Am. J. Hum. Genet. 42:718–725, 1988

Nonsense and Missense Mutations in Hemophilia A: Estimate of the Relative Mutation Rate at CG Dinucleotides

Hagop Youssoufian,* Stylianos E. Antonarakis,* William Bell,† Anne M. Griffin,‡ and Haig H. Kazazian, Jr.*

*Genetics Unit, Department of Pediatrics, and †Division of Hematology, Department of Medicine, The Johns Hopkins University School of Medicine, Baltimore; and ‡Division of Hematology, Department of Medicine, University of North Carolina School of Medicine, Chapel Hill

Summary

Hemophilia A is an X-linked disease of coagulation caused by deficiency of factor VIII. Using cloned cDNA and synthetic oligonucleotide probes, we have now screened 240 patients and found CG-to-TG transitions in an exon in nine. We have previously reported four of these patients; and here we report the remaining five, all of whom were severely affected. In one patient a *TaqI* site was lost in exon 23, and in the other four it was lost in exon 24. The novel exon 23 mutation is a CG-to-TG substitution at the codon for amino acid residue 2166, producing a nonsense codon in place of the normal codon for arginine. Similarly, the exon 24 mutations are also generated by CG-to-TG transitions, either on the sense strand producing nonsense mutations or on the antisense strand producing missense mutations (Arg to Gln) at position 2228. The novel missense mutations are the first such mutations observed in association with severe hemophilia A. These results provide further evidence that recurrent mutations are not uncommon in hemophilia A, and they also allow us to estimate that the extent of hypermutability of CG dinucleotides is 10–20 times greater than the average mutation rate for hemophilia A.

Introduction

Hemophilia A is one of the most common inherited diseases of blood coagulation in man (McKee 1983). It is inherited as an X-linked recessive, and one-third of the cases are thought to represent de novo mutations (Haldane 1935). The disease is caused by a deficiency of coagulation factor VIII (FVIII), and clinical severity is closely correlated with the plasma concentration of FVIII. Nevertheless, considerable heterogeneity exists among different families, and ~10% of patients develop specific antibodies against FVIII, called FVIII inhibitors, that usually appear after therapy with FVIII concentrates (McKee 1983). In general, patients with severe disease (i.e., FVIII levels ≤1 U/dl) are most prone to develop inhibitors.

Received August 11, 1987; revision received November 23, 1987.

Address for correspondence and reprints: Haig H. Kazazian, Jr., M.D., Genetics Unit, CMSC 10-110, The Johns Hopkins Hospital, 600 N. Wolfe Street, Baltimore, MD 21205.

0002-9297/88/4205-0007$02.00

The FVIII gene has been isolated, characterized, and expressed in vitro (Gitschier et al. 1984; Toole et al. 1984; Vehar et al. 1984; Wood et al. 1984). The gene is 186 kb in length and contains 26 exons encoding a 9-kb mRNA and a protein of 2,351 amino acids. The functional epitopes of the FVIII protein molecule are largely unknown. Exon 14 encodes a sequence of 925 amino acids called the B domain, deletion of which does not appear to affect the in vitro synthesis of FVIII (Toole et al. 1986).

The availability of cloned FVIII gene fragments has made possible the study of molecular defects in patients with hemophilia A (Antonarakis et al. 1985; Gitschier et al. 1985, 1986; Youssoufian et al. 1986, 1987). Because of the clinical heterogeneity of this disease, the large size of the FVIII gene, the pool of new mutations, and the loss of mutant alleles in the population within successive generations, it has been suggested that a large number of genetic lesions can cause hemophilia A (Antonarakis et al. 1985; Gitschier et al. 1985), to the extent that each afflicted family may harbor its own distinct mutation. However, we have recently described spontaneous recur-

Table 1

Clinical Characteristics

Patient,[a] Age (Years)	FVIII Level (U/dl)	FVIII Antigen (U/dl)	Inhibitor Titer (BU/ml)
JH-14, 17	<1	<1	1.8–5.0
JH-15, 52	<1	<1	18.0–30.0
JH-16, 40	<1	<1	0.0
JH-18, 34	<1	<1	0.0
JH-19, 28	<1	...	0.0

[a] All Caucasian males.

rences of two different point mutations in severe hemophilia A (Youssoufian et al. 1986). These mutations generated nonsense codons in exons 18 and 22 of the FVIII gene by substitutions of T for C at CG dinucleotides. The occurrence of these mutations at CG dinucleotides was significant because these sites are thought to be mutation hot spots in higher animals (Salser 1977; Mandel and Chambon 1979; Bird 1980; Nussinov 1981; Barker et al. 1984). The mechanism of this phenomenon appears to be methylation of cytosine 5′ to guanine and the subsequent spontaneous deamination of 5-methylcytosine to thymine (Coulondre et al. 1978).

