European Journal of Human Genetics (2000) 8, 500–506

www.nature.com/ejhg

ARTICLE

Identification of novel *USH2A* mutations: implications for the structure of USH2A protein

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Usher syndrome type II is an autosomal recessive disorder, characterised by stable hearing impairment from childhood and progressive retinitis pigmentosa from the late teens. Mutations in the *USH2A* gene, located on 1q41, were recently shown to be responsible for Usher syndrome type IIa. We have investigated the molecular pathology of Usher type II by screening the *USH2A* gene for mutations in 31 unrelated patients from Denmark and Norway. Besides the frequent 2299delG mutation, which accounted for 44% of the disease alleles, a heterogeneous spectrum of mutations was identified. Sixteen new, putative disease-causing mutations were detected, of which 12 were private and four were shared by unrelated patients. The disease-causing mutations were scattered throughout the gene and included six nonsense and seven missense mutations, two deletions and one small insertion. In addition, six non-pathogenic polymorphisms were identified. All missense mutations resulted in major amino acid side-chain alterations. Four missense mutations affected the N-terminal part of USH2A, whereas three missense mutations affected the laminin-type epidermal growth factor-like (LE) domain. The structural consequences of the mutations affecting the LE domain are discussed in relation to the three-dimensional structure of a LE-module of the mouse laminin γ1 chain. *European Journal of Human Genetics* (2000) **8**, 500–506.

Keywords: Hearing impairment; retinitis pigmentosa; Usher syndrome type II; spectrum of mutations; molecular modeling

Introduction

Usher syndrome (MIM 276900) constitutes a group of autosomal recessive disorders characterised by variable degrees of hearing impairment, vestibular dysfunction and progressive visual loss due to retinitis pigmentosa (RP). The condition is clinically and genetically heterogeneous and is classified into three major subtypes: Usher type I, II and III mapping to any one of nine distinct genetic loci.

Usher type II accounts for more than half of all Usher cases, with a prevalence of 2.2/100 000 in Denmark[1] and 3.6/100 000 in Norway.[2] Mutations in the *USH2A* gene, located on 1q41, are responsible for Usher syndrome type IIa. Three deletions were identified in the *USH2A* gene, of which 2299delG[3,4] was found in 29 out of 198 disease alleles (15%). However, Usher type II is genetically heterogeneous since approximately 12% of USH2 families do not show linkage to 1q41.[5,6] Linkage to 3p23–24.2 was recently detected and designated USH2B.[6]

The N-terminal part of the predicted 1546 amino acid residue USH2A protein shares no significant homology with known proteins. However, the deduced protein sequence contains, among others 10 laminin-type epidermal growth factor-like (LE) motifs, each about 50 amino acid residues long and arranged in tandem. The C-terminal part of the protein contains four tandem repeats with similarity to fibronectin type III elements (FN3). These protein domains are frequently observed in extracellular matrix proteins such as growth factors, receptors and cell adhesion molecules.

We have investigated the molecular pathology of Usher type II patients by screening the *USH2A* gene for mutations in a panel of 31 Danish and Norwegian patients. Our aim was to identify the classes of mutations which inactivate the *USH2A* gene or gene product and to investigate the degree of mutational heterogeneity. Such knowledge is essential for patient management and the future design of a rational

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Received 11 November 1999; revised 2 February 2000; accepted 1 March 2000

molecular diagnostic strategy for Usher type II patients. Furthermore, the locations and nature of the mutations may contribute to the understanding of the structure and function of the USH2A protein.

Materials and methods

Patients

Altogether 21 Danish and 10 Norwegian unrelated Usher type II patients were studied. Danish patients were diagnosed at The National Eye Clinic for the Visually Impaired, Hellerup, Denmark. Clinical information about the Norwegian patients was provided by several ophthalmologists and audiologists. All patients had stable moderate hearing impairment and sloping audiographs associated with retinitis pigmentosa, verified by ophthalmological and ERG (electroretinogram) examinations.[7]

Mutation detection

Genomic DNA was isolated from whole blood by use of an automated DNA extractor (341 Nucleic Acid Purification System DNA extractor, Applied Biosystems, Foster City, CA, USA) or by salting out methods.

