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Carrier Detection of Ovine Hemophilia A Using an RFLP Marker, and Mapping of the Factor VIII Gene on the Ovine X-Chromosome

W. Backfisch, S. Neuenschwander, U. Giger, G. Stranzinger, and V. Pliška

Ovine hemophilia A is an X-linked recessive bleeding disorder. For diagnostic purposes, restriction fragment length polymorphism (RFLP) analysis in the region of the factor VIII (F-VIII) gene was carried out using human F-VIII gene probes. The probe St14, known to detect a highly polymorphic region that is closely linked to the F-VIII gene in humans, hybridized nonspecifically with DNA from sheep. Searching for intragenic RFLPs, the entire 9.0-kb coding sequence of the human F-VIII was used as a probe. Using the 1.8-kb SstI/KpnI F-VIII cDNA probe for hybridization, an MspI-RFLP with allelic bands of 5.8 kb (A1) and 4.2 kb (A2) was detected. A1 was in linkage phase with the mutated allele responsible for hemophilia A. The F-VIII locus in the sheep genome was assigned to the long arm of the X-chromosome in the region Xq24-q33, using in situ hybridization with a 3-kb human F-VIII cDNA probe to QFQ banded sheep metaphase chromosomes.

Recently, we have described an inherited coagulopathy caused by a deficiency of the blood coagulation factor VIII (F-VIII) in a family of White Alpine Sheep. Investigations of affected animals showed that clinical signs of bleeding (including severe hemarthrosis), low plasma activity of the F-VIII (<1%), high mortality rate, and X-chromosomal recessive mode of inheritance are comparable to that seen in human patients with severe hemophilia A. Replacement therapy of hemophilic sheep with human F-VIII concentrate resulted in the remission of the coagulopathy and in rapid clinical improvement. Thus, these sheep may become a suitable animal model of human hemophilia A (Neuenschwander et al. 1992; Neuenschwander and Pliška 1990; Pliška et al. 1982). We have preserved this hemophilia model by establishing an animal colony from one female carrier. Because the determination of plasma F-VIII activity does not reliably differentiate between normal females and carriers (Neuenschwander and Pliška 1990), only the birth of a hemophilic lamb confirmed the carrier status of the ewes; however, this breeding and selection practice was lengthy and costly. Heterozygote detection of hemophilia A in humans has been successfully achieved by using restriction fragment length polymorphism (RFLP) and a variable number of tandem repeat (VNTR) markers within or adjacent to the F-VIII gene (Antonarakis and Kazazian 1988). In the study presented here, we used RFLP markers to detect carriers of the F-VIII mutation in the established sheep colony. Furthermore, the F-VIII gene was assigned to a specific region of the ovine X-chromosome by in situ hybridization using a human F-VIII cDNA probe.

Materials and Methods

Animals

A hemophilic ovine colony was established from one carrier ewe (White Alpine Sheep, ewe No. 157; Neuenschwander et al. 1992); it consisted of 99 offspring within four generations. Twelve unrelated normal rams were used for matings. Carrier ewe 711 (Figure 1) and three unrelated rams (B, D, I) were used to test the suitability of individual restriction enzymes for RFLP analysis.

Blood Coagulation Tests

Diagnosis of hemophilia A rested, on one hand, on the occurrence of typical clinical signs of hemophilia A and/or postmortem findings (subcutaneous hematomas, hemoperitoneum, hemarthrosis, prolonged umbilical bleeding, etc.; Neuenschwander and Pliška 1990) and, on the other hand, on coagulation tests using citrated plasma from 2–3-week-old animals. Activated partial thromboplastin time (PTT) and plasma F-VIII activity, relative to a pool of normal ovine plasma, were routinely de-

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Journal of Heredity 1994,85:474–478, 0022-1503/94/$5.00

