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The Genetic Locus for Free Sialic Acid Storage Disease Maps to the Long Arm of Chromosome 6

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Summary

Salla disease (SD), or adult-type free sialic acid storage disease, is an autosomal recessive lysosomal storage disorder characterized by impaired transport of free sialic acid across the lysosomal membrane and severe psychomotor retardation. Random linkage analysis of a sample of 27 Finnish families allowed us to localize the SD locus to the long arm of chromosome 6. The highest lod score of 8.95 was obtained with a microsatellite marker of locus D6S286 at $\theta = .00$. Evidence for linkage disequilibrium was observed between the SD locus and the alleles of three closely linked markers, suggesting that the length of the critical region for the SD locus is in the order of 190 kb.

Introduction

Sialic acid (N-acetylneuraminic acid) is the terminal sugar in the oligosaccharide side chains of several glycoproteins and glycolipids (Gahl et al. 1989). The lysosomal processing of sialic acid consists of two stages, both of which involve sites of genetically determined inborn errors of the metabolism. Terminal sialic acid is cleaved by lysosomal neuraminidase, which is deficient in two rare lysosomal storage diseases, sialidosis (O'Brien 1977) and galactosialidosis (D'Azzo et al. 1982). Subsequently, an active proton-driven and substrate-specific transport system (Mancini et al. 1989) carries the liberated free sialic acid through the lysosome membrane for reprocessing into cytosol. A defect in this transport mechanism leads to the intralysosomal accumulation of free sialic acid (Renlund et al. 1986; Mancini et al. 1991*a*), as evidenced by two phenotypically distinct lysosomal storage disorders—adult-type or Salla disease (SD) and infantile-type sialic acid storage disease (ISSD; MIM 269920). The eponym "Salla disease" refers to the part of northeastern Finland from where the first patients were found (Aula et al. 1979). ISSD, first reported in 1982 (Hancock et al. 1982; Tondeur et al. 1982), is a very rare disorder without any ethnic predilection. The components of the sialic acid–transport mechanism of the lysosomal membrane have not yet been identified or characterized.

Sialuria (MIM 269921) is another rare inborn error with increased free sialic acid in urine (Fontaine et al. 1968). The pathogenesis in sialuria is overproduction of sialic acid because of defective feedback inhibition of the rate-limiting step in the synthesis of sialic acid (Seppälä et al. 1991).

The clinical phenotype of SD is characterized by early-onset, severe developmental delay together with ataxia and some other neurological abnormalities (Renlund et al. 1983). The first signs of the disease include hypotonia and, often, ocular nystagmus presenting during the first 6 mo of life. The life expectancy of severely mentally retarded patients is only slightly reduced. The disease is inherited as an autosomal recessive trait. SD shows exceptional enrichment in the Finnish population, particularly in the northeastern part of the country. Eighty-eight patients have been diagnosed to date in this genetically isolated population, compared with only occasional cases reported in other populations

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(Mancini et al. 1991*b*). ISSD has a more severe clinical course than SD and results in early death in patients with dysmorphic features, edemas, an enlarged liver and spleen, and a failure to thrive. Fewer than 20 cases of ISSD in various ethnic backgrounds have been recorded worldwide (Mancini et al. 1991*b*).

A 15- to 100-fold increase in free sialic acid is typically observed in the urine and in several organs of both SD and ISSD patients, and this is also evident in cell lines cultured from patients (Gahl et al. 1989). Lysosomal membrane preparations from cultured cells of both SD and ISSD patients demonstrate deficient sialic acid–transport activity (Mancini et al. 1991*a*). As stated above, the putative sialic acid–transporting protein of the lysosome membrane—the most probable defective gene product behind these lysosomal diseases—has not yet been isolated, nor has the corresponding gene been cloned. In fact, only a few structural proteins of the lysosomal membrane have so far been isolated and characterized. We have demonstrated elsewhere exclusion of the genes coding for two lysosomal membrane glycoproteins, h-lamp-1 and h-lamp-2, as the defective gene causing SD, by using linkage analysis in Finnish SD families (Schleutker et al. 1991).

Encouraged by the ability that highly informative markers and random linkage analysis have to assign disease loci in the human genome, we decided to map the gene locus of SD in 27 SD families, all of which originate from the genetically isolated Finnish population. Here we report the linkage of SD to a restricted chromosomal region on 6q.

