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Two types of abnormal genes for plasminogen in families with a predisposition for thrombosis

(fibrinolysis/thrombolysis/molecular abnormality/point mutation/genetic diagnosis)

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ABSTRACT **The gene coding for plasminogen has been compared with several abnormal genes from Japanese patients by the polymerase chain reaction and DNA sequence analysis. Two types of abnormal genes coding for plasminogen were identified in these patients. In the type I mutation, a guanosine in GCT coding for Ala-601 near the active-site histidine was replaced by an adenosine resulting in ACT coding for threonine. This mutation was also shown by the loss of a cleavage site for *Fnu*4HI endonuclease, a restriction enzyme that recognizes GCTGC but not ACTGC. In the type II mutation, a guanosine in GTC coding for Val-355 was replaced by a thymidine resulting in TTC coding for phenylalanine. This change was readily shown by digestion with *Ava* II endonuclease, a restriction enzyme that recognizes GGTCC and not GTTCC. The type I mutation has been found to be identical to a plasminogen variant identified in Japanese patients by amino acid sequence analysis and also detected by isoelectric focusing, whereas the type II mutation is a unique amino acid substitution in the connecting region between the third and fourth kringles in plasminogen. DNA sequence analysis also revealed that the abnormal genes carry several silent nucleotide substitutions located primarily within introns and 5′ and 3′ flanking regions.**

Plasminogen circulates in blood as a single-chain glycoprotein that is converted by activators, such as tissue plasminogen activator or urokinase, to an active two-chain form called plasmin. Plasmin then digests the insoluble fibrin clot that has been generated at a site of vascular injury. In addition, plasminogen also plays an important role in other biological reactions, such as inflammation, tissue development, tissue remodeling, and the processing of other molecules. The primary structure of human plasminogen has been established by both amino acid (1–3) and DNA (4–6) sequence analyses. The molecule consists of 791 amino acid residues and includes five homologous kringle structures and a serine protease domain.

In patients with hereditary thrombophilia, deficiencies in plasminogen as well as protein C, protein S, antithrombin III, heparin cofactor II, and tissue plasminogen activator have been identified as the cause of or in part related to a predisposition for thrombosis (for reviews, see refs. 7 and 8). In addition, molecular abnormalities of plasminogen have also been reported in association with venous thrombosis that develops at a relatively younger age (9–13) than in normal individuals. The gene frequency of abnormal plasminogen is significantly high in the Japanese population (0.02) (14–18) as well as other populations (0.01) (19). An amino acid sequence analysis of an abnormal plasminogen isolated from three Japanese patients has demonstrated an amino acid substitution, Ala-601 → Thr, in the serine protease portion of the protein (20–22).

The structure and organization of the gene coding for human plasminogen have been established by nucleotide sequencing employing DNA segments obtained by *in vitro* amplification of leukocyte DNA and isolation of λ phage from genomic libraries (6). These data have made it possible to prepare primers for *in vitro* amplification and sequence analysis of the abnormal genes for plasminogen.