We now describe a novel nonsense mutation in exon 23 of the FVIII gene and two different point mutations in exon 24 (one nonsense and a novel missense), each of which apparently occurred independently in two different families. All five mutations affect CG dinucleotides and generate either TG or CA when the substitutions occur on the coding strand or noncoding strand, respectively. From the proportion of mutations at CG dinucleotides in our survey of 240 patients with hemophilia A, we estimate the propensity of CG dinucleotides to undergo mutations in the coding region of the FVIII gene.

Methods

Hematologic Studies

Of the 240 patients whose DNA was screened for gene defects, 80% were classified as severe and 20% were classified as mild to moderate. All five patients whose defects are presented here had severe hemophilia A associated with FVIII levels <1 U/dl as measured by clotting assays and FVIII antigen levels determined by an immunoradiometric assay (Lazarchick and Hoyer 1978) (table 1). All had received extensive transfusions with FVIII concentrates and cryoprecipitates. In addition, two patients (JH-14 and JH-15) had evidence of FVIII inhibitors as measured by the Bethesda method (Kasper et al. 1975). In each family the mother of the proband was an obligate carrier of hemophilia A.

Restriction-Endonuclease Analysis

Genomic DNA was isolated from peripheral blood leukocytes by standard techniques (Antonarakis et al. 1985). Five micrograms of DNA was digested to completion with one of various restriction enzymes under conditions recommended by the manufacturers. Gel electrophoresis, transfer to nitrocellulose filters, hybridization with nick-translated probes, washing, and autoradiography were performed according to procedures described elsewhere (Antonarakis et al. 1985; Youssoufian et al. 1986). The following cloned FVIII cDNA fragments (provided by Drs. J. Toole and J. Wozney of Genetics Institute, Inc.) were used as probes: (*a*) probe A, a 1.7-kb *Kpn*I cDNA fragment that spans exons 1–12, and (*b*) probe BC, a 6.5-kb *Eco*RI cDNA fragment that spans exons 14–26 (Toole et al. 1984; Antonarakis et al. 1985; Youssoufian et al. 1986).

DNA Polymorphism Analysis

The following polymorphic sites within and closely linked to the FVIII gene were analyzed: (*a*) a *Bcl*I site in intron 18 of the FVIII gene (Gitschier et al. 1985), (*b*) an *Xba*I site in intron 22 of the FVIII gene (Wion et al. 1986), (*c*) a *Bgl*I site in intron 25 of the FVIII gene (Antonarakis et al. 1985), (*d*) a *Bgl*II site detected with the anonymous probe DXS15 (Harper et al. 1984), and (*e*) multiple *Taq*I site polymorphisms detected with the anonymous DNA fragment DXS52 (Oberle et al. 1985). The latter two DNA fragments map at the Xq27-q28 region and are closely linked to the FVIII gene (Goodfellow et al. 1985). These two

Table 2

Clinical Characteristics

Designation of Oligonucleotide	Exon of FVIII	Oligonucleotide Sequence
HEM 23	23 Normal	5′-TATTGCTCGATACATCCGT-3′
HEM 23m	23 Nonsense	5′-TATTGCTTGATACATCCGT-3′
HEM 24-C	24 Normal	5′-GCTCGACTTCACCTCCAAG-3′
HEM 24-T	24 Nonsense	5′-GCTTGACTTCACCTCCAAG-3′
HEM 24-A	24 Missense	5′-GCTCAACTTCACCTCCAAG-3′

probes were provided by Drs. K. Davies and J. L. Mandel, respectively, while the intron 22 probe was provided by Dr. R. Lawn of Genentech, Inc.

Oligonucleotide Analysis

Nineteen-base-long oligonucleotides (table 2) were chemically synthesized, end-labeled using T_4-polynucleotide kinase and γ^{32}P-ATP, further purified by electrophoresis on a 20% polyacrylamide sequencing gel, and extracted from this gel by a crush-soak method (Wallace et al. 1981; Youssoufian et al. 1986). Hybridization to dried agarose gel was performed at the calculated melting temperature (T_m) minus 4 degrees C for ≥3 h, washed in 2 × SSPE/0.1% SDS at 25 C, then washed further in 2 × SSPE at 2 C below T_m for 2 min and subjected to autoradiography. The agarose gel was reused after removal of the first probe by alkaline denaturation.

