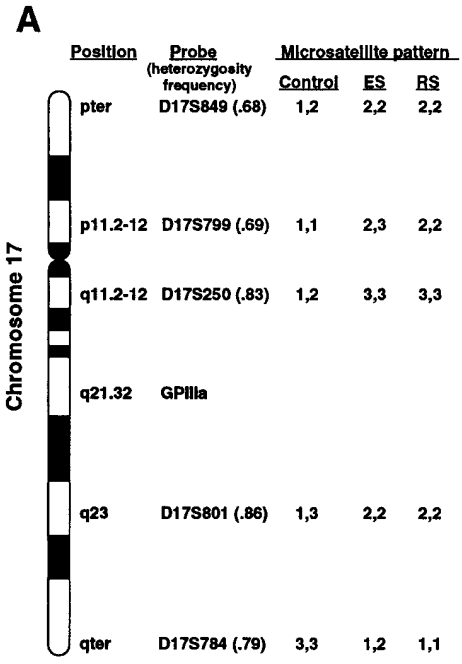


Figure 6. Southern blot demonstrating patient RS has two GPIIIa genes. (*A*) Ethidium stained 0.8% agarose gel containing 7.5 μg of genomic DNA digested with TaqI (lanes *1–3*) or BamHI (lanes *4–6*) from a normal control (lanes *1* and *4*), the patient's mother (lanes *2* and *5*), and the patient (lanes *3* and *6*). Size markers (*M*) are λDNA digested with HindIII and ϕx174 DNA digested with HaeIII. (*B*) The gel in *A* was transferred to a nylon membrane and hybridized with a ^{32}P-labeled 2.0 kb GPIIIa cDNA (lanes *1–6*) or 345 bp fibrillin cDNA probe (lanes *7–12*). Note the patient is homozygous for the uncommon form of the known TaqI polymorphism. This results in the absence of the ~ 4.6- and ~ 2.9-kb fragments, and the double intensity of the ~ 7.5-kb fragment. PhosphorImager analysis of the fibrillin hybridization indicated that relative to the normal control (lanes *7* and *10*), an essentially equivalent amount (±8%) of RS genomic DNA was loaded (lanes *9* and *12*).

tations, and mutations that interfere with pre-mRNA splicing. The unifying factor appears to be a deficiency of functional protein, either due to unstable transcripts or the production of unstable or truncated and dysfunctional protein products. Iwamoto et al. described a patient with a nonsense mutation in exon 28 of the GPIIb gene which resulted in exon 28 skipping (33). One of the mutant alleles described by Kato et al. produced a GPIIb transcript lacking exon 26 secondary to a C→G mutation at the −3 position of the exon 26 splice acceptor site (30). And we have described exon 5 skipping in the GPIIIa gene due to a G to A mutation at position +1 of the splice donor site (31).

The mutation described in this report results in the skipping of exon 9 of the GPIIIa gene. This mutation is unusual in several respects. First, the G→A substitution is 6 bp upstream of the exon 9 splice donor site, a position that shows no apparent sequence preference nor conservation in mammalian genes (61). None of the 24 single base mutations affecting exon splicing identified by Chen and Chasin were located in the −6 position from the splice donor site (44). To consider alternate mutations as the cause for exon 9 skipping, we sequenced the patient's entire exon 9 and flanking regions known to affect splice site selection, but the only abnormality was the G→A substitution. Because none of 206 chromosomes from 103 normal individuals contained this nucleotide change, it does not satisfy the definition of a polymorphism.

The G→A mutation was necessary but not sufficient to effect exon 9 skipping in our patient's GPIIIa gene, and required the presence of an additional distal allelic variation. The patient allele is polymorphic for a known substitution at the 5′