EXPERIMENTAL PROCEDURES

Venous blood was drawn from normal individuals and patients with abnormal plasminogen after informed consent had been obtained. Genomic DNA samples were prepared from leukocytes by standard techniques (23). One to 5 μg of genomic DNA was amplified in 100-μl reaction mixtures as described (24) employing 2.5–5.0 units of *Thermus aquaticus* DNA polymerase (New England Biolabs or Perkin–Elmer/Cetus). After 25 or 30 cycles of amplification, 5 μl of each reaction mixture was applied to a 0.8% or 1.5% agarose gel (International Biotechnologies) containing ethidium bromide (0.5 μg/ml) in 1× TBE buffer (89 mM Tris base/89 mM boric acid/20 mM EDTA, pH 7.8). Oligonucleotides were prepared with a synthesizer purchased from Applied Biosystems. For amplification, 14 pairs of primers were designed from the nucleotide sequence of the normal gene for plasminogen (6) as follows. For amplification and sequencing: pair 1 for exon I, 5′-CAATGAATTCTGGTCCTTATTTCACATCTAAAA and 3′-CACAGAATTCCATGGCATATGTATTTTTAC-TAC; pair 2 for exons II and III, 5′-TAGTAAGCTTCTT-TATTTATGTCCAAATGCCCG and 3′-ATCAAAGCT-TGTCCTAACTCAGAATTCAGTTTTAT; pair 3 for exons IV and V, 5′-AATCGGATCCTGGCTCAGTTTACTG-CAGCCTT and 3′-AAAAGGATCCAGGTTAATTC-TGGGGTTTTGCT; pair 4 for exons VI and VII, 5′-ATTTGAATTCATCCATTTCAGTTTTCTTCTTC and 3′-TGTAAGCTTTTGATTTCAAGAACAGGGC; pair 5 for exons VII, VIII, and IX, 5′-TTTAAGCTTGAAAAA-GAGTCTTATCCATG and 3′-AGAAAGCTTGGGCCCT-CAGTAAGCT; pair 6 for exon X, 5′-GTCAGAATTCTCA-GAGGCTACCGTACT and 3′-CTACGAATTC-TGGGTCTAAGAGAAATTTGG; pair 7 for exons XI, XII, and XIII, 5′-TAGAGAATTCTGGGTGCCCCTGAATAT-TCTC and 3′-GGGCAAGCTTCTCCTCCCCAGAAG-CAGTCT; pair 8 for exons XIV, XV, and XVI, 5′-CCAAAAGCTTATGATTTTACTATTTAGTTCGG and 3′-GGCTAAGCTTGAAGATTGATGCTGTCCCCAA; pair 9 for exons XV, XVI, and XVII, 5′-TCTGGAATTCTGTA-CAATGGAGCAGAACAAA and 3′-ACCGAATTCATG-GATAGGAATTTGCACAGC; pair 10 for exons XVIII and

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XIX, 5′-CCGAAGCTTTGTTCTGGAATATCCTCCT-GAATG and 3′-TTCGAATTCAAAAGCAGCTAAATC-CCCCCAT. For detection of type I and II mutations: pair 11 for exon XV, 5′-AAGCTTCAATTTAACTAAAATT-TGAACTAAAT and 3′-AAGCTTATTCAGGCCCA-CAAAATCATCTGT; pair 12 for exon XV, 5′-TCTGGAAT-TCTGTACAATGGAGCAGAACAAA and 3′-CCACGAAT-TCATCTGTACTGTGTCTTTCCTCT; pair 13 for exon X, 5′-GTCAGAATTCTCAGAGGCTACCGTACT and 3′-CTACGAATTCTGGGTCTAAGAGAAATTTGG; pair 14 for exon X, 5′-TATGGAATTCTGTCTGCTAATACA-GAAAAGAG and 3′-AAATGAATTCCTAGGAAGTTG-GCTTGAAGC.

The amplified DNA samples were digested with *Hin*dIII or *Eco*RI restriction enzyme to generate the proper ends for ligation into sequencing vectors. The digested samples were applied to a 0.8% or 1.5% agarose gel, electroeluted, and then subcloned into M13mp18 or M13mp19 (Bethesda Research Laboratories) with the *Hin*dIII or *Eco*RI end to obtain discrete sequences. The DNA sequence of the insert was then obtained using the dideoxynucleotide method (25) with deoxyadenosine 5′-[α-[^{35}S]thio]triphosphate (Amersham) and buffer gradient gels (26). To obtain the DNA sequences of both haplotypes from the maternal and paternal sides, 11 or more samples of each amplified region of the plasminogen gene were examined. About 10 kilobases out of a total of 53 kilobases for the gene coding for plasminogen have been sequenced in this study. Furthermore, both normal and mutated nucleotide sequences at the same position in a single genomic sample were identified and confirmed by restriction enzyme digestion.

For restriction enzyme analysis for the type I mutation, 10 μl of the amplified DNA sample was incubated with 3 units of *Fnu*4HI (New England Biolabs) for 1 hr or with 6 units for 4 hr at 37°C. For the type II mutation, the sample was incubated with 5 units of *Ava* II (Bethesda Research Laboratories) for 1 hr at 37°C. Five microliters of each sample were applied to the 1.5% agarose (International Biotechnologies) or to 2% NuSieve/1% SeaKem agarose gel (FMC BioProducts) after digestion.