Results

Our screening strategy for FVIII mutations involved restriction analysis with *Taq*I, *Sst*I, and *Eco*RI and successive hybridizations with probe A and probe BC, as described elsewhere (Youssoufian et al. 1986, 1987). To date, we have analyzed the genomic DNA of 240 unrelated, randomly obtained individuals and families with hemophilia A. Some of these families were unselectively obtained from clinic populations, while others were referred for carrier detection or prenatal diagnosis. We have previously reported four point mutations involving *Taq*I restriction sites from a subset of 83 patients of the 240 reported here (Antonarakis et al. 1985; Youssoufian et al. 1986).

Family JH-14

Digestion with *Taq*I and hybridization with probe BC showed a new 3.8-kb fragment in the patient from family JH-14 (fig. 1*A*, III-3; fig. 2*A*, III-3) in place of the normal 1.4-kb fragment (fig. 1*A*). This result localized the absent *Taq*I site to exon 23 of the FVIII gene (fig. 1*C*; see Gitschier et al. 1985). The patient's mother (fig. 2*A*, II-2) also showed this change, whereas the sister, father, and maternal grandmother (figs. 1*A*, 2*A*; data for father not shown) did not. Digestion of the patient's DNA with other restriction enzymes or hybridization with probe A yielded normal patterns, indicating that the change

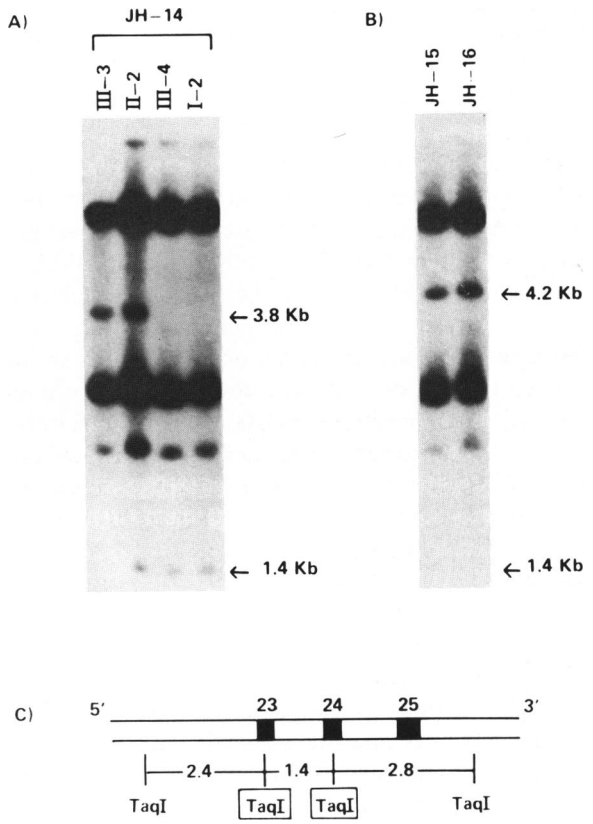

Figure 1 Restriction analysis of DNA cleaved with *Taq*I and hybridized with probe BC. *A*, Family JH-14; *B*, families JH-15 and JH-16; *C*, partial restriction map of FVIII DNA cleaved with *Taq*I. Altered *Taq*I sites are enclosed in rectangles.

