The Molecular Genetic Analysis of Hemophilia A: A Directed Search Strategy for the Detection of Point Mutations in the Human Factor VIII Gene


A directed-search strategy for point mutations in the factor VIII gene causing hemophilia A was used to screen eight potentially hypermutable CpG dinucleotides occurring at sites deemed to be of functional importance. Polymerase chain reaction–amplified DNA samples from 793 unrelated individuals with hemophilia A were screened by discriminant oligonucleotide hybridization. Point mutations were identified in 16 patients that were consistent with a model of 5-methylcytosine (5mC) deamination. Four new examples of recurrent mutation were demonstrated at the following codons: 336 (CGA → TGA), 372 (CGC → TGC), 372 (CGC → CAC), and 1689 (CGC → TGC).

Hemophilia A is an X-linked bleeding disorder affecting approximately 1 in 5,000 males, and is caused by deficiency of factor VIII, a cofactor in the activation of factor X by factor IXa. Characterization of point mutations in the human factor VIII gene has been hampered by its large size and its complex structure; the 9-kb factor VIII mRNA is encoded by a gene comprising 26 exons spanning 186 kb of chromosomal DNA. Hemophilia A is a good model disease for the study of mutation because of its relatively high prevalence, its variable phenotype (implying allelic heterogeneity), and the high proportion of new mutations due to the lowered probability of survival of disease alleles.

Gitschier et al found that the restriction enzyme Taq I (recognition sequence TCGA) detected point mutations in the factor VIII gene of hemophilia A patients. Four of 92 DNA samples exhibited variant Taq I bands on a Southern blot, two of these subsequently proving to be nonsense codons (CGA → TGA). This finding was confirmed by Youssoufian et al, who also provided the first evidence for recurrent mutation due to CG → TG transitions at identical sites in the factor VIII gene. Further examples of recurrent mutation have since been found in five different Taq I restriction sites within exons 18, 22, 23, 24, and 26.

That CpG is a “hotspot” for mutation is evidenced by (a) its under-representation or “suppression” in genomic DNA, (b) the high frequency of polymorphism detected by restriction enzymes containing CpG in their recognition sequences, (c) the high rate of CpG substitution observed in evolutionary studies on vertebrate genes and (d) the high frequency (33%) of CG → TG and CG → CA transitions among point mutations causing human genetic disease. The CpG dinucleotide is the preferred site for cytosine methylation in higher eukaryotes and 5-methylcytosine (5mC) is prone to mutate to thymidine by deamination. As a result of 5mC deamination on the antisense strand, mutations are predicted to occur frequently in the heavily methylated human genome.

The characterization of point mutations in the factor VIII gene is important, not only as a means toward understanding the basis of CpG hypermutability with all its diagnostic implications, but also as a way of relating the structure of the factor VIII protein to its function. Such studies should eventually enable us to provide explanations at the molecular level for the variety of different phenotypes reported to date. There are 70 CpG sites in the factor VIII gene coding sequence, of which 7 occur in Taq I sites. Five of these are CGA (arginine) codons, which a C → T transition would convert to a TGA termination codon (Fig 1). Because these lesions are certain to come to clinical attention, it is not surprising that a high frequency of nonsense mutations have already been found in the factor VIII gene by Taq I site screening. However, the majority of CpG dinucleotides do not fall within available restriction enzyme recognition sequences; these nevertheless may be studied using a combi-
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METHODS

Blood samples. Ten to twenty milliliters EDTA-anticoagulated blood samples were drawn from 793 unrelated individuals with hemophilia A. Approval for this study had been granted by the appropriate local committees; patients were informed that the blood was being drawn for research purposes and that their privacy would be protected. Four hundred sixty-five were severe, 64 were moderately severe, and 119 were mild as determined by factor VIII:C measurement (<1% severe, 1% to 5% moderate, >5% mild). The remainder of samples (145) were from patients whose condition was of undetermined severity. The ethnic distribution of the samples screened was 717 whites/Europeans/Israelis, 56 Pakistanis/Indians, and 20 Chinese.