The activity of plasminogen in the plasma from patients was determined using Testzym kit (Daiichi Kagaku Yakuhin, Tokyo) employing a chromogenic substrate (S-2251; Kabi, Stockholm) after activation with streptokinase, and the concentration of antigen was measured by radial immunodiffusion assay using a goat anti-human plasminogen antibody (Medical Biology Laboratories, Nagoya, Japan) in 0.9% agarose.

Leukocyte DNA was obtained from three unrelated Japanese patients with abnormal plasminogens, named PLG Kagoshima (percent of normal activity/percent of normal antigen = 8/98), PLG Nagoya I (51/65), and PLG Nagoya II (55/98), and eight members of their families. The family members of PLG Kagoshima included a daughter (PLG Kagoshima$_d$, 44/94), and the family members of PLG Nagoya I included two daughters (PLG Nagoya I$_{d2}$, 16/69, and PLG Nagoya I$_{d3}$, 19/96), a brother (PLG Nagoya I$_b$, 40/75), and two grandsons (PLG Nagoya I$_{gs1}$, 55/100, and PLG Nagoya I$_{gs2}$, 60/120). The relatives of PLG Nagoya II included two daughters (PLG Nagoya II$_{d1}$, 60/104, and PLG Nagoya II$_{d2}$, 62/104). Thus, PLG Kagoshima, PLG Nagoya I$_{d2}$, and PLG Nagoya I$_{d3}$ were considered as phenotypic homozygotes, whereas all the other individuals were heterozygotes. PLG Kagoshima$_d$, PLG Nagoya I$_b$, PLG Nagoya I$_{d2}$, PLG Nagoya I$_{d3}$, and PLG Nagoya II had a clinical history of venous thrombosis. PLG Kagoshima was from a consanguineous marriage between a first cousin and a first cousin once removed. PLG Nagoya I$_{d3}$ was previously referred to as plasminogen Nagoya (22).

RESULTS AND DISCUSSION

Type I Mutation in Plasminogen. Initially, exons XIV–XIX coding for the serine protease portion of plasminogen were obtained by the polymerase chain reaction employing oligonucleotide pairs of primers 8, 9, and 10 (Fig. 1). The DNA sequence coding for exon XV was found to differ for normal DNA and PLG Kagoshima DNA. This occurred in the region coding for amino acid 601 that has a sequence of ACT GCT GCC for the normal gene (Fig. 2*A Left*) and ACT ACT GCC for PLG Kagoshima (Fig. 2*A Right*). Indeed, all 23 samples of the amplified DNA from exon XV of PLG Kagoshima showed the same G → A change. This indicates that in this patient, whose parents were related, both alleles that code for plasminogen carried the same mutation, demonstrating that this individual was a genotypic homozygote. This mutation has been called a type I mutation. The nucleotide substitution of an adenosine for a guanosine predicts a threonine (ACT) instead of alanine (GCT) at amino acid 601. This residue is located two amino acids prior to the active site residue of histidine and is the same as that reported for plasminogen variant, PLG V, as determined by amino acid sequence analysis and isoelectric focusing of the abnormal protein (17, 21, 22).

Fifteen additional changes in the nucleotide sequence in the gene coding for plasminogen from PLG Kagoshima were found by DNA sequence analysis when the remaining exons and flanking regions (about 20% of the entire gene for plasminogen) were compared with the normal Caucasian gene. Whereas the normal gene had guanosine and adenosine, 111 and 110 base pairs (bp) upstream from exon II in intron A, these nucleotides were deleted from the gene of the

