Previously we created two strains of factor VIII–deficient mice by insertion of a neo gene into (1) the 3' end of exon 16 and (2) exon 17 of the factor VIII gene. Affected mice of both strains have no plasma factor VIII activity, yet are healthy with no spontaneous bleeding. Factor VIII–deficient females bred with affected males survive pregnancy and delivery. We used reverse transcriptase-polymerase chain reaction of liver RNA to characterize factor VIII mRNA processing. Factor VIII mRNA of the exon 16 knockout strain contains neo sequences plus 17 bp of intron 16 due to use of a cryptic donor site in intron 16. All factor VIII mRNA of the exon 17 knockout strain lacks exon 17 and neo sequences. In VIII protein deficiency, and should be useful in gene correction studies with mouse factor VIII cDNA constructs.

Materials and Methods

Genotype determination. After tail snipping, the tail vein was immediately cauterized by silver nitrate to stop bleeding. DNA was then isolated from the tail tissue. Amplifications were performed on 250 to 500 ng of mouse genomic DNA in a 50-μL vol containing 200 nmol/L each primer, 200 mmol/L each dNTP, 10 mmol/L Tris-HCl (pH 8.3), 1.5 mmol/L MgCl2, and 1 U of Taq polymerase. Two PCR reactions were used for genotyping of the factor VIII knockout mice. To confirm the disruption of exon 16 and exon 17, the forward primer was derived from neo sequence (5'TGTGTC-CGGCCGCTTCTTTC3') and the backward primer was derived from exon 17 (5'GAGCATACTCCTGACTGAC3'). Similarly, to confirm the presence of undisrupted X chromosome, the forward primer was derived from the 5' end of exon 16 (5'TGCAAGGCTGGCTTAATTT3') and the backward primer was derived from exon 17 (5'GAGCATACTCCTGACTGAC3'). Since we did not obtain a polymerase chain reaction (PCR) product across the neo cassette of the disrupted chromosome (Fig 1A), Reactions were denatured at 94°C for 6 minutes and then subjected to 30 steps of cycles. For the neo-exon 17 amplification, each cycle consisted of 94°C for 30s, 55°C for 30s, and 72°C for 30s. For the exon 16-exon 17 amplification, each cycle consisted of 94°C for 30s, 54°C for 30s, and 72°C for 90s. Using a combination of these PCR reactions, the genotypes of normal female, carrier female, affected female, normal male, and affected male could be discerned (Fig 1B).

Factor VIII activity assay. A COATEST VIII C4 (Chromogenix AB, Mölndal, Sweden) chromogenic assay was performed as previously described. Factor VIII activity of the test plasma was determined using a standard curve constructed with serial dilutions of normal mouse plasma.

Reverse transcriptase (RT)-PCR of mouse liver RNA. Whole liver dissected from adult mice was immediately lyophilized in liquid nitrogen to prevent adventitious introduction of RNases into the RNA preparation. Total RNA was isolated by homogenization in 4 mol/L guanidium thiocyanate followed by phenol/chloroform extraction. The RNA concentration was determined by spectrophotometry. The authenticity of the RNA isolated was shown by the absence of RNA bands in samples treated with RNase and by the preservation of 18S and 28S RNA in samples treated with DNase. Five, micrograms of total RNA was primed with 0.5 μg oligo (dT)12-18 primers in a 20-μL reaction volume. The first-strand cDNA synthesis reaction was catalyzed at 42°C by 200 U of superscript II RNase H-reverse transcriptase (GIBCO-BRL, Gaithersburg, MD). To maximize the size of cDNA synthesized, the ratio of the primers to RNA was...
determined empirically for each RNA preparation. After first-strand cDNA synthesis, target cDNA was amplified in a 100-μL reaction volume using two specific oligonucleotides and Taq DNA polymerase. Thermal cycling parameters consisted of a preamplification denaturation of 3 minutes at 95°C followed by 30 cycles of 95°C for 30 seconds, 55° to 65°C annealing for 30 seconds, and extension at 72°C for 45 to 60 seconds per 1 kb of target length. The RT-PCR products were then cloned into the TA-vector (Invitrogen, San Diego, CA) for subsequent characterization. Four pairs of PCR primers were used: one pair amplified a region of exon 14 (P-14a and P-14b); one pair amplified a region of exon 26 (P-26a and P-26b); one pair amplified a region from exon 15 to exon 18, across the knockout exons (MC-15 and MC-18); and one pair amplified from the neo sequence to exon 17 (R-neo and MC-17).