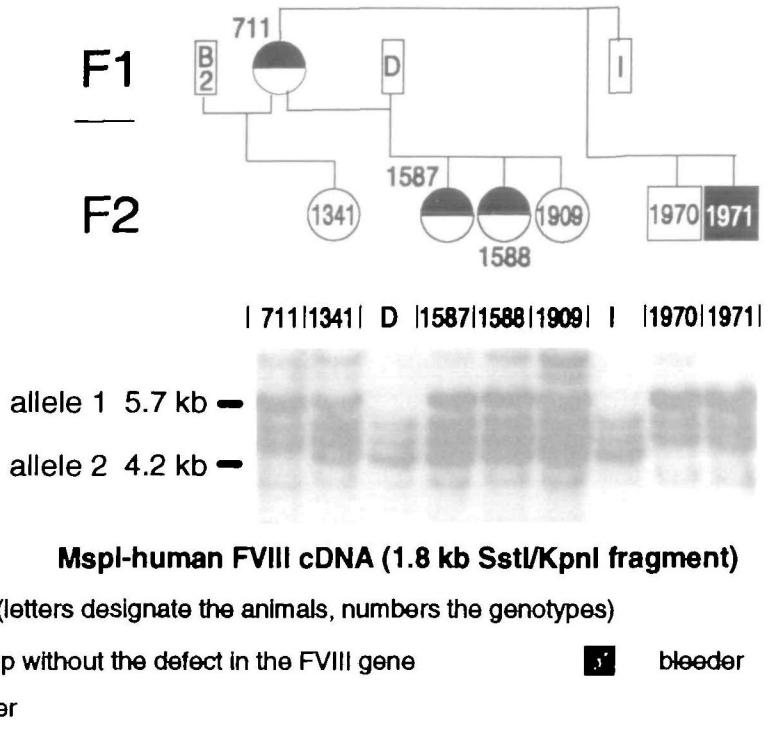

Figure 1. Pedigree of the ewe 711 (F_1 generation) and its offspring Animal numbers correspond to the scheme employed at the Experimental Station of the ETH Zürich, in Chamau ZG, Switzerland. Southern blot hybridization patterns for individual animals are shown. The A-type of the ram B could be deduced from the heterozygous status of its female offspring 1341 (A1-A2) and the homozygous status of the mother ewe 711 (A1) (DNA of this animal was no longer available at the time of our hybridization experiments.)

termined by methods described earlier (Neuenschwander et al. 1992; Neuenschwander and Pliška 1994). Based on these tests, male offspring could be separated into two distinct groups: hemophiliacs with F-VIII activity of <5% of control and prolonged PTT, and normal males with normal F-VIII activity as well as normal PTT. Female "carriers" were defined as animals that produced at least one hemophilic male, had intermediary to normal F-VIII activity, and had normal to slightly prolonged PTT (Neuenschwander et al. 1992).

DNA Probes

St14, a 3.0-kb EcoRI insert in pBR 322, detects a hypervariable VNTR locus in humans that is closely linked to the F-VIII gene (Oberle et al. 1985). The hybrid plasmid pF8.1 contains the entire human F-VIII 9.0-kb cDNA, inserted blunt-ended into the SmaI sites of pUC12. These DNA probes were provided by Dr. I. Oberle, Institut de Chemie Biologique, F-67085 Strasbourg, France (St14), and by Dr. J. Gitschier, Howard Hughes Medical Institute, University of California at San Francisco, San Francisco, CA 94143-0724 (pUC12). Digestion of pF8.1 with SstI and SalI yields a 3.0-kb and a 6.0-kb fragment (inserts) as well as a 2.7-kb fragment (vector). Additional digestion with KpnI cleaves the 3-kb fragment into 1.8-kb and 1.2-kb fragments. The probes were isolated from the vector by preparative low melting agarose gel electrophoresis (Gitschier et al. 1984).

DNA Analysis

DNA samples were prepared by the procedure of Jeanpierre (1987) from fresh or frozen venous blood collected in EDTA-containing tubes. DNA samples (10 μg) were completely digested using the restriction enzymes ApaI, BamHI, BclI, BglII, EcoRI, HaeIII, HindIII, HinfI, KpnI, MspI, PstI, PvuII, RsaI, SalI, and SstI, under the conditions specified by the enzyme supplier (Boehringer Mannheim). The resulting fragments were separated according to their molecular weight by agarose gel electrophoresis (0.6–1.5%). Southern blotting to Hybond-N nylon filters (Amersham) and hybridization were carried out as described by Davies et al. (1988). The filters were washed three times in 2 × SSC (Maniatis et al. 1982) and 1% SDS (sodium dodecylsulphate) at 65°C. Autoradiography was carried out at −80°C using Fuji R X-ray film for 0.5–5 days, with intensifying screens. Data analysis for the detection of genetic linkage in families was performed by the lod-score method according to Morton (1955).

Chromosome Preparation and Identification

Metaphase chromosomes from cultured peripheral blood lymphocytes of a normal ram (G in Figure 2) were prepared according to Fries et al. (1986). Chromosomes were stained by QFQ banding technique (Caspersson et al. 1968) and metaphase spreads were photographed before hybridization. The identification of the chromosomes was based on the ovine karyotypes presented by Long (1985) and DiBerardino et al. (1989).