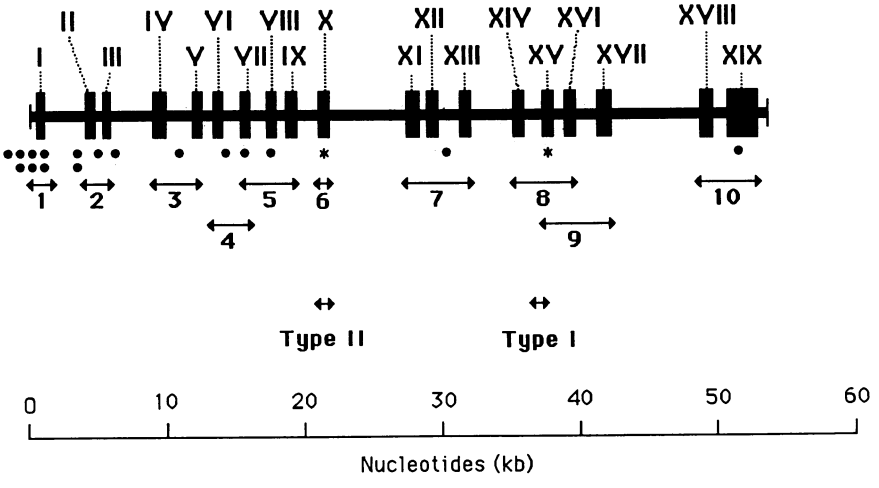

FIG. 1. Regions of the genomic DNA coding for plasminogen that were amplified by the polymerase chain reaction. To detect the molecular defect in abnormal plasminogen, all 19 exons of the plasminogen gene were amplified employing 10 pairs of oligonucleotide primers. For analysis of type I or type II mutations, exons X and XV were amplified separately. Asterisks represent sites of type I and II mutations, and solid circles indicate additional sites of nucleotide substitutions.