DNA isolation. DNA was isolated from lymphocyte pellets made from blood samples by established methods.14,15


TABLE 1

<table>
<thead>
<tr>
<th>Codons</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>-5</td>
<td>CGA</td>
</tr>
<tr>
<td>336</td>
<td>CGC</td>
</tr>
</tbody>
</table>

FIG 1. Location of selected CpG dinucleotides in factor VIII cDNA. Top line: Taq I sites that contain CGA in the reading frame (codon numbering according to Vehar et al26). Second line: Diagram of the factor VIII cDNA coding sequence with predicted domains of the protein.29 Third line: All seven CGA codons not in Taq I sites. Bottom line: Two CGC codons that encode arginine in critical thrombin cleavage sites of the mature protein.

METHODS

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In vitro amplification using the PCR. Specific fragments of the factor VIII gene were amplified by PCR using the following oligonucleotides. Codon -5: DM5 5'GCA AAT AGA GCT CTC CAC CTG CT and DM7 5'GGC AGC TCA CCG AGA TCA CT 3'. Codons 336, 372, and 427: DM494 5'ATG GCA TGG AAG ATG TCA A3 and JP37 5'CAA CAG TGT GTC TCC AAC TTC 3'. Codon 583: DM496 5'TCA GAC AAG AGG AAT GTC ATC CGG T3' and DM497 5'GGA ACT CTG GAT CCT CAA GCT G3'. Codon 795: DM9 5'GAA CCA AGA AGC TTC TCC CA 3'. Codons 1689 and 1696: DM639 5'CCC GGG CAA AGC AAG GTA GGA CTTG3' and DM642 5'GAG CCT CTC CAC TGC AGC A3'.

Oligonucleotides were made or obtained as described below. Between 25 ng and 1 μg of genomic DNA, 0.5 μg of each oligonucleotide, and 1.5 units Taq DNA Polymerase (Cetus, Beaconfield, England) were added to 95 μL buffer containing 1.5 mmol/L MgCl2, 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 0.01% gelatin, and 200 μmol/L each deoxynucleotide 5' triphosphate (dNTP). The reaction mixture was initially incubated at 94°C for 2.5 minutes before undergoing 40 cycles of PCR (denaturation at 94°C for 30 seconds, annealing at 55°C for 15 seconds, extension at 72°C for 2 minutes [codons -5, 583, 795, 1689, and 1696] or 10 minutes [codons 336, 372, and 427]) using a Perkin-Elmer-Cetus Thermal Cycler. Negative controls (PCR mix minus genomic DNA) were included in all amplification experiments as a check for contamination. Ten microliters of the amplification product was electrophoresed on a 2% agarose gel and visualized under ultraviolet light after ethidium bromide staining (Fig 2).

Dot/slot blotting. The amplified DNA was denatured by adding 525 μL. 0.1 mol/L NaOH and incubating at 70°C for 20 minutes,

FIG 2. Agarose gel electrophoresis of PCR-amplified fragments of the factor VIII gene. Lane 1: exon B-intron 8-exon 9 (approximately 700 bp) amplified using JP37 and DM494. Lane 2: exon 1 (124 bp) coamplified with 3' end of exon 14 (213 bp) using DM5 and DM7 (exon 1) and DM9 and DM10 (exon 14). Lane 3: exon 12 (123 bp) amplified using DM496 and 497. Lane 4: 3' end of exon 14 (244 bp) amplified using DM639 and DM642.
then neutralized with 600 μL 0.1 mol/L HCl/10 × saline sodium citrate (SSC) (1 × SSC = 150 mmol/L NaCl/15 mmol/L sodium citrate). Four hundred-microliter DNA samples were applied under suction and in triplicate to Genescreen nylon membranes (DuPont-NEN, Stevenage, England) using a Slot-Blot or Dot-Blot manifold (BRL, Uxbridge, England). After rinsing the slots with 100 μL 5 × SSC, the DNA was covalently bound to the membrane either by exposure to 120 mJ ultraviolet radiation (Stratalinker, Stratagene, La Jolla, CA), or by heating in a vacuum oven at 80°C for 2 hours.

**Discriminant oligonucleotide hybridization (DOH).** For every site to be studied, three oligonucleotide probes were designed to correspond to sequences containing either (a) the wild-type (CpG), (b) a C → T transition (TpG), or (c) a G → A transition (CpA) within that sequence. Oligonucleotides were synthesized on an Applied Biosystems (Warrington, England) 380A Synthesizer, and purified by gel filtration through Sephadex G50 columns (Nick columns, Pharmacia, Uppsala, Sweden).