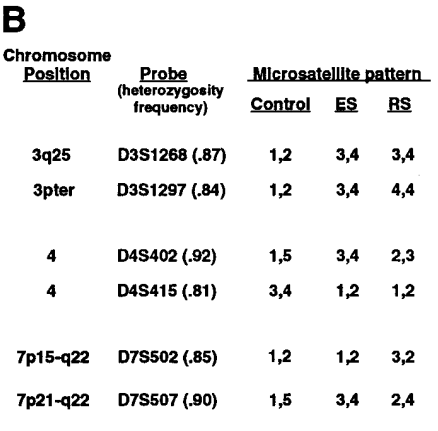


Figure 7. Microsatellite analysis of chromosome 17 (*A*) and chromosomes 3, 4, and 7 (*B*). Each "probe" is actually a set of previously described PCR primers which flank a defined dinucleotide repeat (53–55). Formal sizing of polymorphic fragments was not performed, but the largest was arbitrarily assigned a 1, the next largest, a 2, and so on. Genomic DNA from a normal control, the mother (*ES*), and the patient (*RS*) was analyzed. Data used in preparing this figure were derived from the GDB(TM) Human Genome Data Base at Johns Hopkins University in Baltimore on December 14, 1994 at 11:00 a.m. and May 10, 1995 at 3:30 p.m. eastern time.

end (position 20507) of exon 9 and at several positions in the flanking introns (data not shown). Rather than characterize the effect of each nucleotide change, we introduced the two exon 9 sequence alterations of the mutant allele into the context of the maternal wild-type allele, and showed that the presence of both was required for abnormal splicing. Such cooperativity between widely spaced sequence variants suggested that a perturbation of pre-mRNA secondary structure might be central to the exon skipping phenotype. We used the computer program RNAFOLD to predict the most energy-efficient RNA secondary structure for the four possible combinations of sequence variants in exon 9 (data not shown). An identical structure was predicted when either a C or an A was present at position 20507 (the 5′ polymorphism), indicating that not all nucleotide changes alter structure as predicted by the input parameters of our model. However, the mutant A at position 20624 produced a dramatic alteration in the predicted RNA secondary structure. Interestingly, with the A at position 20624, different structures were obtained for a C or an A at the 5′ end of the exon, with the latter variant predicting a long intraexonic hairpin loop. We are unaware of any naturally occurring alternative exon splicing which results from cooperation between widely spaced sequence variations at the 5′ and 3′ regions of a single exon and which do not involve the consensus splice sequences. It is possible that the A variant at position 20624 alters the GPIIIa pre-mRNA conformation, but only in combination with the A variant at position 20507 does the conformation preclude efficient splicing of exon 9, perhaps by interfering with exon scanning or by reducing the affinity of small nuclear ribonuclear proteins for their *cis*-acting elements. We realize that such modeling may not accurately reflect the situation in vivo, but at least it is consistent with the data shown in Fig. 5. Such effects of secondary exon structure on splicing have been postulated by others (62, 63). Matsuo et al. observed exon skipping despite normal consensus splice sequences when the exon contained a 52-bp deletion, and showed a significant alteration in the predicted secondary structure of the pre-mRNA (64). Carothers et al. found that intraexonic sequence changes could counter the effects of allelic mutations within the splice site consensus (65). Such revertants restored normal splicing. Interestingly, the position of these revertant mutations clustered between 5 and 8 bp upstream of the splice donor site.

We are also intrigued by the utilization of the cryptic splice site (AGGT) 5 bp upstream of the exon 10 splice acceptor site in all transcripts containing the exon 9 deletion. Scrutiny of the intron upstream of GPIIIa exon 10 (Fig. 3) reveals that the cryptic splice site has a preceding polypyrimidine tract that better conforms to the mammalian consensus (61) than does the constitutively utilized splice site. Of note, use of the normal splice acceptor site violates the accepted rule in which the first AG downstream of the branch point is utilized (66, 67). We were unable to detect any transcripts from normal individuals lacking exon 9 or which contained exon 9 and used the cryptic splice site. We have previously presented data indicating that maintenance of an ORF can act as an additional level of scrutiny during splice site selection, based upon the skipping of exons containing nonsense mutations (52) and the restoration of the normal splicing phenotype by revertant mutations that restore the ORF (68). In the majority of these cases, simple skipping of the exon was sufficient to restore the ORF. In the mutant GPIIIa allele characterized in the current study, the majority of detectable GPIIIa transcripts have maintained an ORF after exon 9 skipping by a more complex mechanism: that of utilizing the cryptic splice acceptor 5 bp upstream of exon 10. While far reaching consequences of secondary structure changes in exon 9 could be invoked to account for the simultaneous occurrence of exon skipping and cryptic splice site utilization, in light of our prior work, it is tempting to speculate that the selection of an alternative splice site acceptor for intron 10 is a consequence of the premature termination codon that would result upon exon 9 skipping if the normal acceptor were utilized.

