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Spectrum of Mutations in the Batten Disease Gene, *CLN3*

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Summary

Batten disease (juvenile-onset neuronal ceroid lipofuscinosis [JNCL]) is an autosomal recessive condition characterized by accumulation of lipopigments (lipofuscin and ceroid) in neurons and other cell types. The Batten disease gene, *CLN3*, was recently isolated, and four disease-causing mutations were identified, including a 1.02-kb deletion that is present in the majority of patients (The International Batten Disease Consortium 1995). One hundred eighty-eight unrelated patients with JNCL were screened in this study to determine how many disease chromosomes carried the 1.02-kb deletion and how many carried other mutations in *CLN3*. One hundred thirty-nine patients (74%) were found to have the 1.02-kb deletion on both chromosomes, whereas 49 patients (41 heterozygous for the 1.02-kb deletion) had mutations other than the 1.02-kb deletion. SSCP analysis and direct sequencing were used to screen for new mutations in these individuals. Nineteen novel mutations were found: six missense mutations, five nonsense mutations, three small deletions, three small insertions, one intronic mutation, and one splice-site mutation. This report brings the total number of disease-associated mutations in *CLN3* to 23. All patients homozygous for mutations predicted to give rise to truncated proteins were found to have classical JNCL. However, a proportion of the patients ($n = 4$) who were compound heterozygotes for a missense mutation and the 1.02-kb deletion were found to display an atypical phenotype that was dominated by visual failure rather than by severe neurodegeneration. All missense mutations were found to affect residues conserved between the human protein and homologues in diverse species.

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Introduction

Batten disease (juvenile-onset neuronal ceroid lipofuscinosis [JNCL], or Spielmeyer-Vogt-Sjögren disease [MIM 304200]) is the most common inherited neurodegenerative disease of childhood. Inheritance is autosomal recessive, and its incidence is estimated as being as high as 1/25,000 births, with an increased prevalence in northern-European populations (Zeman 1974). The first clinical symptom is visual failure, which occurs at the age of 4–7 years. Mental retardation, epilepsy, and psychomotor deterioration manifest later, eventually leading to death in the 2d or 3d decade of life. Diagnostic criteria include macular degeneration, the presence of vacuolated lymphocytes in peripheral blood, and the accumulation of autofluorescent lipopigments (ceroid and lipofuscin) in neurons and other cell types, which, on electron microscopy, appear as fingerprint profiles (Santavuori 1988). The biochemical defect underlying the disease is unknown.

Following assignment of *CLN3*, the gene for Batten disease, to chromosome 16 (Eiberg et al. 1989), refinement of localization was achieved by further genetic-linkage studies, analysis of haplotypes, and linkage-disequilibrium mapping (Callen et al. 1991; Gardiner et al. 1990; Mitchison et al. 1993, 1994, 1995; Yan et al. 1993; Lerner et al. 1994). The *CLN3* gene, isolated in 1995, encodes a predicted protein of 438 amino acids that has no homology to any proteins of known function (The International Batten Disease Consortium 1995). Four different mutations were critical in identifying *CLN3*—three intragenic genomic deletions and a point mutation that affects a splice site. A deletion of 1.02 kb is the most common mutation in *CLN3;* it is present on 81% of disease chromosomes (The International Batten Disease Consortium 1995). Our aim in this study was to identify the remaining mutations in our resource of 188 unrelated JNCL patients.

Patients and Methods

Patients

One hundred eighty-eight unrelated patients with diagnostic criteria satisfying the diagnosis of JNCL were included in the study. These patients originated from 18

different countries (43 patients from Finland, 34 patients from the United States, 25 patients from the Netherlands, 23 patients from Germany, 15 patients from the United Kingdom, 14 patients from Norway, 13 patients from Denmark, 5 patients from Sweden, 4 patients from Italy, 3 patients from Greece, 2 patients from Australia, and 1 patient each from Portugal, Austria, Belgium, Newfoundland, New Zealand, Morocco, and Iceland).

One hundred eighty-four patients presented with classical JNCL: disease course characterized by the onset of visual disorder at age 6.2 ± 1.8 years; dementia at age 7.4 ± 2 years; seizures and motor disturbance at age 9.5 ± 3.5 years, with onset of a vegetative state at age 18.4 ± 2.8 years; and death at age 20.2 ± 6.3 years (Wisniewski et al. 1988). Diagnosis was confirmed by the identification of vacuolated lymphocytes and demonstration of ultrastructural fingerprint profiles with or without curvilinear bodies, on electron microscopy (EM) of neural or extraneural tissues.