Oligonucleotide probes (40 ng), were end-labeled by incubation with 70 μCi γ - 32P-adenosine triphosphate (3,000 Ci/m mole; Amersham, England) and 5 U T4 polynucleotide kinase at 37°C for 30 minutes in 50 mmol/L Tris-HCl (pH 7.4), 10 mmol/L MgCl2, 5 mmol/L dithiothreitol (DTT), and 10 mmol/L spermidine. Labeled probes were purified by gel filtration through Sephadex G50 columns (Nick columns, Pharmacia, Uppsala, Sweden).

Membranes were prehybridized for 4 hours at 33°C in 10 mL of 0.1% bovine serum albumin, 0.1% polyvinylpyrolidone, 0.1% ficoll 400, 0.1% sodium dodecyl sulphate, and 200 μg/mL sheared herring sperm DNA. Membranes were then hybridized overnight (16 hours) under identical conditions in the presence of 1 to 2 × 107 disintegrations per minute 32P-labeled oligonucleotide probe. Membranes first were washed at 33°C and then at temperatures empirically determined to optimize discriminant hybridization of the oligonucleotide probes (Table 1). The washing buffer was 3 mol/L tetramethylammonium chloride (TMACl), 50 mmol/L Tris-HCl (pH 8.0), 4 mmol/L EDTA (pH 8.0), and 0.1% sodium dodecyl sulphate. TMACl was used in the hybridization wash because, by binding A-T base pairs selectively, it avoids the preferential melting of A-T over G-C base pairs and thereby removes the effect of base composition on oligonucleotide hybridization.16 Dot blots probed with wild-type and mutant oligonucleotides were autoradiographed after washing, both at nondiscriminant and discriminant temperatures. This permitted assessment of initial hybridization and subsequent differential melting of the oligonucleotide probes and allows us to be confident that we have detected all the potential mutations in our patient sample. Wherever possible, known mutant DNAs were used as positive controls.

**Analysis of restriction fragment length polymorphism (RFLP).** Where identical mutations were detected, four intragenic RFLPs were analyzed. The XbaI and BclI polymorphisms were amplified by PCR using oligonucleotides described by Kogan et al.17 Thirty microliters of the amplification product were digested with XbaI or BclI and electrophoresed through 2% agarose or 12% polyacrylamide gels and visualized under ultraviolet illumination. The BgIII and MspI14 RFLPs were analyzed by digestion of patient DNA with the relevant enzyme followed by Southern blotting and hybridization to probes 131BH (a 1.2-kb BamHI/HindIII fragment of exon 26) or p625.3, respectively.

**Direct sequencing.** PCR-amplified material was purified using GeneClean (Bio 101, La Jolla, CA). Appropriate oligonucleotide probes were synthesized using Applied Biosystems, and fluorescently labeled with 70 μCi γ - 32P-adenosine triphosphate (3,000 Ci/m mole; Amersham, England). The relevant enzyme followed by Southern blotting and hybridization to probes 131BH (a 1.2-kb BamHI/HindIII fragment of exon 26) or p625.3, respectively.

**Table 1. Oligonucleotides and Washing Temperatures Used for Discriminant Hybridization**

<table>
<thead>
<tr>
<th>Codon Number</th>
<th>Exon</th>
<th>DNA Sequence</th>
<th>Amino Acid</th>
<th>Oligonucleotide (5' → 3')</th>
<th>Washing Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>CGA</td>
<td>Arg</td>
<td>JP1 CTGTGCCTTTTGGCATCTGT</td>
<td>63</td>
</tr>
<tr>
<td>336</td>
<td>8</td>
<td>CGA</td>
<td>Arg</td>
<td>JP4 GGAACCCCAACTAGATGGA</td>
<td>64</td>
</tr>
<tr>
<td>372</td>
<td>8</td>
<td>CGC</td>
<td>Arg</td>
<td>JP7 CCTTCTCAAAATTGCTCAG</td>
<td>63</td>
</tr>
<tr>
<td>427</td>
<td>9</td>
<td>CGA</td>
<td>Arg</td>
<td>JP10 AAAGTCGATTATGCGATAC</td>
<td>63</td>
</tr>
<tr>
<td>583</td>
<td>12</td>
<td>CGA</td>
<td>Arg</td>
<td>JP13 TGAACAAGGAAAGCTGACC</td>
<td>64</td>
</tr>
<tr>
<td>795</td>
<td>14</td>
<td>CGC</td>
<td>Arg</td>
<td>JP16 GATGCTCTTGGCAAGAGTC</td>
<td>62</td>
</tr>
<tr>
<td>1,689</td>
<td>14</td>
<td>CGC</td>
<td>Arg</td>
<td>JP19 CAGAGCCCCGGGCTTGT</td>
<td>62</td>
</tr>
<tr>
<td>1,689</td>
<td>14</td>
<td>CGA</td>
<td>Arg</td>
<td>JP22 GAAAAACAGACACT</td>
<td>48</td>
</tr>
</tbody>
</table>