Because the mutant GPIIIa transcript maintained an open reading frame, it would be predicted to translate into protein. GPIIIa stability (and plasma membrane insertion) requires assembly with GPIIb (69). However, no GPIIIa was detected by Western blot analysis. Presumably, the loss of 45 and/or the addition of two amino acids from exon 9 skipping or the 5-bp insertion, respectively, results in a GPIIIa molecule unable to pair with GPIIb. Exon 9 contains residues 350–395 of mature GPIIIa, including an NH$_2$-linked glycosylation site and 2 cysteine residues which are disulfide linked to one another (70); the 5-bp insertion would produce a Val-Ser insertion. Although expression studies are beyond the scope of this work, we speculate that residues GPIIIa 350–395 are important for GPIIb-IIIa assembly and/or stability. This would be consistent with data of Calvete et al. who analyzed proteolytic fragments of the isolated receptor and identified GPIIIa residues 324–366 as one of three interfaces between GPIIb and GPIIIa (71).

Unfortunately, the patient's father is not available for study so we cannot definitively eliminate the possibility of consanguinity in this case. Nevertheless, there is strong evidence that the patient has two identical copies of chromosome 17: (*a*) there are two copies of the GPIIIa gene (Fig. 6); (*b*) based on calculated and determined allelic frequencies, we estimate that there is only a 1 in 5,000 chance that an individual would be homozygous for the six polymorphisms we have studied. These chances become extraordinarily small when considering the patient is also homozygous for the G→A mutation. Many lines of evidence suggest that the chromosome 17 disomy in this patient is uniparental and not the result of consanguinity. First, repeated questioning of the patient's mother consistently revealed no history of consanguinity. Second, the patient was homozygous at all six chromosome 17 markers, but at just one of six markers on chromosomes 3, 4, and 7. There is no reason why, in consanguinity, one chromosome would be selectively affected. Third, crossovers during the first meiotic division make it quite unlikely that related individuals would have identical copies of chromosome 17. Even the two consanguinous unions most likely to cause the inheritance of two copies of the same chromosome 17 (father–daughter or brother–sister) would require an extremely rare series of meioses in which either double crossovers in the intervals of markers we have assayed and/or nonrecombination took place. Taken together, and in the context of the biology of meiotic crossovers, our haplotype studies are not compatible with consanguinity and are most consistent with uniparental isodisomy.

We are unaware of other human diseases caused by disomy of chromosome 17. While a patient with Silver-Russell syndrome due to a paternally inherited balanced translocation [t(17;20)(q25;q13)] has been reported (72), this involved only a portion of chromosome 17. We believe that our patient lacks a paternal chromosome 17, raising issues regarding imprinted

genes on this chromosome. Genomic, or gametic, imprinting refers to the phenomenon in which a gamete-specific modification in the parental generation can sometimes lead to functional differences between maternal and paternal genomes in diploid cells of the offspring (73). Genes become altered in some as yet undefined fashion during gametogenesis such that they are either expressed or not expressed during embryogenesis and in adult life. For any given imprinted gene, it is predictable that only the allele inherited from a parent of a specific gender will be expressed. Human genetic disorders and cancers have been described in which the phenotype results from a mutation in only the expressed parental allele (74, 75). Only a handful of imprinted genes have been described; none are on human chromosome 17 (74). Assuming maternal isodisomy in patient RS, the fact that her phenotype is restricted to Glanzmann thrombasthenia implies chromosome 17 must not harbor genes expressed exclusively from the paternal allele whose loss leads to significant disease. Likewise, chromosome 17 must not harbor genes expressed exclusively from the maternal allele that lead to significant disease when expressed from two copies.