Intronic PCR primers flanking each exon[4] were used for PCR amplification and direct sequencing. All PCR reactions were carried out on a DNA Thermal Cycler/480, (Perkin-Elmer, Norwalk, CT, USA). Hot start PCR was performed in a 30 µl volume containing: 50 ng genomic DNA, 15 pmol of each primer, 0.2 mM dNTPs, 1 × GeneAmp PCR buffer II, 1.5 mM MgCl and 1.5 unit AmpliTaq™ DNA polymerase (PE-Biosystems, Branchburg, NJ, USA).

Sequencing and restriction-enzyme analysis

The PCR products were sequenced with the appropriate primer by the use of PCR-product Presequencing Kit (Amersham Life Science, Buckinghamshire, UK) and ABI PRISM BigDye™ Termination Cycle Sequencing Kit (Perkin-Elmer, PE-Biosystems, Warrington, UK). The sequences were analysed on an ABI 377 automated sequencer unit (Perkin-Elmer, Foster City, CA, USA) and mutations were detected by use of associated software and by manual inspection.

For restriction enzyme analysis of the 2299delG deletion, the missense mutations and the polymorphisms, PCR products were digested over night with the appropriate restriction enzyme and analysed by agarose gel electrophoresis. Primer sequences, conditions for PCR and restriction enzymes are listed in Table 1.

3-D protein model

All LE-domains share a common structural element primarily defined by the eight cysteines (PROSIDE document: PDOC00961). The three-dimensional structure of LE domains in mouse γ1 chain was used to predict structural

Table 1 Diagnostic PCR methods designed to confirm novel sequence changes or to screen controls

Mutation name	*Sequence 5′–3′*	*Annealing temp. (°C)*	*Restriction enzyme*	*Size of bands in bp.* *Normal allele*	*Mutant allele(s)*
2299delG	F. CTGCAATCCTCACTCTGGGCAGCT	64	*Bfa I*	264 + 17	238 + 25 + 17
	R. TCTTATCACAGTTGCTAGGCAGACA				
C163Y	F. TTCTCTCTTTTCCGGTTGCTTCCTCACGT	56	*Pml I*	166 + 27	193
	R. CTGCTGCAGATTTTGTGAGTAGA				
V230M	F. GTGCATCAGACAAAAATCAGCTT	64	*Nla III*	99 + 22	59 + 40 + 22
	R. GTCCTATTTGCACAGTACCACATG				
R334W or	F. TCTCTGGAGATCTTCTCAGATT	60	*Nla III*	170 + 25	N346H: 147 + 25 + 23
N346H	R. ATGAAGTACCAACATCATTATCAT				R334W: 109 + 61 + 25
C536R	F. ACACAACAAGCCAGCCATATACA	58	*Nla III*	68 + 39 + 22 + 20	68 + 61 + 20
	R. TGTTAGGCCAAGATTAAGTTCAT				
G713R	F. GAGGACAGTGTAATTGTAAGAGAC	60	*Pml I*	166 + 26	136 + 30 + 26
	R. CAATAACGTTTGCTTTGCACTTGCACTCAC				
C759F	F. TTAGGTGTGATCACTGCAGTTTTGGAT	60	*Pst I*	90 + 22 + 16	112 + 16
	R. ACACTGCCCAGAGTGAGGACTG				
A/T125	F. GCACTTTTTGTCACAGCTCTGCAGCTG	64	*Pst I*	193 + 21	162 + 31 + 21
	R. AGCTCTTGTGATTTCCAAAAATAAAACCTG				
504A/G	F. ATTCAGGCACTTATTCATAGTACTCCA	63	*Sca I*	162 + 21	137 + 25 + 21
	R. AAGTTTGAACACAATCTGCCCAAGTAC				
1419C/T	F. TCCTGGATACAATAACTTCTATAGTAC	60	*Sca I*	150 + 86	150 + 61 + 25
	R. TGCTCTGACATCTTAATGTGCT				
IVS17–8T/G	F. AAGTAACCCCTTTGTCTGATGAGT	63	*BsmAI*	305 + 73	254 + 73 + 51
	R. GGAAACATTTGCATTCAGAGG				
4371A/G	F. FAM–TTGGTTGTGTGACCAGTGCATCG	64		TET: 167	FAM: 165
	F. TET–AGTTGGTTGTGTGACCAGTGCGTCA				
	R. GAGTTAGTGAGGGAGGAGAAGACA				
K/R1486	F. AGCCATACAGATACTTGAAACC	64	*Afl II*	501	402 + 99
	R. GCTATCAAAGGGCTGAATTAG				