Bases marked in bold type denote the codons screened for C → T and G → A transitions by the discriminant oligonucleotides.
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primers were used for sequencing, either by the method of Green et al., or by using the T7 polymerase kit (Pharmacia).

RESULTS

Screening of patient DNA. A total of 793 unrelated patients with hemophilia A were screened by PCR amplification of DNA sequence surrounding the eight codons of interest (Fig 2), followed by discriminant hybridization with end-labeled oligonucleotides to dot/slot blots of the amplified DNA samples (Fig 3). Six point mutations were detected at codon 336, two at codon 372, one at codon -5, one at codon 427, one at codon 583, one at codon 795, three at codon 1689, and one at codon 1696 (Table 2). The mutation was confirmed in all cases by direct sequencing of PCR-amplified material (data not shown). Mutations were therefore detected in all the codons screened. The phenotypic data for the material (data not shown). Mutations were therefore detected in all the codons screened. The phenotypic data for the occurrence of 5mC in CpG dinucleotides within the factor Vll gene, and the reliability of fragment detection by Taq I remains unknown.

Moreover, whereas in principle the screening of potentially hypermutable CpG dinucleotides at functionally important

degenerations available for study, and no common ancestor was found between this family and those of patients J155 and J242 in the four generations examined. Statistical analysis was therefore performed assuming five recurrent mutations at codon 336, and two at codon 1689. If all codons were equally likely to mutate, the probability of observing at least five mutations in one codon and at least two in another is 12%. Although not statistically significant, this may indicate that codon 336 is hypermutable compared with the other codons.

There is also a skewed distribution of point mutations detected within CpG sites. If we exclude consideration of codon 336 where G → A substitutions may not give rise to hemophilia, eight of the remaining nine-point mutations proven to be independent were C → T transitions (P = .022).

A disproportionate number of these independent mutations occurred in individuals of Indo-Pakistani extraction (3 of 56) as compared with the remainder of the European whites (11 of 717) (Fisher's exact test, P = .074).

DISCUSSION

The detection of high-frequency recurrent C → T and G → A mutations within Taq I restriction sites is consistent with a model of 5mC deamination. Indeed, such “experiments of nature” have provided indirect evidence for the occurrence of 5mC in CpG dinucleotides within the factor VIII gene. However, Southern blot analysis permits only a crude assessment of relative mutation rates at CpG dinucleotides; only seven Taq I sites are available for analysis in the factor VIII gene, and the reliability of fragment detection and resolution is variable. Furthermore, without DNA sequencing, the precise nature of any point mutation detected by Taq I remains unknown. To date, large-scale systematic surveys of CpG mutations in specific genes are lacking. Moreover, whereas in principle the screening of potentially hypermutable CpG dinucleotides at functionally important

![Figure 3](image-url)
positions could prove to be valuable in increasing the efficiency of mutation search procedures, no assessment of the practical utility of this strategy has been reported until now.

There are 36 CpG dinucleotide-containing arginine codons in the factor VIII gene. These include 12 CGA codons and two CGC codons at the two thrombin cleavage sites critical for factor VIII activation as depicted in Fig 1. One of the CGA codons (codon 336) occurs at the cleavage site for activated protein C, which inactivates factor VIII. We have analyzed the five CGA codons located in Taq 1 restriction sites by Southern blotting and will present mutations identified in our patient sample elsewhere.