**Production of factor VIII MoAbs.** Two exon-16 factor VIII knockout mice were immunized by human recombinant factor VIII with complete Freund’s adjuvant. Immediately after the third injection, both mice died, apparently from anaphylactic shock. Spleens were dissected and splenocytes were homogenized through a homogenizer with 80-mesh screen. Splenocytes were pelleted by centrifugation at 3000 g for 15 minutes incubated in 5 mL lysis buffer (0.17 mol/L NH₄Cl, pH 7.5) on ice for 8 minutes to lyse erythrocytes. After addition of 5 mL serum-free HY medium (90% high-glucose Dulbecco’s Modified Eagle Media [D-MEM], 10% NCTC135), cells were repelleted, and 1.1 x 10⁶ splenocytes were mixed with 3 x 10⁷ Sp2/0Ag14 myeloma cells. Fusion was performed according to the procedure of Lane et al.¹³ using Kodak PEG 1450 as fusion agent. Fusion products were resuspended in 300 mL complete HY consisting of 70% HY medium, 20% fusion-tested, fetal calf serum (GIBCO), 4 mmol/L L-glutamine, 0.15 mg/mL oxaloacetate, 50 μg/mL pyruvate, 0.2 U/mL insulin, 5% Origen hybridoma growth supplement (IGEN, Rockville, MD), 100 U/mL penicillin, 0.2 U/mL streptomycin, 0.1 mmol/L Hypoxanthine, and 6 mmol/L azaserine. The suspension was distributed at 150 μL per well into 20 96-well culture plates, fed with 100 μL complete HY medium without azaserine and with 2% Origen on day 6, and yellow supernatants were obtained starting on day 10.

**Enzyme-linked immunosorbent assay (ELISA) screen.** Harvested supernatants were tested by an ELISA screen¹⁴ using a variation of standard procedures. Falcon Pro-Bind ELISA plates (Becton Dickinson Labware, Oxnard, CA) were coated with 50 μL of 0.2 μg/mL human recombinant factor VIII for 2 hours at 20°C, and blocked with 5% instant milk/0.05% Tween-20 (Fisher Biotech, Fair Lawn, NJ) for 2 hours. Plates filled with blocking solution were stored at −20°C and used as needed. Thawed plates were rinsed with Ca/Mg-free phosphate-buffered saline (PBS) (GIBCO), incubated for 1 hour at 25°C with 50 μL of test supernatant, washed three times for 10 minutes with PBS, blocked again for 10 minutes, incubated with 50 μL of a 1/2,000 dilution of peroxidase conjugated goat anti-mouse IgG (BMB; Boehringer Mannheim Corp, Indianapolis, IN) in blocking solution for 30 minutes washed again three times for 10 minutes with PBS, and 100 μL of the ABTS substrate was added to each well. Color development was measured on a Titer Tek Multiscan plate reader (Flow Laboratories Inc, McLean, VA) at 405 nm. Hybridomas from positive wells were cloned by limiting dilution in complete HY without azaserine, and supernatants were restested by ELISA as described above.

**Purification of factor VIII from mouse plasma.** Glycine cryoprecipitation was modified for the production of factor VIII concentrate from mouse plasma.¹⁵ Mouse blood was obtained via cardiac puncture and collected in 0.16 mol/L sodium citrate at a final ratio of 1:10 (anticoagulant:blood). Plasma was frozen at −80°C overnight and warmed to 0°C in an ice bath. One milliliter of mouse plasma was centrifuged at 7,000g for 20 minutes at 0°C the precipitate was washed with 300 μL ice-cold 0.02 mol/L Tris-Cl, pH 7.0, and recentrifuged at 7,000g for 20 minutes. The precipitate was then redissolved in 30 μL of 0.02 mol/L Tris-Cl buffer, pH 7.0 at 20°C, then 75 μL of 2.8 mol/L glycine buffer (2.8 mol/L glycine/0.3 mol/L sodium chloride 0.025 mol/L Tris, pH 6.8) was added and the suspension was held at 20°C for 30 minutes before centrifugation at 7,000g for 10 minutes to remove the precipitate. Twelve to eighteen microliters of supernatant containing mouse factor VIII was used for Western blotting.