F = Forward; R = Reverse; Mismatches to the genomic sequence are underlined.

consequences of three missense mutations. The program 'SwissPdBViewer' was used to view the model, PBD entry 1KLO[8] and all drawings of the structure were made by use of the program RasMol v2.6.SWISS MODEL.[9]

Results

Thirty-one unrelated patients, clinically diagnosed with Usher type II were screened for the 2299delG mutation,[3] revealing six homozygotes and 12 heterozygotes. Twenty-five patients who were not homozygous for 2299delG, were screened for mutations in the coding region of the *USH2A* gene. Exon 2 throughout exon 21 were amplified by PCR from genomic DNA and subjected to automated DNA sequencing. Sixteen different putative disease-causing mutations were identified (Table 2 and Figure 1). In addition, six polymorphisms not associated with disease were detected (Table 3). Disease-alleles were identified in 27 out of 31 patients. Eight patients were homozygotes, 19 were compound heterozygotes, among whom nine disease-alleles remained unidentified (Table 4).

Mutations identified

The mutations, their predicted effect on the *USH2A* gene and their frequencies are summarized in Tables 2, 3 and 4. Their respective locations within USH2A are indicated in Figure 1. The genotypes of the Usher type IIa patients are listed in Table 4.

Nonsense mutations

Six different mutations leading to premature stop codons were found in six patients, all due to C→T transitions. Four mutations, resulting in the substitution of arginines for stop codons, were detected at CpG dinucleotides, known to be hot spots for transitions.[10] Two unrelated Norwegian patients, N1 and N3, had the same mutation at nucleotide 100 (R34X). The mutation at nt. 2797 (Q933X) was identified in two unrelated Danish patients, D21 and D7. The transitions at nucleotide positions 187, 1876, 2023 and 3883 (R63X, D626X, Q675X and R1295X, respectively) were all private.

Deletions and insertion

Besides 2299delG, two deletions and one insertion were predicted to lead indirectly to premature stop codons through shift in the translational reading frame. In two unrelated Danish patients, D14 and D15, a 4 bp insertion was identified, which shifts the reading frame after codon 307 ending at a stop codon at position 323. Patient D3 was homozygous for 2878delAA, which results in a frameshift after codon 959 and ending at a UAA stop codon, three residues downstream. Likewise, the 1965delT mutation causes a frameshift after codon 654 introducing an aberrant amino acid sequence that terminates at a UGA stop codon 100 residues downstream.

Missense mutations

Seven different missense mutations were identified in nine patients. None of the 100 Norwegian control alleles contained the C163Y, N346H, R334W, C536R, G713R or C759F substitutions, whereas the V230M substitution was identified in 1 out of 100 control chromosomes. The N346H substitution was detected in two unrelated patients, D20 and N7, from Denmark and Norway, respectively. Interestingly, in patient D21, a C163Y replacement as well as two different

Table 2 Summary of disease-causing mutations identified in the *USH2A* gene

Name	*Nucleotide change*	*Location*	*Effect on coding sequence*
Nonsense			
R34X	100C→T[b]	EX2	Arg→stop at 34
R63X	187C→T[b]	EX2	Arg→stop at 63
R626X	1876C→T[b]	EX11	Arg→stop at 626
Q675X	2023C→T	EX12	Gln→stop at 675
Q933X	2797C→T	EX13	Gln→stop at 933
R1295X	3883C→T[b]	EX18	Arg→stop at 1295
Deletion or insertion			
921-22insCAGC	Insertion of CAGC between 921 and 922	EX6	FS after Ser307, stop at 323
1965delT	Deletion of T at 1965	EX11	FS after Leu654, stop at 755
2299delG[a]	Deletion of G at 2299	EX13	FS after Ala767, stop at 787
2878delAA	Deletion of AA at 2878	EX14	FS after Val959, stop at 962
Missense			
C163Y	488G→A	EX3	Cys→Tyr at 163
V230M	688G→A[b]	EX4	Val→Met at 230
R334W	1000C→T[b]	EX6	Arg→Trp at 334
N346H	1036A→C	EX6	Asp→His at 346
C536R	1606T→C	EX9	Cys→Arg at 536
G713R	2137G→C	EX12	Gly→Arg at 713
C759F	2276G→T	EX13	Cys→Phe at 759

Mutation designations correspond to those described by Antonarakis and the Nomenclature Working Group;[22] [a]Reported by Weston *et al*;[4] [b]Mutation occurs at CpG dinucleotide; FS = frame shift.