Six of the seven CGA codons that occur outside of Taq 1 sites and both CGC codons were selected for mutation screening using a combination of PCR and DOH, thereby circumventing most of the problems associated with Taq 1 screening. The remaining CGA codon (1966) is flanked by a repetitive A + T rich sequence, which is likely to reduce severely oligonucleotide hybridization specificity and efficiency, and was for this reason omitted from the study. All sense strand C → T transitions within CGA codons should result in a severe phenotype by producing a termination codon (TGA). Antisense strand C → T transitions (except in codon 336), may cause hemophilia by substitution of glutamine (CAA) for arginine. A C → T transition in the antisense strand at codon 336 may result in a thrombophilic phenotype by preventing factor VIII inactivation. 5mC deamination in the two CGC codons located within thrombin cleavage sites would result in the substitution of cysteine for arginine if the mutation occurs on the sense strand, and histidine for arginine if it occurs on the antisense strand. Both are chemically radical substitutions and would be predicted to abolish thrombin cleavage and hence factor VIII activation.

We have screened eight CpG dinucleotides in the factor VIII genes of 793 hemophilic DNAs, and have detected 16 mutations. We have estimated elsewhere that an average CpG dinucleotide (~80% are methylated) in the human genome is subject to a 12-fold higher frequency of mutation than any other dinucleotide. We have calculated on this basis that a total of 14 mutations would be expected in this survey (see Appendix). The observed hypermutability of CpG in factor VIII is thus comparable with that previously calculated for other genes, and is consistent with the model of deamination of 5mC.

Table 3. Haplotype Analysis of Patients With Recurrent Factor VIII Mutations

<table>
<thead>
<tr>
<th>Codon No.</th>
<th>Bcl I</th>
<th>Xba I</th>
<th>Bgl I</th>
<th>Msp I</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>336</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J84</td>
<td>+</td>
<td>+</td>
<td>5.0</td>
<td>4.3</td>
<td>Pakistan</td>
</tr>
<tr>
<td>J88</td>
<td>+</td>
<td>+</td>
<td>5.0</td>
<td>4.3</td>
<td>Pakistan</td>
</tr>
<tr>
<td>J91</td>
<td>+</td>
<td>-</td>
<td>5.0</td>
<td>4.3</td>
<td>Pakistan</td>
</tr>
<tr>
<td>J138</td>
<td>-</td>
<td>+</td>
<td>20</td>
<td>7.5</td>
<td>USSR/Austria</td>
</tr>
<tr>
<td>J278</td>
<td>+</td>
<td>+</td>
<td>5.0</td>
<td>4.3</td>
<td>UK</td>
</tr>
<tr>
<td>H19</td>
<td>+</td>
<td>-</td>
<td>5.0</td>
<td>4.3</td>
<td>FRG</td>
</tr>
<tr>
<td>1689</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J155</td>
<td>+</td>
<td>+</td>
<td>5.0</td>
<td>4.3</td>
<td>UK</td>
</tr>
<tr>
<td>J242</td>
<td>+</td>
<td>+</td>
<td>5.0</td>
<td>4.3</td>
<td>UK</td>
</tr>
<tr>
<td>J403</td>
<td>+</td>
<td>+</td>
<td>5.0</td>
<td>4.3</td>
<td>UK</td>
</tr>
</tbody>
</table>
We have demonstrated four new examples of recurrent mutation of CpG dinucleotides in the factor VIII gene causing hemophilia A. The most dramatic is at codon 336, where five proven independent cases of a C → T transition were found. Only one C → T mutation (H4) has been previously reported at this site.22 The second example of recurrent mutation is provided by codon 1689, where we have detected two independent C → T mutations, one of which causes moderate hemophilia A. This moderate phenotype contrasts with the severe phenotype of patient (H10) reported with this C → T transition.22,23 The reason for this discrepancy is unclear and is under investigation. The G → A transition found converting Arg 372 to His has been reported in one other unrelated family resulting in mild (factor VIII:C 5%) hemophilia A.24 Finally, a further example of recurrent mutation occurred in the 5' cytosine of the CpG dinucleotide at codon 372; the mutation reported here (described in more detail elsewhere)25 has been reported once before in a similarly affected patient.26

Hitherto undescribed point mutations were also detected in codons 5, 427, 583, 795, and 1696; the C → T base changes in these five patients resulted in the creation of premature termination codons. Because each of these patients has undetectable factor VIII:Ag in his plasma, or antibodies to factor VIII (J397), presumably any truncated protein translated is either nonsecreted or unstable in the circulation. All four previously reported point mutations in patients with inhibitors were termination codons located in the factor VIII light chain. Further studies should determine whether the position of the mutation in the factor VIII gene is of relevance to the development of inhibitors in hemophiliacs.