In situ Hybridization

Radioactive labeling of the human 3.0-kb F-VIII cDNA probe was performed by random primed method (Feinberg and Vogelstein 1983, 1984) modified for tritium labeling (Lin et al. 1985). The labeled DNA was separated from nonincorporated nucleoside triphosphates on a Sephadex G 50 column. The DNA was recovered by ethanol precipitation in the presence of salmon sperm DNA (10 μg). The specific radioactivity of the DNA probe was 2.5 × 10 dpm/μg. The procedure for in situ hybridization was based on the method of Harper and Saunders (1981) as modified by Fries et al. (1988). The concentration of the DNA probe was 20 ng/ml.

Analysis of the Silver Grain Distribution

After the QFQ banding, metaphases of the preparations were photographed. The autoradiographic silver grains which contacted individual chromosomes were plotted on the ideogram of the haploid ovine genome (Gunawardana 1991). The different hybridization information concerning autosomes and the sex chromosomes in a histogram was taken into account.

Results

Southern Blot Analysis

Southern blot analysis with the DNA probe St14 yielded only unspecific hybridization with sheep DNA. Genomic blots of digested sheep DNA with 15 different enzymes showed five to 17 distinct bands after hybridization with the full-length F-VIII cDNA probe, depending on the type of the enzymes used (data not shown). The F-VIII gene in sheep seemed to be "hypopolymorphic," similar to that of human (Antonarakis et al. 1985; Gitschier et al. 1985a; Wion et al. 1986). An MspI-RFLP could be

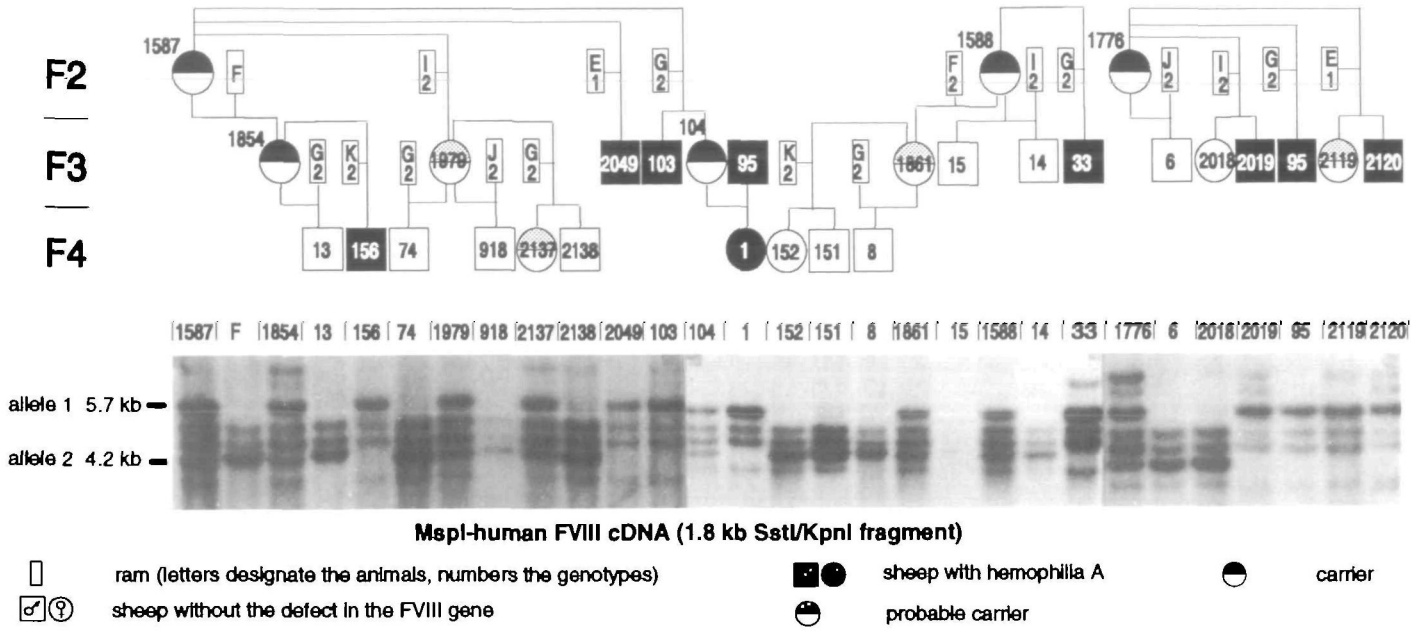

Figure 2. Pedigree of the ewes 1587, 1588, and 1776 (F_2 generation) and their offspring, and the Southern blot hybridization patterns of individual animals

detected using the 1.8-kb SstI/KpnI F-VIII cDNA probe (Figures 2 and 3). The lengths of the allelic RFLP bands were 5.8 kb (allele 1, A1) and 4.2 kb (allele 2, A2), respectively.