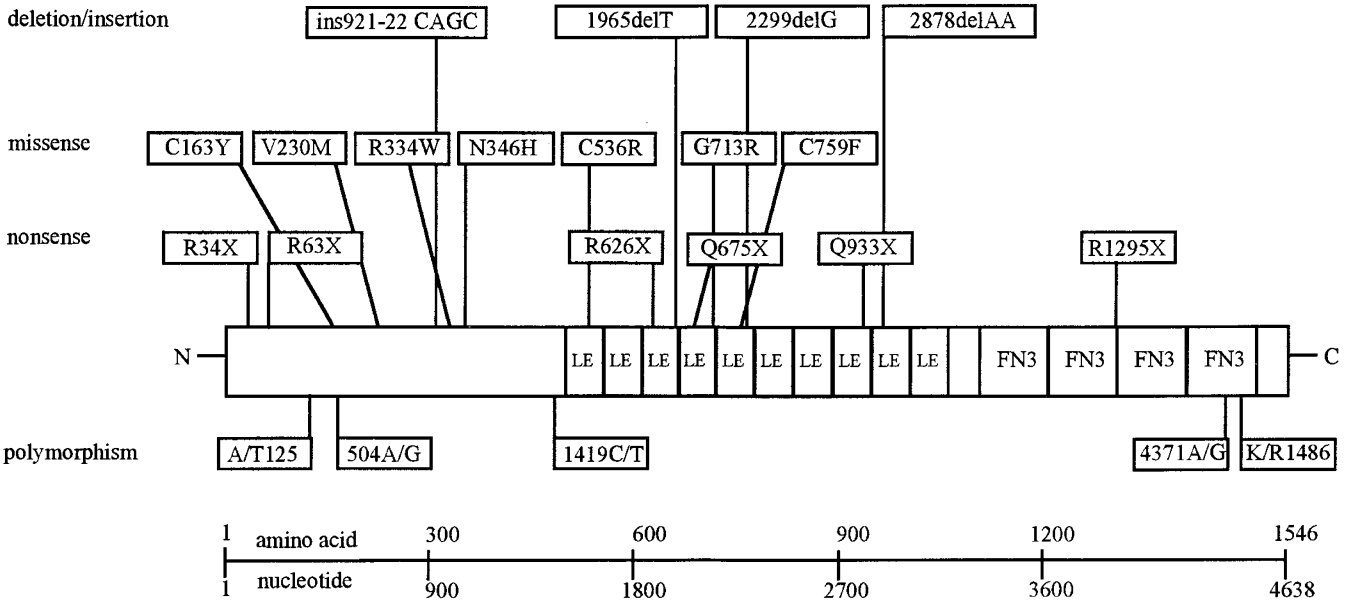

Figure 1 Locations of mutations identified on a schematic presentation of the predicted USH2A protein. Putative functional domains are indicated. All exonic polymorphisms are shown below the diagram; LE = laminin-type EGF-like motif; FN3 = fibronectin type III motif.

nonsense mutations were identified. However, due to lack of DNA from close relatives the *cis-trans* problem could not be solved. The V230M, R334W, C536R, G713R, and C759F substitutions were all private.

Polymorphisms and unclassified variants

In addition to the putative disease causing mutations, five exonic and one intronic polymorphism were identified. Three of the exonic mutations were silent (504A/G, 1419C/T and 4371A/G) and two resulted in amino acid changes (A/T125 and K/R1486). The IVS-8T/G mutation was located in a polypyrimidine tract in close proximity to the acceptor site of intron 8. A similar T to G transversion was reported to affect splicing efficiency in the human β-globin transcript.[11] However, because of homozygosity in several normal healthy individuals, all six mutations were excluded from being disease-causing. The observed frequencies of these polymorphisms among Norwegian normal chromosomes ranged from 0.03 to 0.36 (Table 2). Furthermore, four intronic mutations were observed in several patients. Because, they were presumed to have no effect on RNA-processing they were not tested in the normal control panel but instead regarded as unclassified variants.