In summary, we have described a patient with Glanzmann thrombasthenia and an abnormally spliced GPIIIa mRNA due to the concerted effect of allelic sequence variants. Because the nucleotide substitutions neither created premature termination codons nor altered known splice site sequences, our data support the hypothesis that pre-mRNA secondary structure influences splicing. The choice of a cryptic splice site is consistent with cellular mechanisms favoring transcripts containing an ORF. Finally, we have presented evidence that the thrombasthenic phenotype in this patient may result from uniparental isodisomy for a maternal chromosome containing a mutant GPIIIa gene, a finding which carries certain implications regarding imprinted genes on chromosome 17. Such observations highlight the potential secondary gain that can be afforded by the comprehensive analysis of mutant alleles associated with human disease.

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References

1. George, J.N., J.P. Caen, and A.T. Nurden. 1990. Glanzmann's thrombasthenia: the spectrum of clinical disease. *Blood.* 75:1383–1395.

2. George, J.N., and A.T. Nurden. 1994. Inherited disorders of the platelet membrane: Glanzmann thrombasthenia, Bernard-Soulier syndrome and other disorders. *In* Hemostasis and Thrombosis. Basic Principles and Clinical Practice. R.W. Coleman, J. Hirsh, V.J. Marder, and E.W. Salzman, editors. J.B. Lippincott Co., Philadelphia. 652–672.

3. Glanzmann, E. 1918. Hereditare hamorrhagische thrombasthenie: ein beitrag zur pathologie der blut plattchen. *J. Kinderkr.* 88:1–13.

4. Nurden, A.T., and J.P. Caen. 1974. An abnormal platelet glycoprotein pattern in three cases of Glanzmann's thrombasthenia. *Br. J. Haematol.* 28:253–260.

5. Marguerie, G.A., E.F. Plow, and T.S. Edgington. 1979. Human platelets possess an inducible and saturable receptor specific for fibrinogen. *J. Biol. Chem.* 254:5357–5363.

6. Bennett, J.S., and G. Vilaire. 1979. Exposure of platelet fibrinogen receptors by ADP and Epinephrine. *J. Clin. Invest.* 64:1393–1401.

7. McEver, R.P., N.L. Baenziger, and P.W. Majerus. 1980. Isolation and quantitation of the platelet membrane glycoprotein deficient in thrombasthenia using a monoclonal hybridoma antibody. *J. Clin. Invest.* 66:1311–1318.

8. Nachman, R.L., L.K. Lawrence, and L. Leung. 1982. Complex formation of platelet membrane glycoproteins IIb and IIIa with fibrinogen. *J. Clin. Invest.* 69:263–269.

9. Gogstad, G.O., F. Brosstad, M.-B. Krutnes, I. Hagen, and N.O. Solum. 1982. Fibrinogen-binding properties of the human platelet glycoprotein IIb-IIIa complex: a study using crossed-radioimmunoelectrophoresis. *Blood.* 60:663–671.

10. Bennett, J.S., G. Vilaire, and D.B. Cines. 1982. Identification of the fibrinogen receptor on human platelets by photoaffinity labeling. *J. Biol. Chem.* 257:8049–8054.

11. Coller, B.S., E.I. Peerschke, L.E. Scudder, and C.A. Sullivan. 1983. A murine monoclonal antibody that completely blocks the binding of fibrinogen to platelets produces a thrombasthenic-like state in normal platelets and binds to glycoproteins IIb and/or IIIa. *J. Clin. Invest.* 72:325–338.

12. Bennett, J.S., J.A. Hoxie, S.F. Leitman, G. Vilaire, and D. Cines. 1983. Inhibition of fibrinogen binding to stimulated human platelets by a monoclonal antibody. *Proc. Natl. Acad. Sci. USA.* 80:2417–2421.

13. Poncz, M., R. Eisman, R. Heidenreich, S.M. Silver, G. Vilaire, S. Surrey, E. Schwartz, and J.S. Bennett. 1987. Structure of the platelet membrane glycoprotein IIb. *J. Biol. Chem.* 262:8476–8482.