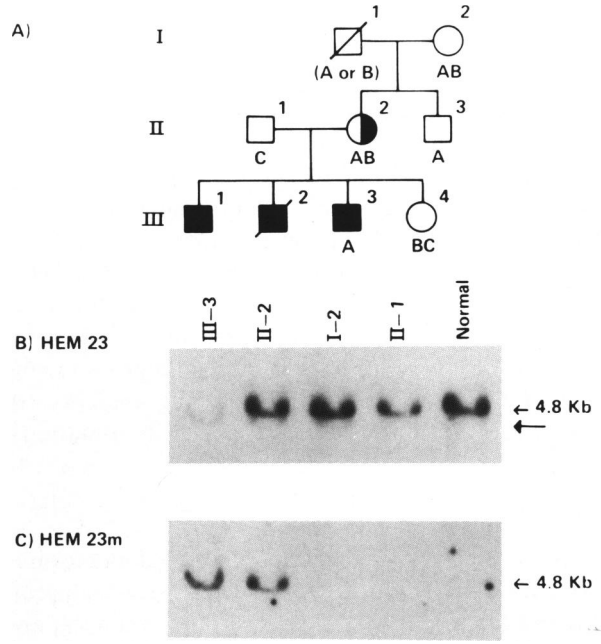

Figure 2 Analysis of the exon 23 mutation. *A*, Pedigree of family JH-14: □, normal male; ○, normal female; ■, hemophilic male; and ◑, carrier female. Letters below symbols indicate X-chromosome haplotypes of polymorphic restriction sites. Haplotype designations: A, + − + + 3; B, − − − − 2; C, + + + + 2. These symbols represent the DNA polymorphic sites in the following order: *Bcl*I, *Xba*I, *Bgl*I, *Bgl*II, and *Taq*I (see Methods). Haplotype of I-1 is uncertain. Also shown are results of oligonucleotide analysis of genomic DNA from members of family JH-14 that was digested with *Eco*RI and hybridized with (*B*) HEM 23 and (C) HEM 23m. See table 1 for sequence of oligonucleotides. Long arrow indicates non-FVIII gene sequences.

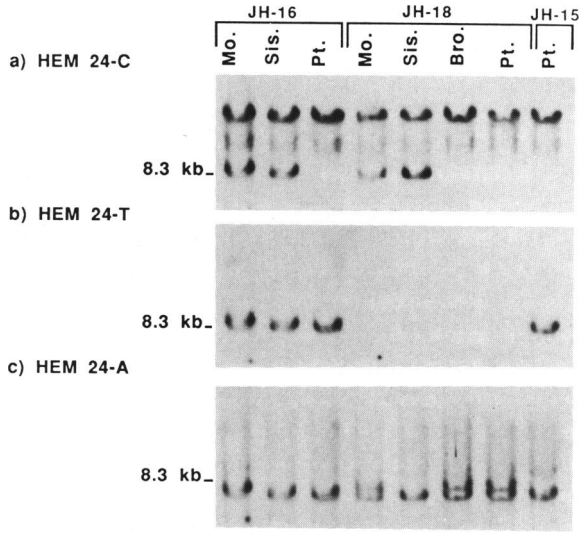

Figure 3 Analysis of the exon 24 mutations. Oligonucleotide analysis of genomic DNA from families JH-15, JH-16, and JH-18 that was digested with *Sst*I and hybridized with (*A*) HEM 24-C, (*B*) HEM 24-T, and (C) HEM 24-A. Bro. = brother; Mo. = mother; and Sis. = sister.

detected with *Taq*I most likely represents a point mutation.

On the basis of previous observations of *Taq*I site mutations (Antonarakis et al. 1985; Gitschier et al. 1985, 1986; Youssoufian et al. 1986), we postulated that this loss of a *Taq*I site is due to a CG-to-TG transition. We synthesized two oligonucleotides, one representing the normal sequence of exon 23 surrounding the *Taq*I site and the other representing the presumed mutant sequence that differs from the normal by the substitution of a T for a C in the *Taq*I site (table 2). Digestion of genomic DNA with *Eco*RI normally produces a 4.8-kb fragment that contains exons 23–25 of the FVIII gene (Gitschier et al. 1984). The normal oligonucleotide probe, HEM 23, does not hybridize to this fragment from the affected male (fig. 2*B*, III-3). However, the DNA from the heterozygous mother, the normal grandmother, and the father shows hybridization of HEM 23 to this fragment (fig. 2*A*, II-2, I-2, and II-1, respectively). A constant, non-FVIII fragment is seen in all individuals. By contrast, the mutant probe, HEM 23m, hybridizes specifically to the 4.8-kb fragment from the DNA of the patient and his mother (fig. 2*C*, III-3 and II-2, respectively) but not to the DNA of other family members or of a normal female control (fig. 3C). Therefore, the change in the *Taq*I site of exon 23 is substitution of a T for a C, which generates a nonsense codon (TGA) in place of the normal arginine codon (CGA) at position 2166 of the FVIII gene.

We attempted to determine the parental origin of the new mutation in the patient's mother by performing DNA polymorphism analysis on various family members. The analysis was incomplete because the maternal grandfather was deceased (see fig. 2*A*). After analyzing five different polymorphic sites within and closely linked to the FVIII gene, it was evident that the X-chromosome haplotype with which this mutation is associated was also shared by the maternal grandmother and uncle (fig. 2*A*, I-2 and II-3, respectively). Therefore, although it is possible that the mutation-bearing X chromosome was contributed by the maternal grandfather, who by chance happened to have this same haplotype, it is more likely that it arose de novo on an X chromosome contributed by the maternal grandmother. It is noteworthy that the ages of the maternal grandfather

and grandmother were 49 and 33 years, respectively, at the time of conception of their carrier daughter.