Subjects, Material, and Methods

Families

Twenty-seven Finnish two-generation SD families were chosen for the study. Diagnosis of SD in the index patient in each family was confirmed by clinical evaluation, increased urinary free sialic acid excretion, and demonstration of enlarged lysosomal bodies by electron microscopy in skin biopsies (Renlund 1984). The family material consisted of 135 individuals, of whom 41 were patients. Two families had three affected children, 10 families had two affected children, and 15 families had one affected child. Most of the families originate from the northeastern part of Finland, where the disease is known to be more prevalent than in other parts of the country. In families 1, 2, and 14, a common ancestor can be traced back to the year 1620 (Renlund et al. 1983).

Samples

Twenty to forty milliliters of venous blood was collected from each individual. A lymphoblastoid cell line was established from all affected patients and from several family members. DNA was generally extracted from venous blood (Vilkki et al. 1988) and occasionally from cultured cells. In two cases DNA was extracted from a second-trimester villus biopsy, which was taken for prenatal diagnosis in order to determine the level of free sialic acid (Renlund and Aula 1987) during the subsequent pregnancy of the mother.

Markers

Eighty-five RFLP markers and 86 microsatellite markers were used before linkage was established. Most of the RFLP markers were provided by Dr. Ray White (University of Utah, Salt Lake City). The microsatellite markers were provided by the Nordic Genome Resource Center in Uppsala, Isogen Bioscience in Amsterdam, and the Généthon Resource Center.

RFLP and Microsatellite Analyses

RFLP studies were carried out using conventional hybridization methods (Vandenpals et al. 1984). PCR was performed in 25 μl using 100 ng genomic DNA as a template, 3.6 pmol of each primer, 0.04 μCi α^{32}P-dCTP, standard buffer and dNTP concentrations in accordance with the manufacturer's instructions (Finnzymes), and 1.0 U DNA polymerase (Finnzymes). Amplification was performed in an MJ RESEARCH PTC-100 thermal cycler for 28 cycles at 94°C for 1 min, at 55°C for 2 min, and at 72°C for 1 min. After PCR, 5 μl of the PCR product and 3 μl of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) were mixed, denaturated at 95°C for 10 min, and electrophoresed in a prewarmed 6% polyacrylamide gel for 2–4 h. After electrophoresis, the gel was fixed on Whatman 3MM paper in a gel dryer. The gels were exposed to Kodak X-OMAT AR film for 1–5 d. The allele sizes were estimated by comparison with M13 sequencing ladder in every gel.

Linkage Analysis

Linkage and allelic association analyses were performed with the LINKAGE program package (version 5.1) (Lathrop et al. 1984), using the MLINK option for pairwise lod scores, the CONTING option for the χ^2 test, and LINKMAP for multipoint analysis (five-point). All pairwise and multipoint calculations were performed under the assumption of equal allele frequencies, a constant sex ratio of 1.6, and complete pen-

etrance. Map distances in centimorgans were calculated using Haldane's (1919) formula. Pairwise lod scores were also calculated under the assumption of linkage disequilibrium between loci D6S286, AFM286za5, D6S251, and SD. For this purpose actual allele frequencies obtained from our family data were used for those alleles that were shown to segregate with the disease allele, while the remaining alleles were assumed to have equal allele frequencies. The prediction of the genetic distance of the SD locus from D6S286 was estimated by applying Luria-Delbrück's analysis to a simplified genetic model. On the basis of linkage disequilibrium analysis, we assumed that (1) all SD alleles descend from a common ancestor in a D6S286/7 haplotype and (2) all SD alleles with a non-D6S286/7 haplotype (17.4%) have undergone recombination between SD and D6S286 during the history of the Finnish population. Consequently we obtained recombination fraction (θ) = .0019 using the formula $1 - e^{-g\theta} = 0.174$, where g = 100 generations. The value θ = .0019 corresponds to a distance of 0.19 cM, which is approximately 190 kb, if we assume the genomewide average that 1 Mb corresponds to a genetic distance of 1 cM. The LINKMAP program was run on a VAX computer.

Results

The high prevalence of SD in the genetically isolated population of Finland and the remote consanguinity observed between some families originating from the northeastern part of the country made the possibility of locus heterogeneity highly unlikely. Furthermore, despite some clinical variation in the phenotypic manifestation of the disease, no evidence of clinical subtypes was observed. All our index patients displayed the typical SD phenotype with mental retardation associated with increased free sialic acid excretion in the urine and enlarged lysosomal vacuoles observed by electron microscopy in skin biopsies. Consequently, we did not allow locus heterogeneity in our linkage analyses. Linkage analysis was carried out under the assumption of complete penetrance, and pairwise lod scores were calculated for all loci using equal allele frequencies.