Pedigree Analysis

The entire colony consisted of 60 females and 39 males. Ten male bleeders have occurred within the male progeny. Within the female progeny, four ewes were identified as carriers of the defective F-VIII gene, based on the occurrence of hemophilic males in their progeny. A female hemophilic animal was produced by mating a hemophilic ram (No. 95; cf. Figure 2) with a female carrier (No. 104).

Based on the Southern blot analysis shown in Figure 1, the carrier ewe 711 has only one band at 5.8 kb (A1) and no band at 4.2 kb (A2), suggesting that this animal is homozygous for the allele A1. The healthy rams D and I have only the 4.2-kb band. The segregation of the A1 allele marker to the clinically normal son 1970 and the bleeder 1971 without an A2 allele indicates a typical X-chromosomal inheritance pattern: (1) Both sons show the same banding pattern as their homozygous mother, and (2) the father's allele A2 does not appear in the profile of the sons. Thus, one of the three combinations of the alleles A1/A2 and H/h (*H* stands for the wild type and h for the mutant allele) shown in Figure 3 exists on the X chromosome within the bleeder family: A1-h, A1-H, and A2-H.

As can be concluded from the segregation of the MspI-RFLP marker and the disease in 14 informative meioses, hemophilia A always cosegregated together with A1 (Figures 1 and 2): thus, A1 in the bleeder family is in linkage phase with the hemophilia locus. No recombination events were observed. Six bleeders (95, 2019, 2120, 33, 103, and 2049) and two carriers (104, 1854) in the F_3 generation inherited the defective F-VIII gene, together with allele A1, from their mothers. Furthermore, in informative matings ewes 1854 and 104 transmitted the disease together with A1 to the offspring 1 and 156 (A1-h). In contrast, the four male lambs 6, 13, 14, and 15 inherited the normal F-VIII allele together with the A2 allele (A2-H). As predicted, the mutated site in the F-VIII gene and the MspI-RFLP marker are closely linked. The lod-score was $Z = 4.21$. The recombination frequency of the linkage group was with 95% probability within the interval (q1; q2) = [0.0; 0.15]. The statistic confidence to predict the status of a carrier or a noncarrier ewe was 85%. This low predictive value was due to the limited number of studied animals. Considering the fact that the MspI-RFLP is an intragenic marker, the confidence should be much higher.

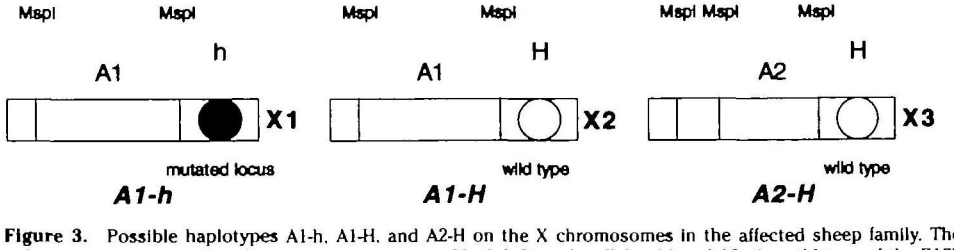

Figure 3. Possible haplotypes A1-h, A1-H, and A2-H on the X chromosomes in the affected sheep family. The polymorphic cleavage site of the restriction enzyme MspI defines the alleles A1 and A2; the wild type of the F-VIII gene is designated by H, and the mutated site by h

In situ Hybridization

The silver grain distribution in 88 metaphases from a normal ram was analyzed. In 40 metaphases (45.5%), the X chromosome was labeled with one or more grains. From 934 grains analyzed, 67 (7.2%) were located on the X chromosome. The histogram of the grain distribution is shown in Figure 4. The relative length of the X chromosome in the diploid chromosome set in males is 2.64% (Hediger 1988). Thirty-six grains (3.9%) were concentrated in the region Xq24-q33, with the relative length of roughly 0.6% of the total chromosome length in a male diploid chromosome set. A maximum of 19 grains were located at the passage of band q31 to q32. The second highest not flanking columns with eight grains were found at various loci of several autosomes. Consequently, the