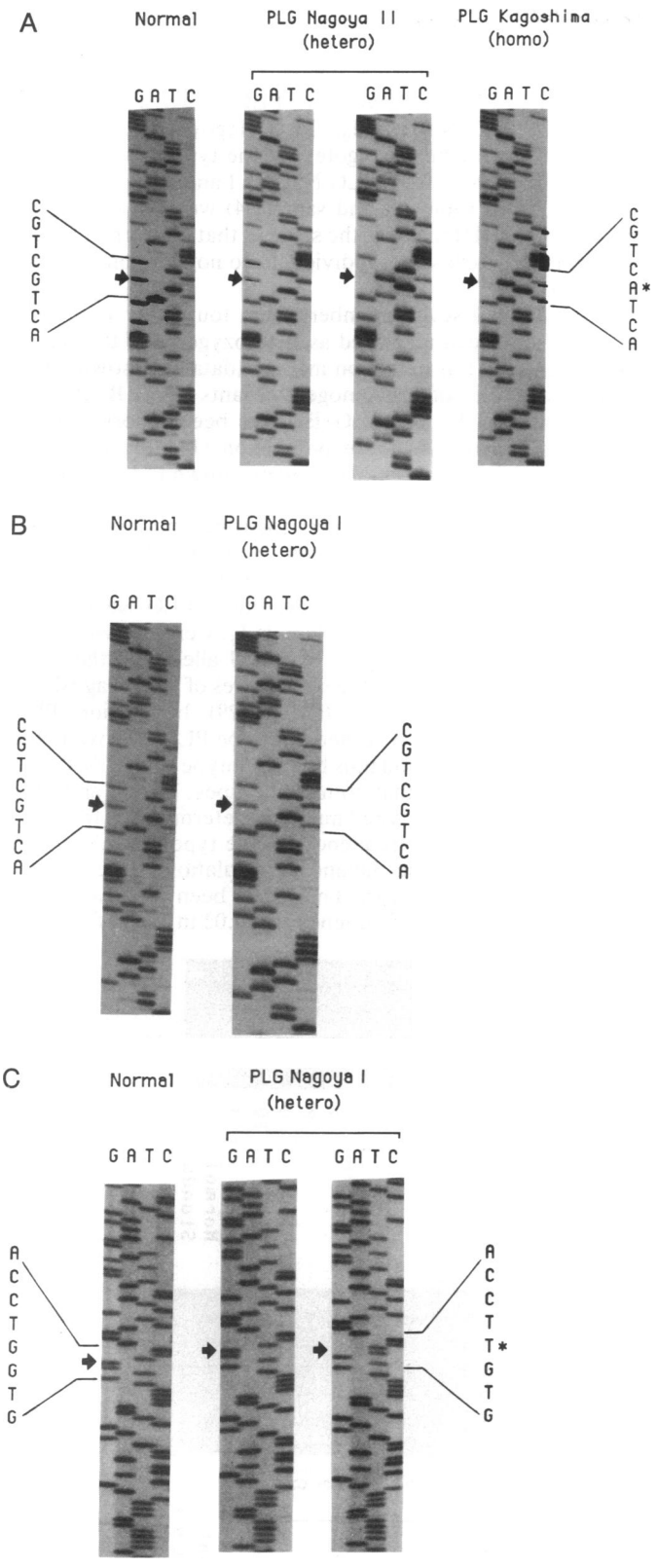

FIG. 2. (*A*) DNA sequence of a portion of exon XV from normal and two abnormal plasminogen genes. (*Left*) Normal gene. (*Center*) PLG Nagoya II. (*Right*) PLG Kagoshima. The arrows indicate the normal guanosines or abnormal adenosines and the asterisk indicates the abnormal adenosine. (*B*) DNA sequence of a portion of exon XV from a normal individual (*Left*) and PLG Nagoya I (*Right*). The arrows indicate the normal guanosines. (*C*) DNA sequence of a portion of exon X from a normal individual (*Left*) and PLG Nagoya I (*Center* and *Right*). The arrows indicate the normal guanosines or abnormal thymidine and the asterisk indicates the abnormal thymidine.

patients with the type I mutation. The remaining nucleotide substitutions were as follows: 84 bp downstream from exon III in intron C, adenosine (guanosine in normal); 109 bp downstream from exon III in intron C, adenosine (guanosine in normal); 608 bp downstream from exon IV intron D, guanosine (adenosine in normal); 333 bp upstream from exon V in intron D, adenosine (guanosine in normal); 116 bp downstream from exon V in intron E, cytidine (guanosine in normal); 103 bp in exon VII, thymidine (cytidine in normal); 155 bp in exon VIII, cytidine (thymidine in normal); 14 bp downstream from exon VIII in intron H, guanosine (adenosine in normal); 158 bp in exon XI, guanosine (adenosine in normal); 30 bp downstream from exon XI in intron K, adenosine (guanosine in normal); 185 bp downstream from exon XI in intron K, adenosine (guanosine in normal); 1065 bp downstream from exon XII in intron L, adenosine (guanosine in normal); 1307 bp downstream from exon XII in intron L, guanosine (thymidine in normal). Twelve of these changes were located within introns. Three nucleotide substitutions, however, were present within exons VII, VIII, and XI, but these did not change the amino acid sequence of the protein (see Table III in ref. 6). These data indicated that the amino acid sequence for the abnormal plasminogen present in PLG Kagoshima was identical to that of the normal molecule except for Ala-601. Thus, it was concluded that this single amino acid change results in the loss of activity in the plasminogen present in PLG Kagoshima.

When 12 DNA samples spanning exon XV from PLG Nagoya II or PLG Kagoshima$_d$ were sequenced, one-half of each of the samples had the same sequence as the normal gene, and the other half had the same sequence as that of PLG Kagoshima (Fig. 2*A Center*). These results were consistent with the conclusion that PLG Nagoya II and PLG Kagoshima$_d$ were heterozygotes for the type I mutation.

When the DNA samples from exon XV from two phenotypic homozygotes (PLG Nagoya I$_{d2}$ and PLG Nagoya I$_{d3}$) were analyzed, half of each of their 12 DNA samples showed the same sequence as the normal gene, and the other half had the same sequence as that of PLG Kagoshima. A change of G → A in the 3′ noncoding region 48 nucleotides downstream from the stop codon (210 bp in exon XIX) was also found in half of the samples from these two phenotypic homozygotes. It seemed unlikely, however, that the last change resulted in an abnormality. These experiments also indicated that the two daughters of PLG Nagoya I are compound genotypic heterozygotes afflicted by the type I mutation as well as a second DNA abnormality.