**Western blot.** Western blot was performed on human recombinant factor VIII and mouse cryoprecipitated factor VIII. Samples

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**Fig 1. Genotyping of factor VIII knockout mice.** (A) By PCR, a 680-bp fragment was amplified from the normal X chromosome using primers outside of the disrupted region. Either a 420-bp or a 150-bp fragment was amplified from exon 16- or exon 17-disrupted X chromosomes using primers containing the neo sequence (neo-R). (B) Products from the two PCR reactions were combined and loaded on a 1% agarose, 2% Nusieve gel. All possible genotypes (normal female, carrier female, affected female, normal male, and affected male) were easily determined.
RESULTS

Affected mice have a mild phenotype. The oldest factor VIII-deficient mice are now about 1 year old, and they are healthy without spontaneous bleeding, illness, or reduction in activity. The only disease phenotype observed has been excessive bleeding after tail snipping. Roughly 2/3 of affected males die within 2 hours of tail snipping, whereas all wild-type and carrier mice survive without difficulty. Female carriers were bred with affected males of the F2 generation to produce factor VIII-deficient females in the F3 generation. Further breeding of these deficient females with affected males produced litters in which 100% of the animals have factor VIII deficiency. Three factor VIII-deficient females have been bred with affected males. All three survived pregnancy and delivery, and had litters of 3 to 5 pups. All newborn mice of these litters had defective factor VIII genes as determined by PCR of genomic DNA isolated from the tail snips and less than 1% factor VIII activity by COATEST assay.

FVIII mRNA processing in deficient mice. Using an RT-PCR assay of total liver RNA, exon 26 was present in liver RNA in both exon 16 and exon 17 knockout mice. Although transcription initiated from the B promoter located in intron 22 is not ruled out, the data suggest that factor VIII mRNA in these mice is stable (Fig 2A). While the amount of exon 14 (an exon 5' to the neo cassette insertions) appeared normal in exon 17 knockout mice, no RT-PCR product derived from exon 14 was observed in exon 16 knockout mice. When primers crossing the neo insertions were used, two amplified fragments in exon 17 knockout mice were found and they were smaller than those of normal mice. In this PCR, no amplified fragments were seen in exon 16 knockout mice (Fig 2A). PCR using primers from the neo cassette to exon 17 gave a 200-bp product in exon 16 knockout mice, but no PCR product in exon 17 knockout mice. All of these results can be explained if (1) neo cassette sequences are difficult...
to reverse transcribe and amplify by PCR with our primers because of their high G + C content (64%) and (2) neo cassette sequences remain in transcripts of exon 16 knockout mice but are deleted from transcripts of exon 17 knockout mice. To determine the structure of the various transcripts, RT-PCR fragments were then subcloned, sequenced, and the splice sites were compared with mouse cDNA sequence. In exon 16 knockout mice, the donor site in intron 16 was deleted during insertion of the neo gene. Essentially all factor VIII mRNA of this strain contains the neo sequence plus 17 bp of intron 16 due to use of a cryptic donor site in intron 16 (ATG/GTAAGC) (Fig 2B). In exon 17 knockout mice, the neo cassette was inserted into the exon. All factor VIII mRNA of this strain lacks both exon 17 and neo cassette sequences. Two donor sites are used in the skipping of exon 17; the intron 16 donor site and a cryptic donor site 46 bp from the 3' end of exon 16 (AAG/GTCTT) (Fig 2B).

Production of MoAbs that react to mouse factor VIII. We decided to produce antibodies to mouse factor VIII because no commercial antibody is available that cross-reacts with mouse factor VIII and we needed a means to measure small amounts of mouse factor VIII protein. Human recombinant factor VIII was used as an antigen in deficient mice to generate MoAbs against mouse factor VIII. The rationale for using human factor VIII injections into deficient mice was that all epitopes of human factor VIII would be recognized as foreign, and some epitopes would be shared between human and mouse factor VIII. The shared epitopes should stimulate production of antibody that cross-reacts with mouse factor VIII.