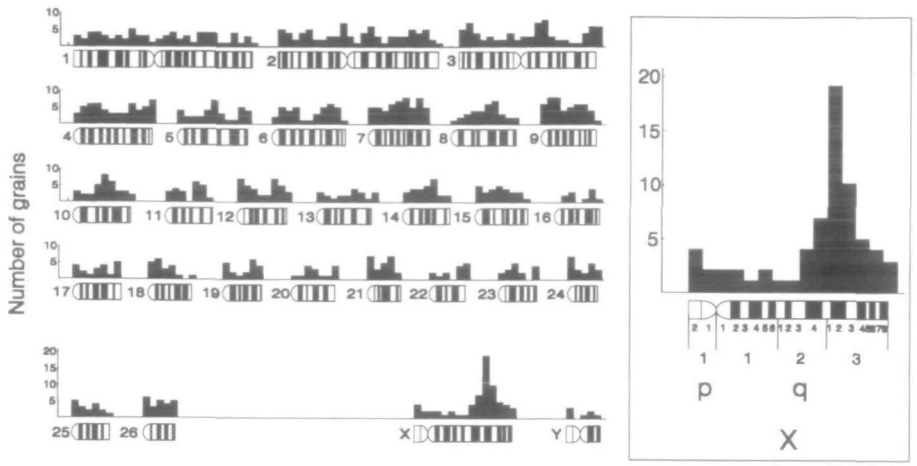

Figure 4. Localization of the F-VIII gene in sheep Histogram of silver grain distribution in 88 metaphases after hybridization with a human 3kb F-VIII cDNA probe A peak was found on the X chromosome in the region Xq24-q33

highest grain column was 2.4 times higher than the second highest nonflanking column.

Discussion

Cloning of the F-VIII genes of human hemophiliacs within individual families reveals many different mutations associated with this disease (Tuddenham et al. 1991). The type of mutation correlates with the severity of clinical signs. Point mutations causing incomplete transcription of the gene by introduction of a stop codon into the coding sequence (Antonarakis et al. 1985; Gitschier et al. 1985b; Youssoufian et al. 1986) resulted in a severe form of hemophilia A. Substitution of a single amino acid by a point mutation found at least in one patient caused only a mild coagulopathy (Gitschier et al. 1986). Base insertions (Kazazian et al. 1988) and deletions (Gitschier et al. 1985b; Youssoufian et al. 1987), causing mostly severe coagulopathies, have also been detected. The molecular basis of mutations in hemophilic animals, on the other hand, has not yet been determined. Undoubtedly, the comparison of wild types as well as mutant F-VIII gene in humans and animals may offer an interesting contribution to the phylogeny and structure-function relationship of coagulation factors. Results presented in this communication document at least a certain homology within the F-VIII gene in human and in sheep: the human probe used in our study interacted with the sheep DNA both in Southern blot analysis and in in situ hybridization, also indicating a conservative evolution of this blood clotting factor.

The results of this family study demonstrated the value of RFLP markers for the carrier detection of X-linked, recessively inherited hemophilia A. Programmed mating and indirect genotype diagnosis allow one to predict the status of each ewe within the family shortly after birth, with a high degree of reliability. No recombination between an intragenic marker and a mutated locus in the F-VIII gene was described in humans (Antonarakis and Kazazian 1988). The distance of two loci of 1cM (i.e., 10 bp) is equivalent to $q = 0.01$. The recombination frequency between the 5′ and 3′ end of the human F-VIII gene with about 200 kb length corresponds to the value $q = 0.002$. If the length of the F-VIII gene of sheep is similar to that in humans, the status of the animals, based on the MspI-RFLP, could be predicted with a minimal confidence of 99.8%. Clearly, the use of this RFLP marker has greatly improved the accuracy of carrier detection as well as permitted a neonatal diagnosis of hemophilia in this colony. This is, however, valid solely within the investigated family.

Mapping the ovine F-VIII gene by in situ hybridization we have found the highest cumulation of silver grains in the Xq24-q33 chromosomal region, following the band numbering by Hediger (1988). This result confirms the localization of F-VIII on the X-chromosome. Confirmation of the specific assignment should be done with a homologous F-VIII probe.

The MspI-RFLP is the first marker physically mapped on the X chromosome in sheep. Using this marker, the F-VIII gene could be genetically mapped in the future, provided that further polymorphic loci will be located in its vicinity.

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Received April 14, 1993
Accepted March 30, 1994