Type II Mutation in Plasminogen Gene. The sequence of 12 DNA samples prepared from exon XV of PLG Nagoya I, a heterozygote, was the same as that of the normal gene (Fig. 2*B*). Furthermore, when the DNA sequences of the other exons (XIV, XVI, XVII, XVIII, and XIX) coding for the catalytic chain of PLG Nagoya I were analyzed, a single nucleotide substitution of G → A was found in half of the samples from exon XIX. This change also occurred 48 nucleotides downstream from the stop codon in the 3′ noncoding region of exon XIX and was the same as that found in the two daughters of PLG Nagoya I. These data suggested that a second type of molecular defect was probably located in the 5′ end of the gene in the regulatory region or the region coding for the amino-terminal portion of the protein.

Exons I–XIII from the 5′ end of the gene from PLG Nagoya I were then amplified using oligonucleotide pairs 1–7 (Fig. 1). When exon X was amplified by primer pair 6 and the *Eco*RI fragment was sequenced, 5 of 11 samples showed a normal sequence of GTG GTC CAG (Fig. 2*C Center*) whereas the remaining six showed GTG TTC CAG (Fig. 2*C Right*). The nucleotide change G → T leads to the amino acid substitution Val-355 → Phe. This change has been called a type II mutation and occurs three amino acid residues prior to the

first disulfide bond in kringle four. The same results were obtained for PLG Nagoya I_{d2} and PLG Nagoya I_{d3}. Seventeen additional nucleotide changes were observed by DNA sequence analysis in PLG Nagoya I, PLG Nagoya I_{d2}, and PLG Nagoya I_{d3}. Whereas the nucleotides at 510, 454, 430, 286, 59, and 32 bp upstream from nucleotide 1 (6) in the 5′ flanking region of the normal gene were adenosine, cytidine, adenosine, adenosine, thymidine, and cytidine, respectively, those in the type II gene were guanosine, thymidine, guanosine, guanosine, guanosine, and thymidine, respectively. The remaining nucleotide substitutions were as follows: 28 bp downstream from exon I in intron A, guanosine (thymidine in normal); 115 bp upstream from exon III in intron B, adenosine (guanosine in normal); 84 bp downstream from exon III in intron C, adenosine (guanosine in normal); 333 bp upstream from exon V in intron D, adenosine (guanosine in normal); 116 bp downstream from exon V in intron E, cytidine (guanosine in normal); 112 bp downstream from exon VI in intron F, adenosine (thymidine in normal); 158 bp in exon XI, guanosine (adenosine in normal); 30 bp downstream from exon XI in intron K, adenosine (guanosine in normal); 185 bp downstream from exon XI in intron K, adenosine (guanosine in normal); 1307 bp downstream from exon XII in intron L, guanosine (thymidine in normal); 210 bp in exon XIX, adenosine (guanosine in normal). These nucleotide substitutions, however, appear to be silent changes that are located in the 5′ flanking noncoding region, in the introns, or in exon XI where they coded for the same amino acid that is present in the normal molecule (6). Consequently, it was concluded that the amino acid substitution Val-355 → Phe was responsible for the type II mutation. PLG Nagoya I_{d2} and PLG Nagoya I_{d3} are compound genotypic heterozygotes carrying both the type I and type II mutations as these data indicate.

The genes for plasminogen from Japanese patients with type I and type II mutations contained 16 and 18 nucleotide substitutions, respectively, when their DNA sequences were compared to the normal Caucasian gene. Seven of these changes were common in the genes of patients with type I and type II mutations, indicating that there are substantial differences in the DNA sequences of different populations (Caucasians and Orientals). Differences between Caucasians and Orientals have been observed for other genes, such as the gene coding for factor IX (27). In the genes from patients with type II mutation, there were six mutations in the 5′ flanking region. These nucleotide substitutions may affect the efficiency of the expression of type II abnormal gene, although none were located in the two potential clusters for regulatory elements for transcription of plasminogen (6).