Table 3 Polymorphisms and unclassified variants identified in the *USH2A* gene

	Nucleotide change	*Location*	*Effect on coding sequence*	*Frequency among normal chromosomes*
Polymorphisms				
A/T125	A or G at 125	EX2	Ala or Thr at 125	70/30
504G/A	G or A at 504	EX3	Silent	72/28
1419C/T	C or T at 1419	EX8	Silent	62/38
IVS17–8T/G	T or G	IVS17	ND	77/23
4371A/G	G or A at 4371	EX20	Silent	97/3
K/R1486	A or G at 4457	EX21	Lys or Arg at 1486	64/36
Unclassified variants				
IVS3–80T/C	T or C	IVS3	ND	ND
IVS9+34C/A	C or A	IVS9	ND	ND
IVS15+35A/G	A or G	IVS15	ND	ND
IVS18–44A/G	A or G	IVS18	ND	ND

ND = not determined.

Table 4 Genotypes of the Usher type IIa patients

Patient/Genotype	*Mutation*	*Mutation*	*Mutation*
Heterozygotes			
D8	2299delG	ND	
D9	C759F	ND	
D11	2299delG	ND	
D12	1965delT	ND	
D20	N346H	ND	
D24	2299delG	ND	
N1	R34X	ND	
N7	N346H	ND	
N15	2299delG	ND	
Compound heterozygotes			
D5	2299delG	V230M	
D7	2299delG	Q933X	
D10	2299delG	R63X	
D15	2299delG	921–22insCAGC	
D16	2299delG	C536R	
D17	2299delG	G713R	
D21[a]	Q675X	Q933X	C163Y
D23	2299delG	R626X	
D25	2299delG	R334W	
N3	R34X	R1295X	
Homozygotes			
D2	2299delG		
D3	2878delAA		
D4	2299delG		
D14	921–22insCAGC		
D18	2299delG		
D19	2299delG		
N4	2299delG		
N5	2299delG		

D and N indicate Danish and Norwegian origin; [a]patient D21: three putative disease-causing mutation were identified. The *cis-trans* problem could not be solved, due to lack of DNA from close relatives; ND = not detected.

Discussion

We have identified pathogenic *USH2A* alleles in 27 unrelated Usher type II patients. Putative disease-causing mutations were identified in 45 of the 62 disease alleles studied (73%). Sixteen new disease-causing mutations were characterised. These included six nonsense, seven missense, two deletions and one insertion. In addition, five exonic and one intronic non-pathogenic polymorphisms were identified.

Characteristics of the mutations

Since Usher type II is a recessive disorder, the mutations were predicted to be pathogenic due to loss of USH2A function. The nonsense mutations, deletions and insertions leading directly or indirectly to premature termination of translation were predicted to disrupt the function of the USH2A protein. All missense mutations resulted in major changes in the side-chain properties of the corresponding amino acid residue. Regarding the C163Y, V230M, R334W and N346H substitutions, they are all located in the N-terminal part of the protein; however, their disruptive effect on the function of the USH2A protein is not yet known. Among the seven missense mutations reported here, three (C536R, C759F and G713R) result in changes of highly conserved residues in the laminin-type epidermal growth factor-like (LE) domain. Hence, Usher type II is caused both by mutations that in homozygous or compound heterozygous states cause complete absence, as well as mutations that cause alterations in single amino acid side chains of the 1546 a.a., USH2A protein.