14. Bray, P.F., J.-P. Rosa, G.I. Johnston, D.T. Shiu, R.G. Cook, C. Lau, Y.W. Kan, R.P. McEver, and M.A. Shuman. 1987. Platelet glycoprotein IIb: chromosomal location and tissue expression. *J. Clin. Invest.* 80:1812–1817.

15. Fitzgerald, L.A., B. Steiner, S.C. Rall, Jr., S.-S. Lo, and D.R. Phillips. 1987. Protein sequence of endothelial glycoprotein IIIa derived from a cDNA clone. *J. Biol. Chem.* 262:3936–3949.

16. Rosa, J.-P., P.F. Bray, O. Gayet, G.I. Johnston, R.G. Cook, K.W. Jackson, M.A. Shuman, and R.P. McEver. 1988. Cloning of glycoprotein IIIa cDNA from human erythroleukemia cells and localization of the gene to chromosome 17. *Blood.* 72:593–600.

17. Heidenreich, R., R. Eisman, S. Surrey, K. Delgrosso, J.S. Bennett, E. Schwartz, and M. Poncz. 1990. Organization of the gene for platelet glycoprotein IIb. *Biochemistry.* 29:1232–1244.

18. Zimrin, A.B., S. Gidwitz, A. Lord, E. Schwartz, J.S. Bennett, G.C. White III, and M. Poncz. 1990. The genomic organization of platelet glycoprotein IIIa. *J. Biol. Chem.* 265:8590–8595.

19. Villa-Garcia, M., L. Li, G. Riely, and P.F. Bray. 1994. Isolation and characterization of a TATA-less promoter for the human β_3 integrin gene. *Blood.* 83:668–676.

20. Bray, P.F., and M.A. Shuman. 1990. Identification of an abnormal gene for the GPIIIa subunit of the platelet fibrinogen receptor resulting in Glanzmann thrombasthenia. *Blood.* 75:881–888.

21. Bray, P.F. 1994. Inherited disease of platelet glycoproteins: considerations for rapid molecular characterization. *Thromb. Haemostasis.* 72:492–502.

22. Plow, E.F., and M.H. Ginsberg. 1989. Cellular adhesion: GPIIb-IIIa as a prototypic adhesion receptor. *Prog. Hemostasis Thromb.* 9:117–156.

23. Hynes, R.O. 1992. Integrins: modulation, and signaling in cell adhesion. *Cell.* 69:11–25.

24. Loftus, J.C., T.E. O'Toole, E.F. Plow, A. Glass, A.L. Frelinger, and M.H. Ginsberg. 1990. A β_3 integrin mutation abolishes ligand binding and alters divalent cation-dependent conformation. *Science (Wash. DC).* 249:915–918.

25. Bajt, M.L., M.H. Ginsberg, A.L. Frelinger III, M.C. Berndt, and J.C. Loftus. 1992. A spontaneous mutation of integrin $\alpha_{IIb}\beta_3$ (platelet glycoprotein IIb-IIIa) helps define a ligand binding site. *J. Biol. Chem.* 267:3789–3794.

26. Lanza, F., A. Stierl, D. Fournier, M. Morales, G. André, A.T. Nurden, and J.-P. Cazenave. 1992. A new variant of Glanzmann's thrombasthenia (Strasbourg I). Platelets with functionally defective glycoprotein IIb-IIIa complexes and a glycoprotein III $^{214}Arg \rightarrow ^{214}Trp$ mutation. *J. Clin. Invest.* 89:1995–2004.

27. Chen, Y.-P., I. Djaffar, I. Pidard, B. Steiner, A.-M. Cieutat, J.P. Caen, and J.-P. Rosa. 1992. $Ser^{752} \rightarrow Pro$ mutation in the cytoplasmic domain of integrin β_3 subunit and defective activation of platelet integrin $\alpha_{IIb}\beta_3$ (glycoprotein IIb-IIIa) in a variant of Glanzmann thrombasthenia. *Proc. Natl. Acad. Sci. USA.* 89:10169–10173.

28. Djaffar, I., and J.-P. Rosa. 1993. A second case of variant Glanzmann's thrombasthenia due to substitution of platelet GPIIIa (integrin β3) Arg214 by Trp. *Hum. Mol. Genet.* 2:2179–2180.