There may be differences in mutation rates between the CpG dinucleotides examined in this study. We also have noted a probable difference in the overall mutation rate in the eight sites studied between patients of Indo-Pakistani and Caucasian origin. These differences may reflect partial methylation resulting from differential methylation of CpG dinucleotides, either between different germ cells of an individual or between different individuals in a population. The observed between-site differences in mutation rates could be caused by a higher level of methylation (and therefore of 5mC deamination) at some codons than others (eg, codon 336). Similarly, the racial differences could result from a higher overall level of methylation in these codons in Indo-Pakistani as compared with whites. These racial differences, and the fact that sperm remains the sole tissue in which differential methylation of particular CpG dinucleotides has not been demonstrated,27 suggests that we are observing differences in the methylation status of specific sites between individuals.

There is also a disparity in frequency between C → T and G → A mutations within the eight codons studied. Previous studies also have reported a significantly higher number of C → T over G → A substitutions.8,9 There would appear to be three possible reasons to account for this finding: (a) hemimethylation of CpG sites so that the sense strand cytosine is more frequently methylated than the antisense strand cytosine, (b) a strand preference in mutation repair efficiency, or (c) the relative absence of phenotypic effect of G → A substitutions leading to a bias in mutation detection. These explanations are not mutually exclusive.

Arginine residues encoded by CpG-containing codons are a common feature of proteolytic cleavage sites in coagulation factor proteins. Indeed, examples of recurrent mutation at CpG dinucleotides have already been reported in the two factor XIa cleavage sites of factor IX at residues Arg 14528,29 and Arg 180,31 in the factor Xa cleavage site of thrombin at the Arg 271 residue,32,33 and in the thrombin cleavage site of protein C.34 Potentially hypermutable CpG-containing arginine codons are also present in the proteolytic cleavage sites of factors V, VII, X, XI, XII, XIIIa subunit, and β-fibrinogen.35 If these CpG dinucleotides are methylated in the germline, then they may well represent hotspots for mutation leading to removal of the cleavage sites and a consequent bleeding disorder. Recurrent mutation at specific CpG sites has also been shown to be a cause of antithrombin III deficiency, heparin cofactor II deficiency, and dysfibrinogenemia.36-37

If it is eventually demonstrated that CpG mutability correlates with methylation status, it will become advisable to establish the methylation status of specific CpG sites in a given gene before any labor-intensive screening of patient samples is undertaken. Perhaps when the cellular role of cytosine methylation is understood, we shall be nearer to predicting patterns of gene methylation and hence of mutability.

ACKNOWLEDGMENT

We thank the following clinicians, who provided individual blood samples: Drs F. Hill, M. McEvoy, H. Daly, D. Mitchell, R. Vaughan-Jones, M. Winter, D. Bevan, S. Height, G. Savidge, G. Scott, D. Prangnell, M. Adelman, J. Hayes, J. Shirley, M. Stevens, T.K. Lam, and A. Li. We gratefully acknowledge the secretarial assistance of Margaret Runnicles and the statistical advice of Caroline Doré.

APPENDIX

An Estimate of the Number of Mutations Expected When Screening 8 CpG Dinucleotides in 793 Patients

<table>
<thead>
<tr>
<th>Approximate Figures</th>
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<tbody>
<tr>
<td>Proportion of hemophilia A cases due to point mutations3</td>
</tr>
<tr>
<td>Relative mutability of CpG dinucleotide10</td>
</tr>
<tr>
<td>Numbers of CpG dinucleotides not screened</td>
</tr>
<tr>
<td>Number of non-CpG dinucleotides in the factor VIII gene</td>
</tr>
<tr>
<td>Average proportion of base changes giving rise to an amino acid substitution</td>
</tr>
</tbody>
</table>

\[
\text{Approximate Figures} = \frac{8 \times 12}{(8 \times 12) + 0.66 \times (62 \times 12) + 6,982} = 13.9
\]
REFERENCES


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JK Pattinson, DS Millar, JH McVey, CB Grundy, K Wieland, RS Mibashan, U Martinowitz, K Tan-Un, M Vidaud and M Goossens