Four patients presented with an atypical disease course. Patient L39Pa (who died at age 25 years), from the Netherlands, had a severe visual and moderate seizure disorder, with typical motor dysfunction, but had minimal speech retardation and remained conversant until the terminal stages of the disease. Patients L204Pa and L285Pa are both under the care of one physician (Dr. Pirkko Santavuori, University of Helsinki Clinic and Children's Hospital, Helsinki). Patient L204Pa, now 15 years old, presented with visual failure at age 6 years when ophthalmological examination revealed severe retinal dystrophy and mild macular changes. This finding was considered to be slightly atypical of JNCL. To date, the patient has not experienced any epileptic fits. Vacuolated lymphocytes were found in repeated examinations, and the EM findings from a rectal biopsy revealed the presence of both curvilinear and fingerprint patterns. Patient L285Pa, now 30 years old, presented with visual failure at age 6 years and became virtually blind at age 13 years. This patient displays no other clinical signs of the disease, has completed high school education, and now runs a business. Vacuolated lymphocytes were found in repeated examinations, and EM findings of a rectal biopsy found inclusions typical of neuronal ceroid lipofuscinosis. Patient L46Pb, from the United Kingdom, now 22 years old, presented with visual loss at age 10 years and had severe visual impairment by age 12 years. The patient remained well until the onset of seizures at age 18 years; however, the patient has no evidence of motor dysfunction or regression. The patient has now completed college education and is now in full-time employment. Diagnosis of JNCL was confirmed by both the presence of vacuolated lymphocytes and the EM finding of fingerprint inclusions in lymphocytes. Genomic DNA was extracted directly from peripheral blood or from lymphoblastoid cell lines, by use of standard methods.

Table 1

***CLN3* Primers Used for Exon Amplification**

Exon	Sense (5′→3′)	Antisense (5′→3′)	Length (bp)
1	aaaggtacaggcctcagggt	agctctcattcccctcaggt	381
2	acctgagggaatgagagct	tgggttcagctccttttgc	285
3	attgaagggcataggtaaga	actttaccccaccttgtccc	266
4	tcaagtgaaggcagagctgg	agtcccagctgggtagtgaa	251
5	cctgtgtttgtagcaggcct	aaggtcggtctctactctcagc	281
6	tggtcaggagctgagaaagg	gaatccctttcctctgggag	275
7	ggagcctctatgagctgatactg	ggaacattcaggaggacctagg	250
8	tgtcccatggtcagcctag	ttctctccttggacccctct	241
9	gcagtgagctacccatcttt	aggaaaaggccaaacccag	301
10	aatccagtggcatggaagttg	ctacgaccaagggaacaat	287
11	tcgggaaaggtggacagt[a]	ggtattgctgagcgtgactc	318
12	tcgggaaaggtggacagt[a]	aggtgaaacggatgcgac	526
13	tttgaactcctctttttctgg	acactttccactgatagtggga	376
14	tcctaaaaccagggacccct	ttcagtcccagacatccctg	303
15	agggatgtctgggactgaag	ggcatgatgccaggaaga	374

[a] Same primer.

1.02-kb–Deletion Assay

Three PCR-based methods were used to detect the 1.02-kb genomic deletion: primers F2 and P3, described previously (The International Batten Disease Consortium 1995; Munroe et al. 1996), were used to amplify DNA surrounding the deletion; in cases in which, because of the age and quality of patient DNA, long-range PCR was not possible, either primers F2 and R1 (5′-agtgagggagaggaaggtga-3′) or primers that amplify exon 7 (table 1) were used to check for the absence of exon 7. Positive controls for PCR of other *CLN3* exons were included.

PCR Amplification of Exons

Primers to amplify each exon and the surrounding intron sequence (table 1) were designed from genomic DNA sequence of *CLN3* (Mitchison et al. 1997). PCR was performed in a final volume of 100 μl, by use of 100 ng of genomic DNA, 0.2 μM each primer, 0.25 mM each dNTP, 1.5 mM $MgCl_2$ and 1.5 units of AmpliTaq (Perkin-Elmer). A "hot" start was performed, followed by 30 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C and then by a final cycle of 10 min at 72°C, by use of Hybaid OmniGene. The resulting products were electrophoresed in 1% agarose gels and, after ethidium bromide staining, were visualized with a UV transilluminator.

SSCP Analysis

Two different systems were used for the detection of SSCPs. The first used the Phastsystem (Pharmacia), which has been described elsewhere (The International Batten Disease Consortium 1995). Gels were electrophoresed for 300 Vh at 4°C and for 200 Vh at 15°C

in this study. The second method used a radioactive protocol, and samples were analyzed on MDE™ high-resolution gels (AT Biochem).