A total of 171 randomly distributed polymorphic DNA loci, 85 biallelic RFLPs, and 86 microsatellite polymorphisms were analyzed before a significant lod score was obtained with microsatellite marker Mfd131 on locus D6S251 (Wilkie et al. 1993). Only one obligatory recombination event was recorded between SD and this marker, and pairwise linkage analysis revealed a maximum lod score of 8.57 (θ = .02). Linkage was subsequently confirmed with five other microsatellites identifying the nearby loci D6S257, D6S286, AFM286za5, AFM319vh5 (Weissenbach et al. 1992), and COL9A1 (Warman et al. 1993). The segregation of the alleles of three of the most informative loci in the 27 Finnish SD families is shown in figure 1. No obligatory recombinations were observed between the SD locus and loci D6S286, AFM286za5, and AFM319vh5, and pairwise linkage analysis (MLINK) revealed maximum lod scores of 8.95, 5.26, and 3.19, respectively, at θ = 0 for each marker. The maximum lod scores for markers COL9A1 and D6S257, also assigned to this chromosomal region, were 6.08 (θ = .05) and 2.71 (θ = .12), respectively (table 1.).

For multipoint linkage analyses, the known order of three markers D6S257–D6S286–AFM286za5 was used, as well as a constant sex ratio of 1.6 (Chen et al. 1992). The genetic distances between these three fixed loci have been reported elsewhere to be 9.9 and 2.0 cM, respectively. Locus D6S251 was placed distal to AFM286za5.

The SD locus was placed on the genetic map formed by using the fixed order of the four most informative markers (D6S257–D6S286–AFM286za5–D6S251) in multipoint linkage analysis. The maximum lod score of 13.78 was obtained precisely at locus D6S286. LINKMAP analysis sets the position of the SD locus 9.9 cM distal to D6S257 on the male map (fig. 2) and 15.8 cM on the female map. Because of the lack of informativeness of locus AFM319vh5 (heterozygosity 0.37), it was excluded from the final multipoint linkage analysis. The COL9A1 locus was also omitted from multipoint analysis because the genetic distances between D6S286, D6S251, and COL9A1 have not yet been confirmed.

The recombinations between SD and the five linked loci observed in our families are presented in figure 3. The recombinations in family 6 localize the SD locus between loci D6S251 and D6S257 on the chromosomal region, representing 15.0 cM on the male map and 24.0 cM on the female map.

Linkage Disequilibrium

In order to further restrict the critical chromosomal region for the SD locus, we tested the linked markers for possible linkage disequilibrium in SD chromosomes. When the allele distribution of loci D6S286, D6S251, and AFM286za5 was analyzed in parental SD chromosomes versus non-SD chromosomes, a statistically significant deviation from random distribution was observed. In the case of locus D6S286, 38 (83%) of 46 SD chromosomes contained allele 7, in contrast to