Structural consequences of the mutations affecting the LE domains

The availability of the three-dimensional structure of the mouse laminin γ1 chain is an important source for predicting the consequences of amino acid substitutions in the LE domains. Based on crystallographic data the LE domains have been shown to be independently folded protein modules. They are described as four looped structures (a–d), stabilised by four disulfide bridges (Figure 2).[8,12] The modules have a distinct variability with respect to the size, structure and arrangement of the loops. The eight invariant cysteine residues principally define the primary structure of the LE domain and form disulfide bonds with the following pattern: Cys1-Cys3 (loop a), Cys2-Cys4 (loop b), Cys5-Cys6 (loop c) and Cys7-Cys9 (loop d) (Figure 2). In addition, loop d of the preceding LE module and the loop b of the following LE module form a hook-like association through hydrogen bonds and hydrophobic interactions.[8] The C536R and C759F substitutions disrupt the disulfide bond formation to C520 (C2) and C747 (C1), respectively (Figure 2). Both substitutions were predicted to result in abnormal folding of the LE domain, thereby affecting the functional properties of the protein. The G713R replacement of the hydrophobic side chain of glycine with the positive charged side of arginine, is likely to strongly destabilise the loop structure as suggested by the conservation of the glycine in the multiple alignment (Figure 2) The LE domains are thought to function as a rigid, rod-shaped, mechanical spacer that keeps the N-terminal and C-terminal regions apart.[12] In addition, the LE domains may be involved directly in inter-molecular interactions. Thus, disruption of rigidity as well as disruption of binding properties could explain loss of function of these mutant proteins.

Limitations in mutation detection

Putative disease-causing mutations were identified in 27 out of 31 (87%) patients studied. In four patients, no alterations were found which could be associated with disease and in nine patients the second disease allele remained unidentified. This relates to general problems in mutation detection such as technical limitations and genetic heterogeneity. Although splice site mutations have been reported to account for 15% of all mutations involved in human genetic disease,[13] no putative splice site mutations were observed in this study, despite that all PCR primers were designed to include all exon/intron boundaries. Likewise, in human genetic disease large rearrangements such as deletions and insertions