Direct DNA Sequencing

Amplified exon products to be sequenced were desalted and concentrated by use of Microcon-100 columns (Amicon). Sequencing was performed with the same primers as were used for exon amplification, by use of the *Taq* FS Dye Terminator Cycle sequencing kit (Perkin-Elmer), and automated analysis was done with the ABI 373A sequencer. All exons were sequenced in both directions, and sequence comparisons were performed by use of Sequence Navigator software (Perkin-Elmer) and manual scanning. In one laboratory, the exons were sequenced manually (Sanger et al. 1977) with Sequenase T7 DNA polymerase (United States Biochemicals).

RNA Extraction and Analysis

Cytoplasmic RNA was isolated by use of standard methods (Sambrook et al. 1989). RNA was reverse transcribed by use of oligo(dT) and Superscript reverse transcriptase (Gibco-BRL). Primers 6972 (5′-aaattgttggctcctcttgg-3′) and 6700 (5′-gcgctctctgcttcttcttc-3′) were used to amplify the RNA-cDNA duplex from patient L121/BA. Primers 6795 (5′-ttgatccttgtcacctgtcg-3′) and 6797 (5′-attcagaaggcatgahtgcc-3′) were used to amplify the RNA-cDNA duplex from patient BB, followed by amplification using nested primers 6972 and 6333 (5′-ggctgggagcacagttcat-3′). All products were subcloned and sequenced.

Restriction-Endonuclease Analysis

Amplified exon products were digested according to the manufacturer's recommendations. Samples were electrophoresed in 1% agarose gels and, after ethidium bromide staining, were visualized by a UV transilluminator.

Results

1.02-kb–Deletion Screen

One hundred eighty-eight unrelated patients with JNCL were screened for the 1.02-kb deletion in order to determine the frequency of the common disease-causing mutation and to identify patients carrying other mutations. The 1.02-kb deletion was found to be present on 85% of Batten disease chromosomes. Seventy-four percent (139) of the patients were homozygous for the 1.02-kb deletion, 22% (41) were heterozygous, and 4% (8) carried other undefined mutations on both chromosomes. All results were concordant with the observed haplotypes for alleles at markers *D16S299* and *D16S298*. (Mitchison et al. 1993, 1994, 1995; Lerner et al. 1994). Forty-nine patients carried mutations other than the 1.02-kb deletion; the mutations in three of these have been reported elsewhere (The International Batten Disease Consortium 1995).

Mutational Analysis

Forty-six patients were screened for new mutations. All 15 exons of the gene were amplified: SSCP analysis was performed on exons 7 and 8, and any band shifts were subsequently sequenced. All remaining exons were sequenced in both directions. Nineteen novel mutations were identified: six missense mutations, five nonsense mutations, three small deletions, three small insertions, one intronic mutation, and one splice-site mutation (table 2 and fig. 1). None of the missense mutations or the intron mutation was found on 90 chromosomes of unaffected control individuals, which suggests that these changes do not represent frequent polymorphisms. Inheritance of the mutation in family members was checked by use of a restriction-digest test, if one was available. If the mutation did not affect a restriction site, DNA from family members was sequenced. Mendelian inheritance of mutations was observed (table 2).

Cell lines were available from the patients with the intronic and the splice-site mutations; therefore RNA was tested to confirm the predicted effects of the mutations. Patient L121Pa/BA, heterozygous for the 1.02-kb deletion and the intronic substitution (IVS6-13G→C), was subjected to reverse-transcriptase PCR (RT-PCR) analysis using primers that amplified exons 5–9. Three PCR products were amplified: the expected normal size (409 bp), a product of 265 bp, and a product of 220 bp. Sequencing revealed the largest band to be the correctly spliced normal sequence, whereas the 265-bp product was found to be missing exon 7, and the 220-bp product was missing exons 7 and 8 (corresponding to the 1.02-kb deletion). Proband BB, heterozygous for the 1.02-kb deletion and the splice-site change (IVS14-1G→T), was also subjected to RT-PCR analysis, with primers that amplified exons 5–15. Two PCR products were amplified. Sequencing revealed one product to be missing exons 7 and 8 (corresponding to the 1.02-kb deletion). The other product was shown to have incorrectly spliced exon 15. A cryptic splice-acceptor site in exon 15 was used, which resulted in two in-frame stop codons being introduced: GCCCTGGAGtgatgagcaccgggag (sequence in uppercase is exon 14; sequence in lowercase is exon 15; and the two stop codons are underlined). The predicted translation product is a truncated protein of 399 amino acids.