Families JH-15, JH-16, JH-18, and JH-19

A different *Taq*I site alteration was observed in four other unrelated patients with hemophilia A. When DNA from the patients of families JH-15, JH-16, JH-18, or JH-19 was digested with *Taq*I and hybridized with probe BC, a new 4.2-kb fragment was found in place of a normal 1.4-kb fragment (fig. 1*B;* data for JH-18 and JH-19 not shown). The result localized the altered *Taq*I site to exon 24 (fig. 1*C*). No other changes were noted with other combinations of restriction enzymes and probes (data not shown). Obligate carriers from each family also showed this *Taq*I site change (data not shown). These results were suggestive of a point mutation, possibly a C-to-T transition at CG dinucleotide either on the sense strand (CG-TG) or the antisense strand. The latter mutation yields a CG-CA substitution on the sense strand. The former mutation has been described elsewhere in an inhibitor-positive patient with severe hemophilia A (Gitschier et al. 1985). To determine the precise nucleotide change, three oligonucleotides that flank the exon 24 *Taq*I site but differ from each other by the single substitution of either a T for a C or an A for a G within the *Taq* I site (table 2) were synthesized. The normal oligonucleotide, HEM 24-C, does not hybridize to the 8.3-kb fragment of *Sst*I-cleaved DNA from the patients of these families (fig. 3*A*). With this probe three constant non-FVIII sequences are observed in all individuals. The oligonucleotide bearing the first mutant sequence, HEM 24-T, hybridizes specifically to the 8.3-kb *Sst*I fragment from the affected males from families JH-15 and JH-16, as well as to that of females from family JH-16 who are heterozygous for this mutation (fig. 3*B*). Therefore, the exon 24 mutation in these two patients is a C-to-T transition at codon 2228, generating a nonsense codon in place of arginine, and is identical to the exon 24 mutation described elsewhere (Gitschier et al. 1985). On the other hand, the oligonucleotide bearing the second presumed mutant sequence, HEM-A, hybridizes to the 8.3-kb *Sst*I fragment from affected males in the JH-18 and JH-19 families, as well as to that of carrier females (fig. 3*C;* data not shown for JH-19). Thus, the mutation in the latter two families is a CG-to-CA change at codon 2228, a mutation generating a glutamine in place of arginine at this position in the protein. No FVIII:C Ag could be demonstrated in the plasma of any of these patients.

To demonstrate that these four mutations represent independent events, we analyzed intragenic polymorphic sites (*Bcl*I and *Bgl*II) and the two extragenic sites that are closely linked to the FVIII gene (see Methods). The affected X chromosome in JH-15 differed from that of JH-16 at the intragenic *Bcl*I site, and the affected X chromosome in JH-18 differed from that of JH-19 at both intragenic sites (data not shown). These differences in intragenic polymorphic sites, in conjunction with pedigree analysis showing lack of relationship, constitute strong evidence for independent origins of these four exon 24 mutations.

Discussion

In this study we have described a novel mutation in exon 23 of the FVIII gene, two independent occurrences of a novel missense mutation in exon 24, and two further independent occurrences of an exon 24 nonsense mutation described elsewhere (Gitschier et al. 1985). All five of these mutations involve CG-to-TG or CG-to-CA transitions.

Considerable evidence indicates that the missense mutation described here is causally related to hemophilia A. This mutation in codon 2228 has not been observed in more than 600 normal or hemophilic X chromosomes (Antonarakis et al. 1987; S. E. Antonarakis, H. Youssoufian, and H. H. Kazazian, Jr., unpublished observations). The independent recurrence of this mutation in association with severe hemophilia A lends further credence to a causal role. Moreover, since the arginine residue at position 2228 is conserved in both copies of the C domain (Gitschier et al. 1984; Toole et al. 1984), the substitution of a neutral amino acid, glutamine, for the positively charged arginine could produce an important structural alteration in the FVIII protein.

It has become clear that CpG dinucleotides play a prominent role in mutagenesis in higher animals (Salser 1977; Mandel and Chambon 1979; Bird 1980; Nussinov 1981; Barker et al. 1984). Evidence in support of this proposal includes the significant underrepresentation of this dinucleotide in vertebrate genomes (Nussinov 1981), a higher frequency of polymorphism observed with restriction enzymes (e.g., *Taq*I or *Msp*I) that contain CpG in their recognition sequences (Barker et al. 1984), and recurrent point mutations in exons 18 and 22 of the FVIII gene (Youssoufian et al. 1986). Furthermore, mutations

that involve CpG dinucleotides have been described in α_1-antitrypsin (Kidd et al. 1983), adenosine deaminase (Bonthron et al. 1985), insulin (Shibasaki et al. 1985), antithrombin III (Duchange et al. 1986), factor IX (Bentley et al. 1986), and protein C (Romeo et al. 1987). Thus, C-to-T transitions at CpG dinucleotides have been observed in a variety of heritable disorders, although a systematic evaluation of their relative importance as a cause of mutation has not been available.