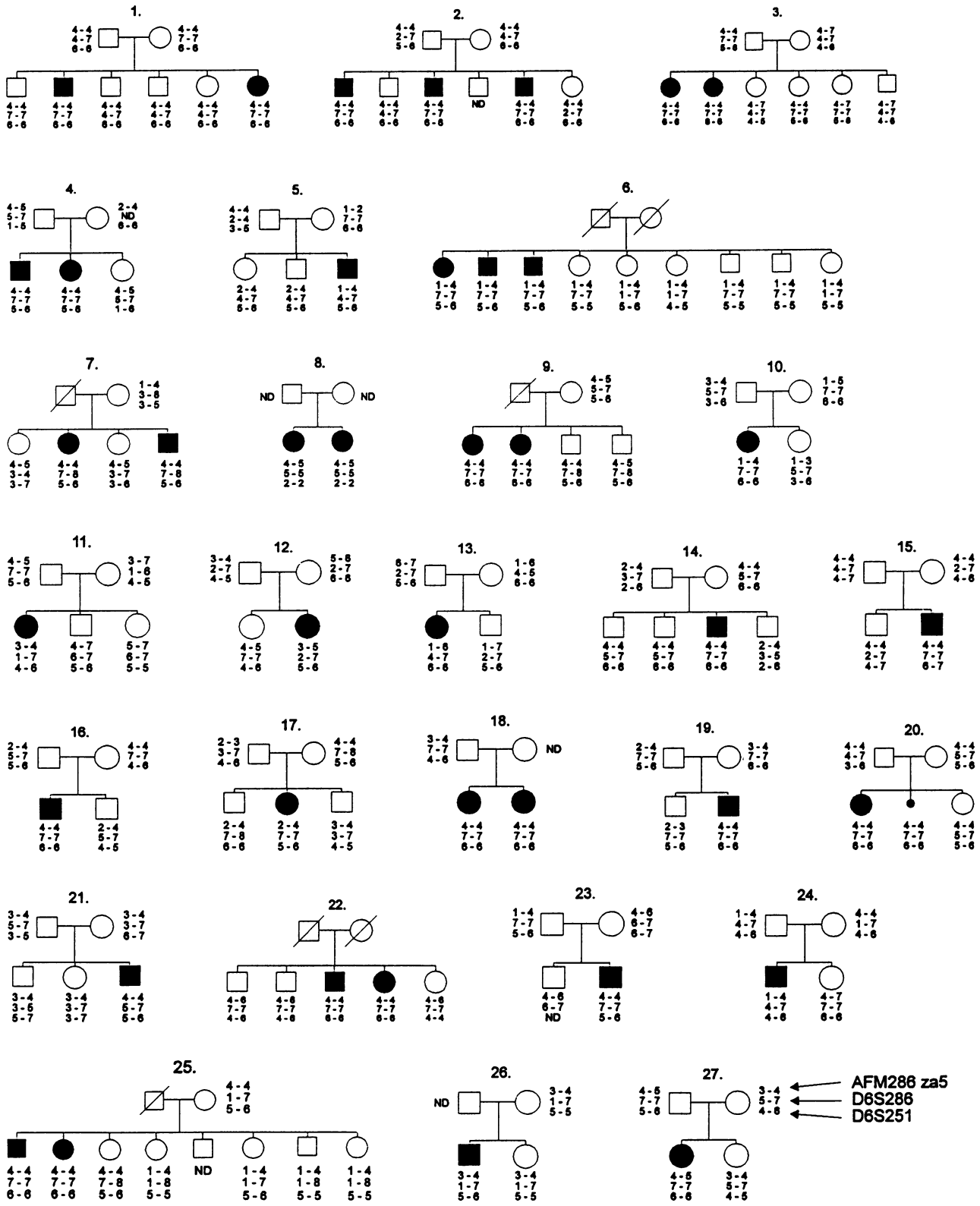

Figure 1 Pedigrees of the 27 SD families studied. The alleles in loci AFM286za5, D6S286, and D6S251 are given for each individual. ND = not determined.

Table 1

Pairwise Lod Scores between SD and Six Marker Loci on Chromosome 6 at Different Values of θ

Locus[a]	Lod Score at θ = .000	.001	.01	.05	.10	.15	.20	.25	.30	Z_{max} (θ)	90% Confidence Limit
D6S257	−∞	−10.36	−2.63	1.75	2.66	2.61	2.23	1.71	1.18	2.71 (.12)	
D6S286	8.95	8.92	8.70	7.70	6.44	5.21	4.04	2.95	1.98	8.95 (.00)	.00; .04
D6S286 +LD	12.99	12.97	12.84	12.05	10.91	9.71	8.47	7.18	5.85	12.99 (.00)	.00; .05
AFM286za5	5.26	5.25	5.13	4.54	3.79	3.04	2.33	1.69	1.12	5.26 (.00)	.00; .07
AFM286za5 +LD	8.63	8.62	8.54	8.04	7.29	6.47	5.62	4.74	3.83	8.63 (.00)	.00; .08
D6S251	−∞	7.81	8.55	8.12	7.00	5.77	4.54	3.37	2.29	8.57 (.02)	.00; .08
D6S251 +LD	−∞	13.32	14.04	13.51	12.29	10.94	9.53	8.06	6.54	14.04 (.01)	.00; .07
COL9A1	−∞	2.42	5.15	6.08	5.55	4.66	3.69	2.71	1.82	6.08 (.05)	.01; .13
AFM319vh5	3.19	3.18	3.11	2.80	2.39	1.98	1.58	1.19	.82	3.19 (.00)	.00; .12

[a] LD = linkage disequilibrium has been incorporated, as described in the text.