The two exon 16 knockout mice that were immunized by human recombinant factor VIII both died of anaphylaxis after the third injection indicating a strong immune response to human recombinant factor VIII. We were fortunate to have Sp2 myelomas in culture, and used them for a fusion with splenocytes from the two immunized mice. Hybridoma cell lines were grown and the supernatants containing antibodies were tested for the presence of anti-human factor VIII by ELISA. Sixty-six positive cell lines were obtained. These 66 lines were then assayed by Western blot for their ability to detect factor VIII in cryoprecipitated plasma of normal mice. Two of these anti-human factor VIII antibodies cross-reacted with mouse factor VIII light chain. Of these MoAbs, one was identified as an IgM while the other was an IgG1.

Factor VIII light chain is undetectable in knockout mice. Figure 3 shows the factor VIII protein levels in normal mice, female carriers, and the factor VIII-deficient mice as determined by Western blot using anti-mouse factor VIII IgG1. Factor VIII was cryoprecipitated from the plasma of normal mice, carrier females, and the two knockout strains. Cryoprecipitate derived from 100 μL of plasma was loaded in each lane. Factor VIII light chain was undetectable in the plasma of either factor VIII knockout strain (Fig 3). When cryoprecipitate from normal plasma was diluted with cryoprecipitate from deficient plasma, the factor VIII in a 10% normal:90% deficient sample was easily detected, indicating that this procedure should detect factor VIII protein in 10 μL of normal mouse plasma or 20 μL of whole blood (data not shown). Because 200 μL of whole blood is readily obtained by tail snipping, this protein assay should be useful in the detection of factor VIII after gene correction.

DISCUSSION

The lack of spontaneous bledding coupled with long-term survival of factor VIII knockout mice implies that the phenotype of severe factor VIII deficiency in mice is significantly milder than in human beings. The fact that affected female mice are able to proceed normally through pregnancy and delivery suggests that mice do not require factor VIII to prevent bleeding after delivery. It also suggests that there may be either an alternative to the use of factor VIII as a cofactor of factor IX or an alternative pathway which promotes clotting in the absence of factor VIII except in extreme situations, such as a snipped tail. Because mice without factor VIII are healthy, we are able to produce a line of hemophilic mice and dispense with the need to genotype the pups. Additionally, the ability to produce a factor VIII-deficient line of mice should be quite useful for gene correction studies, providing more test animals per breeding.

The mRNA processing events observed in the knockout strains indicate that factor VIII deficiency is due to truncated protein in exon 16 knockout mice and to both truncated protein and deleted protein in exon 17 knockout mice. In exon 16 knockout mice, translation of the RNA containing a neo cassette leads to nonsense codons in the neo cassette sequence. In exon 17 knockout mice, translation of the two RNAs lacking exon 17 sequences leads to frameshifting in the case of the RNA with simple skipping of exon 17, and
deletion of exon 17-encoded amino acids with addition of 16 new amino acids in the case of the RNA formed by use of the cryptic donor site in intron 16. In humans and mice, exons 16 and 17 encode a portion of the A3 domain that is at the N terminus of the light chain. Thus, factor VIII heavy chain is probably made in the knockout mice, but its stability is unknown.

By Western blot, both anti-mouse factor VIII antibodies recognize a protein of 70 kD, the molecular weight of the factor VIII light chain. It is interesting that so few anti-human factor VIII antibodies crossreact with mouse factor VIII (only 2 of 66). However, extensive further screening of the antibodies produced by the hybridoma cell lines with cryoprecipitated mouse plasma would probably show other MoAbs not detected by the initial screening. In any case the small fraction of cross-reacting antibodies suggests that an important immunogenic epitope of the human protein is not conserved in mouse factor VIII. As discussed above, factor VIII heavy chain could be produced in both knockout strains. Whether this truncated form of factor VIII is stable is unknown. Since our two antibodies to mouse factor VIII cDNA is used.

VI11 heavy chain could be produced in both knockout strains. To the light chain of the protein, we do not know whether cryoprecipitation will be a boon to gene correction studies. MoAbs not detected by the initial screening. In any case the conserved in mouse factor VIII. As discussed above, factor VIII-deficient mice. In any case, our ability to detect small amounts of intact factor VIII in mouse plasma after simple cryoprecipitation technique for the preparation of factor VIII concentrate of high purity and high stability. Vox Sang 52:10, 1987.

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