Within the coding region of the FVIII gene there are five *Taq*I sites that can potentially yield nonsense codons (Gitschier et al. 1984). Mutations at all five of these sites have now been described (Antonarakis et al. 1985; Gitschier et al. 1985, 1986; Youssoufian et al. 1986; and present report), and all of them involve C-to-T transitions. In addition, two G-to-A transitions in exons 24 and 26 have also been associated with hemophilia A (Gitschier et al. 1986; and present report). Thus, for the 10 potential C-to-T transitions at these five sites, seven mutations of this type have now been observed. In our sample of 240 patients, we have observed five of these seven mutations and have characterized nine independent occurrences of these five mutations.

Because a relatively large number of CG-to TG-or CG-to-CA mutations have now been characterized in the FVIII gene, it is possible to estimate the excess contribution of C-to-T transitions leading to hemophilia A. This estimate requires an assessment of the total number of different mutations that produce hemophilia A, a number that may be estimated as follows: (1) For nonsense mutations, examination of the coding sequence of the FVIII gene (Gitschier et al. 1984) reveals that, following single nucleotide changes, 793 codons can potentially yield nonsense codons. (2) For frameshift mutations, by analogy with the β-thalassemias (Kazazian and Antonarakis 1987), the number of frameshifts may be roughly equal to the number of nonsense mutations. (3) For missense mutations, a reasonable comparison can again be made with known β-chain variants. β-Globin has 146 codons, and there are 110 β-chain variants that produce a clinical phenotype (unstable hemoglobin, abnormal oxygen affinity, hemoglobin M, or sickling) in the heterozygous state (Bunn and Forget 1986, p. 382), indicating that the frequency of clinically recognizable missense mutations per codon is ~.75 for this gene. In the case of FVIII, the frequency of deleterious missense mutations per codon is likely to be somewhat smaller. Of the 2,351 amino acids that make up the protein, at least 581 (derived from the B domain) are thought to be dispensable for in vitro synthesis and procoagulant activity (Toole et al. 1986). Therefore, of the remaining 1,770 codons, we estimate that approximately 1,300 missense mutations may be clinically significant. (4) Transcriptional and processing mutations include splice-site mutations (three per nucleotide at the donor GT and acceptor AG splice sites of each intron plus an unknown number of mutations in consensus sequences at splice sites) and others that affect promoter and other 5′ elements, for a total of approximately 350. (5) Gene deletions from our own patient population have a prevalence of 6% in this disease (Youssoufian et al. 1987). Therefore, the sum total of potential mutations to hemophilia A by this calculation is approximately 790 + 790 + 1,300 + 350 + 200 = 3,430. We believe that this estimate is quite conservative. For example, it is possible that missense mutations in the B domain may well have deleterious effects; the inclusion of these potentially deleterious mutations would further raise the total number of mutations.

Since we have surveyed five sites that can yield seven C-to-T transitions capable of producing hemophilia A, the expected frequency of C-to-T mutations in these patients is 7/3,430. This figure is in contrast to the observed frequency of 9/240 (or 3.75%), giving an increased relative mutation rate of CG to TG or of CG to CA of approximately 18. Even if one completely eliminates the most speculative number in the calculation—i.e., that of missense mutations—one gets an increased relative mutation rate of 10. A reasonable range on this estimate of the increase in relative mutation rate is 10–20.

It is postulated that one-third of the mutations in X-linked lethal disorders are due to de novo events (Haldane 1935). In the case of the exon 23 mutation, the carrier mother most likely acquired this mutation as a germ-line, de novo event. To date, at least 13 of 29 mutations characterized in our sample of 240 patients have occurred de novo within 2 generations (Youssoufian et al. 1986, 1987; H. Youssoufian, S. E. Antonarakis, and H. H. Kazazian, Jr., unpublished data).

Acknowledgments

We thank Drs. K. Davies, R. M. Lawn, J. L. Mandel, J. Toole, and J. Wozney for their generous gifts of probes, Dr. A. Chakravarti for helpful discussions, Dr. L. W. Hoyer for

measuring FVIII antigen, and E. Pasterfield for manuscript preparation. The study was supported by National Institutes of Health grants (to S.E.A., W.B. and H.H.K.) and by a Johns Hopkins institutional grant (to S.E.A.).