Detection of the Type I Mutation by Restriction Enzyme Digestion. When the genomic DNA samples were amplified employing a pair of primers specifically for exon XV (pair 12), a discrete band of 360 bp was obtained for each sample, as predicted from the sequence of the normal gene for plasminogen (6). Since the G → A change in exon XV of the type I plasminogen abnormality destroys a *Fnu*4HI site (GCNGC) that is present in the normal gene, the amplified samples from the patients and normal individuals were digested with *Fnu*4HI endonuclease. The 360-bp fragment from normal DNA was cleaved into two fragments of 230 and 130 bp (Fig. 3*A*, lane normal), whereas that of the DNA from PLG Kagoshima remained unchanged (Fig. 3*A*, lane sample 10). The *Fnu*4HI digest of the 360-bp fragment from abnormal PLG Nagoya I_{d2}, PLG Nagoya I_{d3}, PLG Nagoya I_{gs1}, PLG Nagoya I_{gs2}, PLG Nagoya II, PLG Nagoya II_{d1}, PLG Nagoya II_{d2}, and PLG Kagoshima$_d$ showed a mixed pattern corresponding to normal DNA and to that from PLG Kagoshima (Fig. 3*A*). Prolonged digestion with a higher concentration of the enzyme gave exactly the same results. In additional experiments employing another set of primers (pair 11), identical results were obtained (data not shown). These experiments indicated that PLG Kagoshima was a homozygote for the type I mutation, whereas PLG Nagoya I_{d2}, PLG Nagoya I_{d3}, PLG Nagoya I_{gs1}, PLG Nagoya I_{gs2}, PLG Nagoya II, PLG Nagoya II_{d1}, PLG Nagoya II_{d2}, and PLG Kagoshima$_d$ were heterozygotes for the type I mutation. In contrast, the DNAs from PLG Nagoya I and PLG Nagoya I_b (Fig. 3*A*, lanes sample 1 and sample 4) were cleaved completely by *Fnu*4HI that was the same as that for normal DNA. Accordingly, the last two individuals do not contain the type I mutation.

An additional seven members from four other unrelated families have been identified as heterozygotes of the type I abnormality by this detection method (data not shown). The gene frequencies of plasminogen variants (PLG B, PLG1′, PLGN*3, PLG V, and PLG*B) have been reported to be about 0.02 in the Japanese population (14–18). The gene products of these variant alleles were shown to be nonfunctional (15–18, 28). All five alleles seem to be identical, judging from the patterns of isoelectric focusing and similar gene frequency in the same population. However, the identity of these five alleles should be determined by a direct sequencing of either protein or DNA. In this study, PLG Kagoshima, PLG Kagoshima$_d$, and PLG Nagoya I_{d3} were shown to be a homozygote or heterozygote of type I allele. On the other hand, it was reported that the phenotypes of PLG Kagoshima and PLG Kagoshima$_d$ were PLG B (29). In addition, PLG Nagoya I_{d3} in our study turned out to be PLG Nagoya in the previous report (22), and thus her phenotype was PLG V (17). Therefore, it is clear that both phenotypes, PLG B and PLG V, are identical to the type I mutation determined at the DNA level. Since the gene frequency for the type I mutation was relatively high in the Japanese population, especially in residents in western Japan, it may have been due to a founder effect. Since the gene frequency is ≈0.02 in Japan for type I

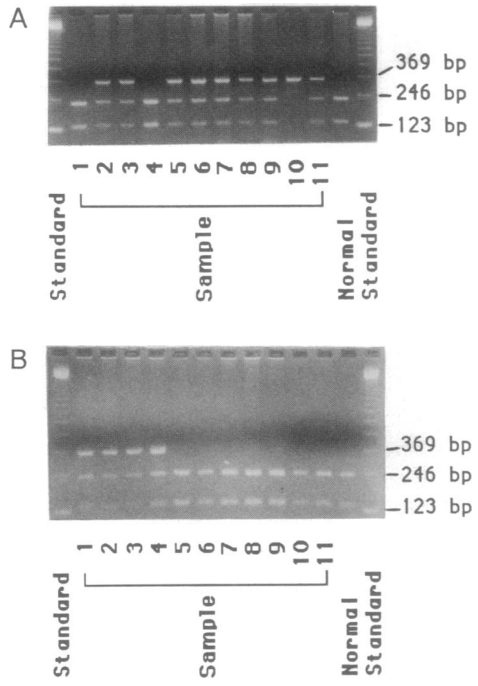

FIG. 3. (*A*) *Fnu*4HI digest of the amplified genomic DNAs for the type I mutation. (*B*) *Ava* II digest of the amplified genomic DNAs for the type II mutation. Molecular size standards (123-bp ladder) are from Bethesda Research Laboratories. Samples 1–11 refer to PLG Nagoya I, PLG Nagoya I_{d2}, PLG Nagoya I_{d3}, PLG Nagoya I_b, PLG Nagoya I_{gs1}, PLG Nagoya I_{gs2}, PLG Nagoya II, PLG Nagoya II_{d1}, PLG Nagoya II_{d2}, PLG Kagoshima, and PLG Kagoshima$_d$, respectively.