15 (33%) of 46 non-SD chromosomes (table 2). In the case of locus D6S251, 36 (74%) of 49 SD chromosomes carried allele 6, which was present in only 14 (29%) of 49 non-SD chromosomes, and in locus AFM286za5, 36 (78%) of 46 SD chromosomes demonstrated allele 4, in contrast to 18 (39%) of 46 non-SD chromosomes. No statistically significant evidence for nonrandom allelic association was detected in the case of other linked loci. When pairwise linkage analyses were performed between the SD locus and loci D6S286, D6S251, and AFM286za5, with the observed linkage disequilibrium used in the calculations, the maximum lod scores for D6S251, D6S286, and AFM286za5 were 14.04, 12.99, and 8.63, respectively (table 1). When the Luria-Delbrück equation (Luria and Delbrück 1943; Hästbacka et al. 1992), originally developed for analyzing mutations in bacterial populations, was used to estimate the distance between the SD locus and those markers demonstrating linkage disequilibrium, it was predicted that the critical region for SD is approximately 190 kb from locus D6S286.

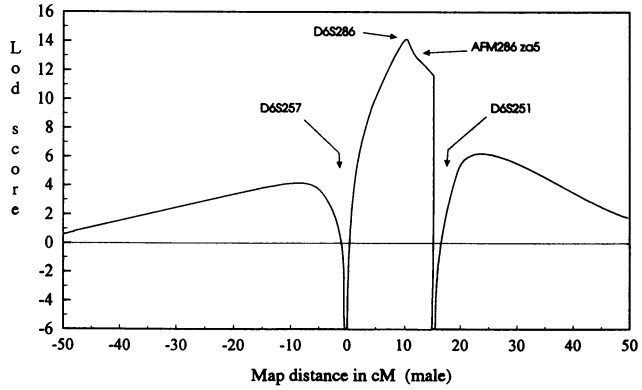

Figure 2 Multipoint linkage analysis in the 27 SD families. The figure illustrates the male map computed by the LINKMAP program under the assumption of a constant sex ratio of 1.6. D6S257 was chosen as the starting point (zero).

Discussion

Using random linkage analysis we have assigned the locus of SD, a free sialic acid storage disorder, to the long arm of chromosome 6. The observed recombination events, together with multipoint linkage analysis, define the critical region, flanked by markers D6S257 and D6S251, as a 15.0-cM genetic region on the male map and to a 24.0-cM region on the female map. Loci D6S257, COL9A1, and D6S251 have previously been cytogenetically localized to 6cen, 6q12-q14, and 6q14-q16.2, respectively, which sets the SD locus within the area 6cen-6q14 (fig 3). A search of the Genome Data Base for assignments of genetic loci in this chromosomal region did not reveal any genes coding for lysosomal proteins or any other candidate genes that could be considered to be defective in SD.

We found no evidence for locus heterogeneity in SD in our linkage analyses of Finnish SD families. Mapping of the SD locus now offers the possibility to study whether ISSD, the other phenotype associated with free sialic acid–transport defects, is linked to the same locus at 6q. This should be feasible in light of the collaboration aimed at collecting appropriate family material from different populations.