29. Chen, Y.-P., T.E. O'Toole, J. Ylanne, J.-P. Rosa, and M.H. Ginsberg. 1994. A point mutation in the integrin β_3 cytoplasmic domain ($S^{752} \rightarrow P$) impairs bidirectional signaling through $\alpha_{IIb}\beta_3$ (platelet glycoprotein IIb-IIIa). *Blood.* 84: 1857–1865.

30. Kato, A., K. Yamamoto, S. Miyazaki, S.M. Jung, M. Mori, and N. Aoki. 1992. Molecular basis for Glanzmann's thrombasthenia (GT) in a compound heterozygote with the GPIIb gene: a proposal for the classification of GT based

on the biosynthetic pathway of glycoprotein IIb-IIIa complex. *Blood.* 79:3212–3218.

31. Jin, Y., H. Dietz, A.T. Nurden, and P.F. Bray. 1993. SSCP is a rapid and effective method for the identification of mutations and polymorphisms in the gene for GPIIIa. *Blood.* 82:2281–2288.

32. Simsek, S., H. Heyboer, L.G. de Bruihne, R. Goldschmeding, H.T.M. Cuijpers, and A.E.G., von dem Borne. 1993. Glanzmann's thrombasthenia caused by homozygosity for a splice defect that leads to deletion of the first coding exon of the glycoprotein IIIa mRNA. *Blood.* 81:2044–2049.

33. Iwamoto, S., E. Nishiumi, E. Kajii, and S. Ikemoto. 1994. An exon 28 mutation resulting in alternative splicing of the glycoprotein IIb transcript and Glanzmann's thrombasthenia. *Blood.* 83:1017–1023.

34. Poncz, M., S. Rifat, B.S. Coller, P.J. Newman, S.J. Shattil, T. Parrella, P. Fortina, and J.S. Bennett. 1994. Glanzmann thrombasthenia secondary to a Gly273→Asp mutation adjacent to the first calcium-binding domain of platelet glycoprotein IIb. *J. Clin. Invest.* 93:172–179.

35. Wilcox, D.A., J.L. Wautier, D. Pidard, and P.J. Newman. 1994. A single amino acid substitution flanking the fourth calcium binding domain of α_{IIb} prevents maturation of the $\alpha_{IIb}\beta_3$ integrin complex. *J. Biol. Chem.* 269:4450–4457.

36. Chen, F., B.S. Coller, and D.L. French. 1993. Homozygous mutation of platelet glycoprotein IIIa (β_3) Cys^{374}→Tyr in a Chinese patient with Glanzmann thrombasthenia. *Blood.* 82:163*a* (Abstr.).

37. Wilcox, D.A., C.M. Paddock, J. Gill, and P.J. Newman. 1995. Glanzmann thrombasthenia resulting from a single amino acid substitution between the second and third calcium-binding domains of GPIIb. Role of the GPIIb amino terminus in integrin subunit association. *J. Clin. Invest.* 95:1553–1560.

38. Schlegel, N., O. Gayet, M.C. Morel-Kopp, B. Wyler, M.F. Hurtaud-Roux, C. Kaplan, and J. McGregor. 1995. The molecular genetic basis of Glanzmann's thrombasthenia in a gypsy population in France: identification of a new mutation on the α_{IIb} gene. *Blood.* 86:977–982.

39. Tomiyama, Y., H. Kashiwagi, S. Kosugi, M. Shiraga, Y. Kanayama, Y. Kurata, and Y. Matsuzawa. 1995. Abnormal processing of the glycoprotein IIb transcript due to a nonsense mutation in exon 17 associated with Glanzmann's thrombasthenia. *Thromb. Haemostasis.* 73:756–762.

40. Peretz, H., N. Rosenberg, S. Usher, E. Graff, P.J. Newman, B.S. Coller, and U. Seligsohn. 1995. Glanzmann's thrombasthenia associated with deletion-insertion and alternative splicing in the glycoprotein IIb gene. *Blood.* 85:414–420.