References

Antonarakis, S. E., P. G. Waber, S. D. Kittur, A. S. Patel, H. H. Kazazian, Jr., M. A. Mellis, R. B. Counts, G. Stamatoyannopoulos, E. J. W. Bowie, D. N. Fass, D. D. Pittman, J. M. Wozney, and J. J. Toole. 1985. Hemophilia A: molecular defects and carrier detection by DNA analysis. N. Engl. J. Med. 313:842–848.

Antonarakis, S. E., H. Youssoufian, and H. H. Kazazian, Jr. 1987. Molecular genetics of hemophilia A in man. Mol. Biol. Med. 4:81–94.

Barker, D., M. Schafer, and R. White. 1984. Restriction sites containing CpG show a higher frequency of polymorphism in human DNA. Cell 36:131–138.

Bentley, A. K., D. J. G. Rees, C. Rizza, and G. G. Brownlee. 1986. Defective propeptide processing of blood clotting factor IX caused by mutation of arginine to glutamine at position −4. Cell 45:343–348.

Bird, A. P. 1980. DNA methylation and the frequency of CpG in animal DNA. Nucleic Acids Res. 8:1499–1504.

Bonthron, D. T., A. F. Markham, D. Ginsburg, and S. H. Orkin. 1985. Identification of a point mutation in the adenosine deaminase gene responsible for immunodeficiency. J. Clin. Invest. 75:894–897.

Bunn, H. F. and B. G. Forget, eds. 1986. Human hemoglobin variants. *In* Hemoglobin: molecular, genetic and clinical aspects. W. B. Saunders, Philadelphia.

Coulondre, C., J. H. Miller, P. J. Farabaugh, and W. Gilbert. 1978. Molecular basis of base substitution hotspots in *Escherichia coli*. Nature 274:775–780.

Duchange, N., J.-F. Chasse, G. N. Cohen, and N. M. Zakin. 1986. Antithrombin III tours gene: identification of a point mutation leading to an arginine → cysteine replacement in a silent deficiency (abstract). Nucleic Acids Res. 14:2408.

Gitschier, J., D. Drayna, E. G. D. Tuddenham, R. I. White, and R. M. Lawn. 1985. Genetic mapping and diagnosis of haemophilia A achieved through a *Bcl* I polymorphism in the factor viii gene. Nature 314:738–740.

Gitschier, J., W. I. Wood, T. M. Goralka, K. L. Wion, E. Y. Chen, D. H. Eaton, G. A. Vehar, D. J. Capon, and R. M. Lawn. 1984. Characterization of the human factor VIII gene. Nature 312:326–330.

Gitschier, J., W. I. Wood, M. A. Shuman, and R. M. Lawn. 1986. Identification of a missense mutation in the factor VIII gene of a mild hemophiliac. Science 232:1415–1416.

Gitschier, J., W. I. Wood, E. G. D. Tuddenham, M. A. Shuman, T. M. Goralka, E. Y. Chen, and R. M. Lawn. 1985. Detection and sequence of mutations in the factor VIII gene of haemophiliacs. Nature 315:427–430.

Goodfellow, P. N., K. E. Davies, and H. H. Ropers. 1985. Report of the Committee on the Genetic Constitution of the X and Y Chromosomes. Cytogenet. Cell Genet. 40:296–352.

Haldane, J. B. S. 1935. The rate of spontaneous mutation of a human gene. J. Genet. 31:317–326.

Harper, K., R. M. Winter, M. E. Pembrey, D. Harley, K. E. Danes, and E. G. D. Tuddenham. 1984. A clinically useful DNA probe closely linked to hemophilia A. Lancet 2:6–8.

Kasper, C. K., L. Aldort, D. Aronson, R. B. Count, J. R. Edson, J. Fratantoni, D. Green, J. W. Hampton, M. W. Hilgartner, P. H. Levine, C. W. McMillan, J. G. Pool, S. S. Shapiro, N. R. Shulman, and J. Van Eyes. 1975. A more uniform measurement of factor VIII inhibitor. Thromb. Diath. Haemorrh. 34:869–872.

Kazazian, H. H., Jr., and S. E. Antonarakis. 1987. The varieties of mutation. Pp. 43–67 *in* B. Childs, N. A. Holtzman, H. H. Kazazian, Jr., and D. L. Valle, eds. Progress in medical genetics: new series, vol. 7: Molecular genetics in medicine. Elsevier, New York.

Kidd, V. J., R. B. Wallace, K. Itakura, and S. L. C. Woo. 1983. α_1-Antitrypsin deficiency detection by direct analysis of the mutation in the gene. Nature 304:230–234.