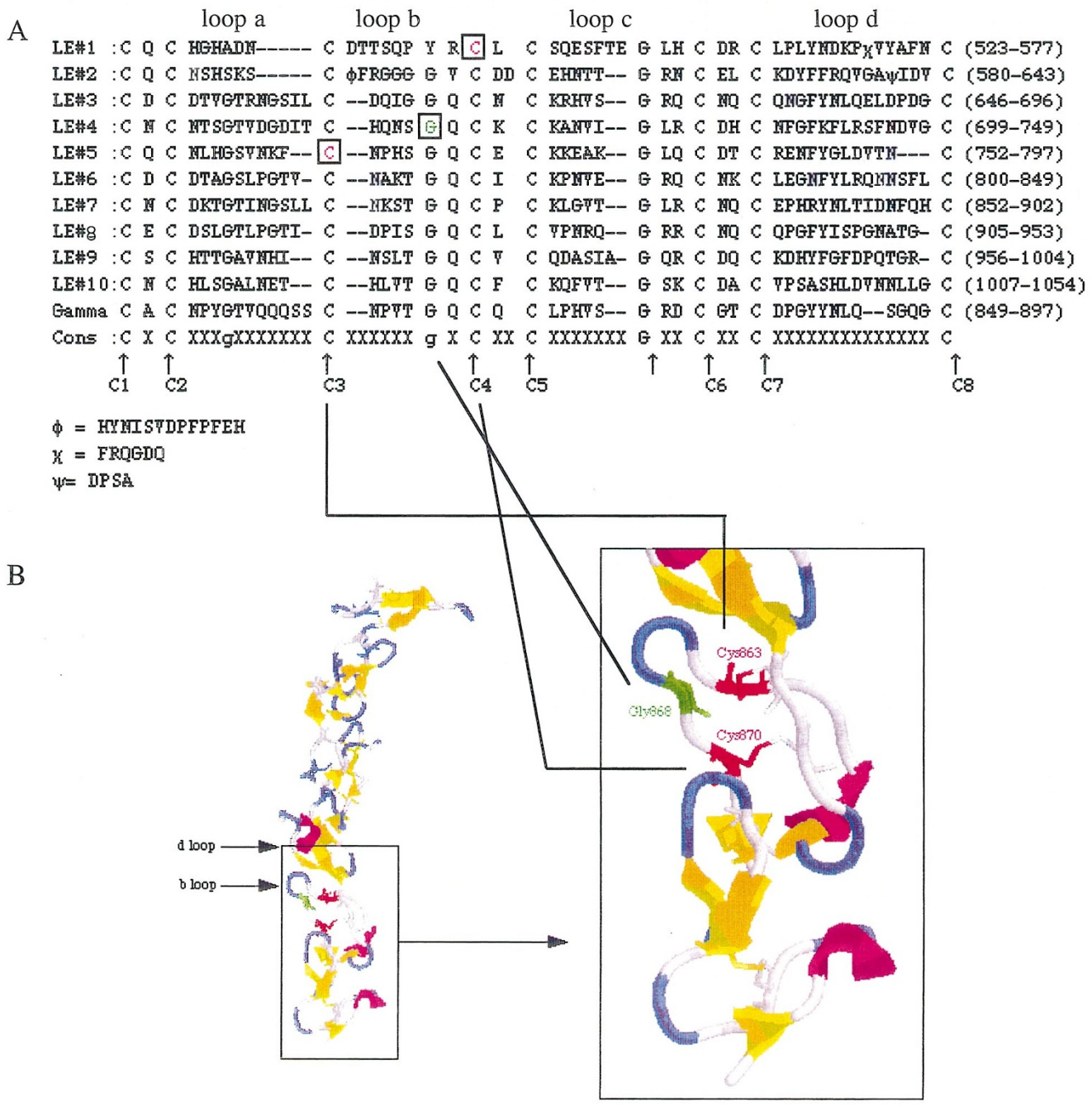

Figure 2 **A** **Alignment** A multiple amino acid alignment of the 10 LE (LE No. 1–LE No. 10) domains from the USH2A protein and the third LE domain in mouse laminin γ1 chain. The corresponding amino acid residues are in brackets. The position of the cysteine residues (1–8) and conserved glycine residues are aligned, gaps and φ, χ and ψ are introduced to show maximal homology. The consensus sequence is the bottom line (Cons). The loop regions are indicated above: loop a (C1–C3), loop b (C2–C4) loop c (C5–C6) and loop d (C7–C8). Residues C536 and C759 are in red, residue G713 is in green. **B** **Structure** The three-dimensional structure of the mouse laminin γ chain 1. Strands are yellow, helixes red and turns blue. Loops d and b are indicated by arrows. The side chains involved in substitutions are coloured as in the alignment. Cys863, Gly868 and Cys870 in the mouse laminin γ1 chain structure correspond to Cys536, Gly713 and Cys759, respectively in the human USH2A.

usually account for a large fraction of disease-causing mutations. Southern blot technique will be required to address whether such mutations are involved in USH IIa.

In four patients who met the clinical criteria for Usher type II no *USH2A* mutations could be identified. One of these patients belong to a previously characterised Norwegian Saami family in which the segregation of 1q41 markers are consistent with the *USH2A* locus (lod score 3.09).[14] We cannot exclude the possibility that another Usher locus could be closely linked to the *USH2A* locus. As for the remaining three patients linkage to USH2A has not been confirmed.

To date, two genes have been found to be responsible for Usher syndrome. These are *MYO7A* and *USH2A* in Usher type I and Usher type II, respectively. So far, 46 mutations likely to be disease-causing have been identified in *MYO7A* and no single mutation appears to predominate,[15–20] except for a probable founder mutation (C31X) which accounted for 50% of the mutations in the Danish Usher type I patients.[20] In Usher type IIa patients, however, the 2299delG mutation, reported in patients from Scandinavia (Denmark, Norway and Sweden), Northern and Southern Europe, North America, UK and China dominate the mutational spectrum (frequency ranging from 0.15 to 0.44).[3,21]

The 2299delG mutation occurs at high and similar frequency among Danish (0.45) and Norwegian (0.42) Usher IIa patients. Preliminary results suggest that Danish and Norwegian 2299delG alleles share the same intragenic haplotype based on six SNP markers but deviate with respect to flanking microsatellite markers (results not shown). Further haplotype analysis is required to determine whether the 2299delG has spread by founder effect and/or recurrent mutational events.

Acknowledgements

We would like to thank the families for their collaboration and the clinical colleagues who have contributed to this study. We thank Marianne Schwartz, Department of Clinical Genetics, University Hospital, Copenhagen for technical assistance with the Danish patients' samples. This work has been supported by grants from The Norwegian Foundation for Health and Rehabilitation, grant 1998/257 (BD), and The John and Birthe Meyer Foundation (BD, ØN, LT) and by the National Institutes of Health NIDCD grant P01 DCO1813-06 and Usher grants from the Foundation Fighting Blindness (WK, MDW).

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