41. Sharp, P.A. 1987. Splicing of messenger RNA precursors. *Science (Wash. DC).* 235:766–771.

42. Wu, J.Y., and T. Maniatis. 1993. Specific interactions between proteins implicated in splice site selection and regulated alternative splicing. *Cell.* 75: 1061–1070.

43. Talerico, M., and S.M. Berget. 1990. Effect of 5′ splice site mutations on splicing of the preceding intron. *Mol. Cell. Biol.* 10:6299–6305.

44. Chen, I.-T, and L.A. Chasin. 1993. Direct selection for mutations affecting specific splice sites in a hamster dihydrofolate reductase minigene. *Mol. Cell. Biol.* 13:289–300.

45. Nakai, K., and H. Sakamoto. 1994. Construction of a novel database containing aberrant splicing mutations of mammalian genes. *Gene.* 141:171–177.

46. Jin, Y., H.C. Dietz, and P.F. Bray. 1993. A mutation in the GPIIIa gene results in an mRNA splicing abnormality and suggests maintenance of reading frame is a deliberate process. *Blood.* 82:210*a* (Abstr.).

47. Faraday, N., P. Goldschmidt-Clermont, K. Dise, and P.F. Bray. 1994. Quantitation of soluble fibrinogen binding to platelets by fluorescence activated flow cytometry. *J. Lab. Clin.* 123:728–740.

48. Coller, B.S., D.A. Cheresh, E. Asch, and E. Seligsohn. 1991. Platelet vitronectin receptor expression differentiates Iraqi–Jewish from Arab patients with Glanzmann thrombasthenia in Israel. *Blood.* 77:75–83.

49. Djaffar, I., D. Vilette, P.F. Bray, and J.-P. Rosa. 1991. Quantitative isolation of RNA from human platelets. *Thromb. Res.* 62:127–135.

50. Bray, P.F., G. Barsh, J.P. Rosa, X.Y. Luo, E. Magenis, and M.A. Shuman. 1988. Physical linkage of the genes for platelet membrane glycoprotein IIb and IIIa. *Proc. Natl. Acad. Sci. USA.* 85:8683–8687.

51. Eldadah, Z.A., T. Brenn, H. Furthmayr, and H.C. Dietz. 1995. Expression of a mutant human fibrillin allele upon a normal human or murine genetic background recapitulates a Marfan cellular phenotype. *J. Clin. Invest.* 95:874–880.

52. Dietz, H.C., and R.J. Kendizior, Jr. 1994. Maintenance of an open reading frame as an additional level of scrutiny during splice site selection. *Nature Genet.* 8:183–188.

53. Weber, J.L., A.E. Kwitek, P.E. May, M.R. Wallace, F.S. Collins, and D.H. Ledbetter. 1990. Dinucleotide repeat polymorphisms at the D17S250 and D17S261 loci. *Nucleic Acids Res.* 18:4640*a* (Abstr.).

54. Weissenbach, J., G. Gyapay, C. Dib, A. Vignal, J. Morissette, P. Millasseau, G. Vaysseix, and M. Lathrop. 1992. A second-generation linkage map of the human genome. *Nature (Lond.).* 359:794–801.

55. Gyapay, G., J. Morissette, A. Vignal, C. Dib, C. Fizames, P. Millasseau, S. Marc, G. Bernardi, M. Lathrop, and J. Weissenbach. 1994. The 1993-94 Généthon human genetic linkage map. *Nature Genet.* 7:246–339.

56. Fasman, K.H., A.J. Cuticchia, and D.T. Kinsbury. 1994. The GDB(TM) human genome data base anno 1994. *Nucleic Acids Res.* 22:3462–3469.

57. Cuticchia, A.J., K.H. Fasman, D.T. Kinsbury, R.J. Robbins, and P.L. Pearson. 1993. The GDB(TM) human genome data base anno 1993. *Nucleic Acids Res.* 21:3003–3006.

58. Zucker, M., and P. Stiegler. 1981. Optimal computer folding of large RNA sequences using thermodynamics and auxiliary information. *Nucleic Acids Res.* 9:133–148.

59. Lapalme, G., R.J. Cedergren, and D. Sankoff. 1982. An algorithm for the display of nucleic acid secondary structure. *Nucleic Acids Res.* 10:8351–8356.