At an earlier stage of the study, when data from only

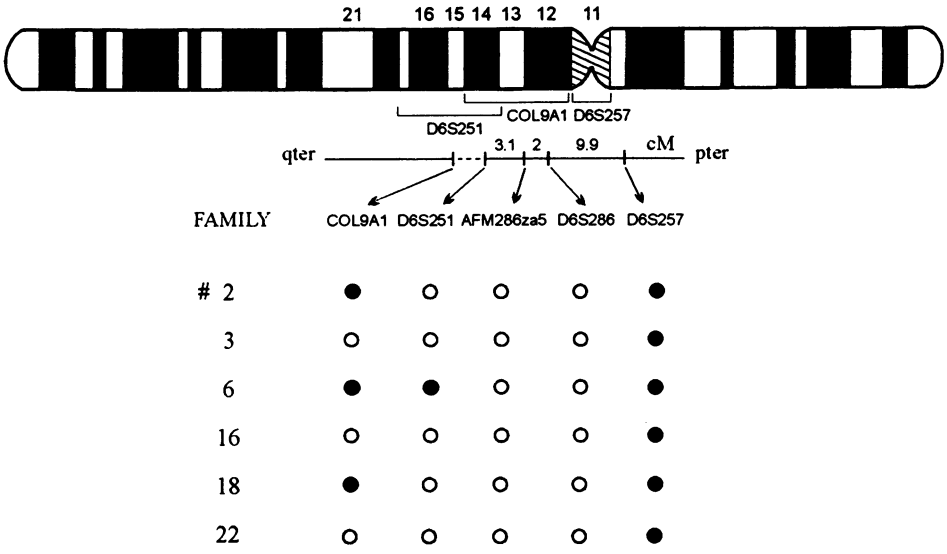

Figure 3 Delineation of the SD region at 6q. The recombinations detected in the 27 SD families are marked with blackened circles under the corresponding marker. The recombinations observed in family 6 define the genetic region of the SD locus. The cytogenetic assignment of loci D6S257, COL9A1, and D6S251 is indicated. The genetic distance between COL9A1 and D6S251 is unknown.

64 markers (most of them biallelic RFLPs) were available, we used the exclusion program EXCLUDE (Edwards 1987) to pinpoint the most likely chromosomal area for linkage. The analysis yielded the highest probability score (68%) on the long arm of chromosome 2 (Haataja et al. 1992), but further analyses with additional markers later excluded that area as the locus of SD. The EXCLUDE analysis suggested a very low probability of assignment to chromosome 6, but at that time only two chromosome 6 RFLP markers had been analyzed.

Table 2

Linkage Disequilibrium Shown by the Allele Distribution in Locus D6S286 in Parental Chromosomes Carrying the SD Mutation and in Non-SD Chromosomes

D6S286 Allele	No. (%) of SD Chromosomes	No. (%) of Non-SD Chromosomes
1	1 (2.2)	4 (8.7)
2	1 (2.2)	5 (10.9)
3	...	4 (8.7)
4	3 (6.4)	6 (13.0)
5	1 (2.2)	8 (17.4)
6	1 (2.2)	1 (2.2)
7	38 (82.6)	15 (32.6)
8	1 (2.2)	3 (6.5)
Total	46 (100.0)	46 (100.0)

NOTE.—$P < .001$.

The linkage disequilibrium observed between the SD locus and the alleles of the three closest markers D6S286, AFM286za5, and D6S251 made it possible to further restrict the critical chromosomal region for SD. Linkage disequilibrium has been observed elsewhere in some other recessively inherited autosomal diseases with a similar enrichment in the isolated Finnish population. Linkage studies of infantile neuronal ceroid lipofuscinosis (INCL) (Järvelä 1991; Hellsten et al. 1993), diastrophic dysplasia (Hästbacka et al. 1992), and cartilage-hair hypoplasia (Sulisalo et al. 1993) revealed that the disease allele was strongly associated with specific alleles of the linked markers. Linkage disequilibrium offers additional scope for gene localization, particularly in family material from an isolated population such as this. It is likely that the present Finnish population of approximately 5 million descended from a small number of founders ~2,000 years ago. It is probable that one or several of the founders carried the SD mutation in a D6S286/7 haplotype, which has since spread during the approximately 100 subsequent generations. If the assumption of 190 kb, based on the Luria-Delbrück equation, holds for the most probable chromosomal region of the SD locus, it would be well within the scope of molecular cloning techniques and could be covered by one YAC clone or by three or four cosmid

contigs. Isolation of the transport protein(s) from lysosome membrane preparations by using liposome-reconstituted vesicles and protein chemistry would be an alternative approach to cloning the gene defective in SD (Mancini et al. 1992).

The detection of linkage in SD and the observed allelic association have direct implications for prenatal diagnosis and carrier identification of SD in Finland. Until now, an assay of free sialic acid in a chorionic villus biopsy and cultured amniotic fluid cells has been the method of choice for prenatal diagnosis of free sialic acid storage disorders (Vamos et al. 1986; Renlund and Aula 1987). In SD, however, in which the increase in intracellular free sialic acid is less severe than in ISSD, the results of sialic acid assays are not always unequivocal. Segregation analyses of informative markers in SD families will now provide the possibility of prenatal diagnosis to families with at least one previously affected child. Further definition of the linkage disequilibrium with new markers should reveal even stronger allelic association than that observed with the D6S286/7 allele in the present study. In that case, the linked marker allele could serve directly as a diagnostic marker, such as the HY-TM1/15 allele in INCL (Vesa et al. 1993) or the CSF1R/1-1 haplotype in diastrophic dysplasia (Hästbacka et al. 1993). Furthermore, the identification of carriers has for the first time made it possible to improve genetic counseling for family members of patients affected with SD.

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