mutation, the expected number of genotypic homozygotes with the plasminogen variant was calculated to be ≈50,000 from a total population of 125 million. Very few homozygotes, however, have been found thus far (9, 10, 29), suggesting that a complete loss of plasminogen is essentially incompatible for life. This is also supported by the fact that only heterozygotes and no homozygotes with plasminogen deficiencies have been reported (30–35). Some homozygotes such as PLG Kagoshima may have some alternative pathway for fibrin degradation, since this patient has been free of thrombosis thus far.

Detection of the Type II Mutation by Restriction Enzyme Digestion. When exon X coding for amino acid residue 355 was amplified (pair 13), a discrete band of about 390 bp was obtained for each sample, as predicted from the sequence of the gene for normal plasminogen (6). Because the G → T change in exon X of the type II mutation eliminates an *Ava* II site (GGA/TCC) that was present in the normal gene, the samples from the patients and normal individuals were digested with *Ava* II endonuclease. The 390-bp fragment of normal DNA was cleaved into three fragments (about 230, 130, and 30 bp) (Fig. 3, lane normal). The 30-bp fragment, however, was too small to be detected by the agarose gel system used in this study. The *Ava* II digest of the 390-bp fragment from PLG Nagoya I (Fig. 3*B*, lane sample 1), PLG Nagoya I_{d2} (lane sample 2), PLG Nagoya I_{d3} (lane sample 3), and PLG Nagoya I_b (lane sample 4) showed a mixture of 360-, 230-, and 130-bp fragments. The 360-bp fragment is unique for the type II mutation. On the other hand, the DNAs from PLG Nagoya I_{gs1}, PLG Nagoya I_{gs2}, PLG Nagoya II, PLG Nagoya II_{d1}, PLG Nagoya II_{d2}, PLG Kagoshima, and PLG $Kagoshima_d$ were cleaved completely into the same fragments generated from normal DNA. Another set of primers (pair 14) was employed to confirm the results obtained by the first set, and a 400-bp band was observed for each sample as predicted (data not shown). Identical results were obtained when the amplified fragment of 400 bp was digested with *Ava* II endonuclease (data not shown). Thus, PLG Nagoya I, PLG Nagoya I_{d2}, PLG Nagoya I_{d3}, and PLG Nagoya I_b were heterozygotes of the type II mutation. The type II mutation was of particular interest in that two patients previously considered as phenotypic homozygotes (PLG Nagoya I_{d2} and PLG Nagoya I_{d3}) have been found to be compound genotypic heterozygotes of type I and type II mutations. The gene frequency of type II mutation may be low, and this allele and the relatively frequent variant allele (type I) were accidentally combined.

For patients with type II mutation, the DNA sequence of all exons, except for exon X, was the same as that of the normal individuals. A single-point mutation of G → T in exon X results in the amino acid substitution Val-355 → Phe. Since no other change in the amino acid sequence was present, the substitution of phenylalanine for valine in human plasminogen may be responsible for the molecular defect of the type II mutation. This suggests that Val-355 is important for the three-dimensional structure of the human protein and this substitution interferes with the interaction of enzyme and substrate. Other possibilities may also exist, however, such as an interference with the activation cleavage site that is located between Arg-561 and Val-562 in plasminogen. This mutation may also affect the stability of the plasminogen molecule, since both the activity and antigen level of plasminogen in the heterozygotes of the type II mutation were slightly lower than those in the heterozygotes of the type I mutation. At the present time, it is not possible to determine whether or not the differences in the activity and antigen of plasminogen are statistically significant, since only one family of the type II mutation has been identified.

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