Lazarchick, J., and L. W. Hoyer. 1978. Immunoradiometric measurement of the factor VIII procoagulant antigen. J. Clin. Invest. 62:1048–1052.

McKee, P. A. 1983. Haemostasis and disorders of blood coagulation. Pp. 1531–1560 *in* J. B. Stanbury, J. B. Wyngaarden, D. S. Fredrickson, J. L. Goldstein, and M. S. Brown, eds. The metabolic basis of inherited disease. McGraw-Hill, New York.

Mandel, J. L., and P. Chambon. 1979. DNA methylation: organ specific variations in the methylation pattern within and around ovalbumin and other chicken genes. Nucleic Acids Res. 7:2081–2103.

Nussinov, R. 1981. Eukaryotic dinucleotide preference rules and their implications for degenerate codon usage. J. Mol. Biol. 149:125–131.

Oberle, I., D. Drayna, G. Camerino, R. White, and J. L. Mandel. 1985. The telomeric region of the human X chromosome long arm: presence of a highly polymorphic DNA marker and analysis of recombination frequency. Proc. Natl. Acad. Sci. USA 82:2824–2828.

Romeo, G., H. J. Hassan, S. Staempfli, L. Roncuzzi, L. Cianetti, A. Leonard, V. Vicente, P. M. Mannucci, R. Bertina, C. Peschle, and R. Cortese. 1987. Hereditary thrombophilia: identification of nonsense and missense mutations in the protein C gene. Proc. Natl. Acad. Sci. USA 84:2829–2832.

Salser, W. 1977. Globin mRNA sequences: analysis of base pairing and evolutionary implications. Cold Spring Harbor Symp. Quant. Biol. 42:985–1002.

Shibasaki, Y., T. Kawakami, Y. Kanazawa, Y. Akanuma, and F. Takaku. 1985. Posttranslational cleavage of proinsulin is blocked by a point mutation in familial hyperproinsulinemia. J. Clin. Invest. **76**:378–380.

Toole, J. J., J. L. Knopf, J. M. Wozney, L. A. Sultzman, J. L. Buecker, D. D. Pittman, R. J. Kaufman, E. Brown, C. Shoemaker, E. C. Orr, G. W. Amphlett, W. B. Foster, M. L. Coe, G. J. Knutson, D. N. Fass, and R. M. Hewick. 1984. Molecular cloning of a cDNA encoding human anti-haemophilic factor. Nature **312**:342–347.

Toole, J. J., D. D. Pittman, E. C. Orr, P. Murtha, L. C. Wasley, and R. J. Kaufman. 1986. A large region (95 KDa) of human factor VIII is dispensable for *in vitro* procoagulant activity. Proc. Natl. Acad. Sci. USA **83**: 5939–5942.

Vehar, G. A., B. Keyt, D. Eaton, H. Rodriguez, D. P. O'Brian, F. Rotblat, H. Oppermann, R. Keck, W. I. Wood, R. N. Harkins, E. G. D. Tuddenham, R. M. Lawn, and D. J. Capon. 1984. Structure of human factor VIII. Nature **312**:337–342.

Wallace, R. B., M. J. Johnson, T. Hirose, T. Miyake, E. Kawashima, and K. Itakura. 1981. The use of synthetic oligonucleotides as hybridization probes. II. Hybridization of oligonucleotides of mixed sequence to rabbit β-globin DNA. Nucleic Acids Res. **9**:879–894.

Wion, K. L., E. G. D. Tuddenham, and R. M. Lawn. 1986. A new polymorphism in the factor VIII gene for prenatal diagnosis of hemophilia A. Nucleic Acids Res. **14**:4535–4542.

Wood, W. I., D. J. Capon, C. C. Simonsen, D. L. Eaton, J. Gitschier, B. Keyt, P. H. Seeburg, D. H. Smith, P. Hollingshead, K. L. Wion, E. Delwart, E. G. D. Tuddenham, G. A. Vehar, and R. M. Lawn. 1984. Expression of active human factor VIII from recombinant DNA clones. Nature **312**:330–337.

Youssoufian, H., S. E. Antonarakis, S. Aronis, G. Tsiftis, D. G. Phillips, and H. H. Kazazian, Jr. 1987. Characterization of five partial deletions of the factor VIII gene. Proc. Natl. Acad. Sci. USA **84**:3772–3776.

Youssoufian, H., H. H. Kazazian, Jr., D. G. Phillips, S. Aronis, G. Tsiftis, V. A. Brown, and S. E. Antonarakis. 1986. Recurrent mutations in haemophilia A give evidence for CpG mutation hotspots. Nature **324**:380–382.