60. Mitchell, G.A., J.E. Looney, L.C. Brody, G. Steel, M. Suchanek, J.F. Engelhardt, H.F. Willard, and D. Valle. 1988. Human ornithine-δ-aminotransferase. cDNA cloning and analysis of the structural gene. *J. Biol. Chem.* 263: 14288–14295.

61. Padgett, R.A., P.J. Grabowski, M.M. Konarska, S. Seiler, and P.A. Sharp. 1986. Splicing of messenger RNA precursors. *Annu. Rev. Biochem.* 55: 1119–1150.

62. Steingrimsdottir, H., G. Rowley, G. Dorado, J. Cole, and A.R. Lehmann. 1992. Mutations which alter splicing in the human hypoxanthine guanine phosphoribosyltransferase gene. *Nucleic Acids Res.* 20:1201–1208.

63. Menichini, P., A. Inga, G. Fronza, R. Iannone, P. Degan, P. Campomenosi, and A. Abbondandolo. 1994. Defective splicing induced by 4NQO in the hamster *hprt* gene. *Mutat. Res.* 323:159–165.

64. Matsuo, M., H. Nishio, Y. Kitoh, U. Francke, and H. Nakamura. 1992. Partial deletion of a dystrophin gene leads to exon skipping and to loss of an intra-exon hairpin structure from the predicted mRNA precursor. *Biochem. Biophys. Res. Commun.* 182:495–500.

65. Carothers, A.M., G. Urlaub, D. Grunberger, and L.A. Chasin. 1993. Splicing mutants and their second-site suppressors at the dihydrofolate reductase locus in Chinese hamster ovary cells. *Mol. Cell. Biol.* 13:5085–5098.

66. Smith, C.W., E.B. Porro, J.G. Patton, and B. Nadal-Ginard. 1989. Scanning from an independently specified branch point defines the 3′ splice site of mammalian introns. *Nature (Lond.).* 342:243–247.

67. Smith, C.W., T.T. Chu, and B. Nadal-Ginard. 1993. Scanning and competition between AGs are involved in 3′ splice site selection in mammalian introns. *Mol. Cell. Biol.* 13:4939–4952.

68. Dietz, H.C., D. Valle, C.A. Francomano, R.J. Kendzior, R.E. Pyeritz, and G.R. Cutting. 1993. The skipping of constitutive exons in vivo induced by nonsense mutations. *Science (Wash. DC).* 259:680–683.

69. O'Toole, T.E., J.C. Loftus, E.F. Plow, A.A. Glass, J.R. Harper, and M.H. Ginsberg. 1989. Efficient surface expression of platelet GPIIb-IIIa requires both subunits. *Blood.* 74:14–18.

70. Calvete, J.J., A. Henschen, and J. González-Rodríguez. 1991. Assignment of disulphide bonds in human platelet GPIIIa. A disulphide pattern of the β-subunits of the integrin family. *Biochem. J.* 274:63–71.

71. Calvete, J.J., K. Mann, M.V. Alvarez, M.M. López, and J. González-Rodríguez. 1992. Proteolytic dissection of the isolated platelet fibrinogen receptor, integrin GPIIb/IIIa: localization of GPIIb and GPIIIa sequences putatively involved in the subunit interface and in intrasubunit and intrachain contacts. *Biochem. J.* 282:523–532.

72. Ramirez-Duenas, M.L., C. Medina, R. Ocampo-Campos, and H. Rivera. 1992. Severe Silver-Russell syndrome and translocation. *Clin. Genet.* 41:51–53.

73. Barlow, D.P. Imprinting: a gamete's point of view. 1994. *Trends Genet.* 10:194–199.

74. Nicholls, R.D. 1994. New insights reveal complex mechanisms involved in genomic imprinting. *Am. J. Hum. Genet.* 54:733–740.

75. Rainier, S., L.A. Johnson, C.J. Dobry, A.J. Ping, P.E. Grundy, and A.P. Feinberg. 1993. Relaxation of imprinted genes in human cancer. *Nature (Lond.).* 362:747-749.