Discussion

A high percentage (96%) of patients were found to have the 1.02-kb deletion on at least one chromosome, whereas 49 patients were found to carry other mutations, including three that have been described elsewhere

Table 2

Novel Mutations Identified in *CLN3*

Family[a]	Haplotype[b]	Mutation[c]	Nucleotide Change[d]	Amino Acid Change/ Predicted Consequence[d]	Location[e]	Parental Origin of Mutation[f]	Restriction-Site Change	No. of Families with Mutation	Country/ies of Origin
L39	56[g]/46[h]	Missense	c.302T→C	Leu101Pro	Exon 5	Maternal	*Bsi*HKAI (loss)	1	The Netherlands
L227	56[g]/54[h]	2-bp insertion	c.374-375insCC	Frameshift after Pro126	Exon 6	Maternal	...	1	United Kingdom
L1	44[h]/44[h]	1-bp deletion	c.424delG	Frameshift after Leu141	Exon 6	Both	*Bst*NI (loss)	5	The Netherlands (3), United States (2)[i]
L121/BA	56[g]/64[h]	Intron change	IVS6-13G→C	Aberrant splicing[j] Truncated protein	Intron 6	Paternal	...	1	United States
L29	56[g]/66[h]	Nonsense	c.482C→G	Ser161STOP	Exon 7	Maternal	*Sau*3A (gain)	1	Sweden
L259	45[g]/32[h]	Nonsense	c.485C→G	Ser162STOP	Exon 7	Maternal	*Nla*III (gain)	1	Denmark
L46	56[g]/64[h]	Missense	c.509T→C	Leu170Pro	Exon 7	Maternal	...	1	United Kingdom
L116	66[h]/66[h]	2-bp deletion	c.558delAG	Frameshift after Ser185	Exon 8	Both	*Alw*NI (loss)	1	Italy
L250	n3[h]/n3[h]	1-bp insertion	c.586-587insG	Frameshift after Gly195	Exon 8	ND	...	1	United Kingdom
L189	44[k]/34[h]	Nonsense	c.631C→T	Gln211STOP	Exon 8	Maternal	*Acc*I (gain)	1	Italy
L285	n6[g]/n6[h]	Missense	c.883G→A	Glu295Lys	Exon 11	Maternal	...	1	Finland
L209	63[h]/63[h]	1-bp insertion	c.944-945insA	Frameshift after Ser314	Exon 12	Both	*Hinc*II (gain)	4	Italy (2), Iceland, United States[i]
L243	26[g]/43[h]	Nonsense	c.979C→T	Gln327STOP	Exon 13	Maternal	*Bfa*I (gain)	1	Denmark
L216	56[g]/66[h]	Missense	c.988G→T	Val330Phe	Exon 13	Maternal	...	1	Norway
L10	56[g]/66[h]	Missense	c.1000C→T	Arg334Cys	Exon 13	Paternal	*Bsr*BI (loss)	3	The Netherlands (3)
L204	56[g]/45[h]	Missense	c.1001G→A	Arg334His	Exon 13	Paternal	*Bsr*BI (loss)	4	Finland, United Kingdom, Germany, United States[i]
L8	56[g]/54[h]	Nonsense	c.1054C→T	Gln352STOP	Exon 13	Maternal	*Pst*I (loss)	2	The Netherlands, United States[i]
BB	56[g]/26[h]	Splice site	IVS14-1G→T	Aberrant splicing[j] Truncated protein	Intron 14	Maternal	...	1	United States[i]
L61	56[g]/63[h]	1-bp deletion	c.1272delG	Frameshift after Ser423	Exon 15	ND	...	1	United Kingdom

[a] Family in which mutation originally was found.
[b] Formed by markers *D16S299* and *D16S298*.
[c] None of the missense or the intronic mutations is present on 90 normal chromosomes, by sequencing.
[d] Numbering is according to Antonarakis's (1996) recommendations for a nomenclature system for human genome mutations (http://ariel.ucs.unimelb.edu.au:80/~cotton/antonara.htm).
[e] Numbering is that of Mitchison et al. (1997).
[f] "ND" denotes that it was not possible to confirm the parental origin of the mutation.
[g] 1.02-kb Deletion is present.
[h] Novel mutation is present.
[i] Both parents are of northern-European ethnic origin.
[j] Confirmed by RT-PCR analysis and sequencing.

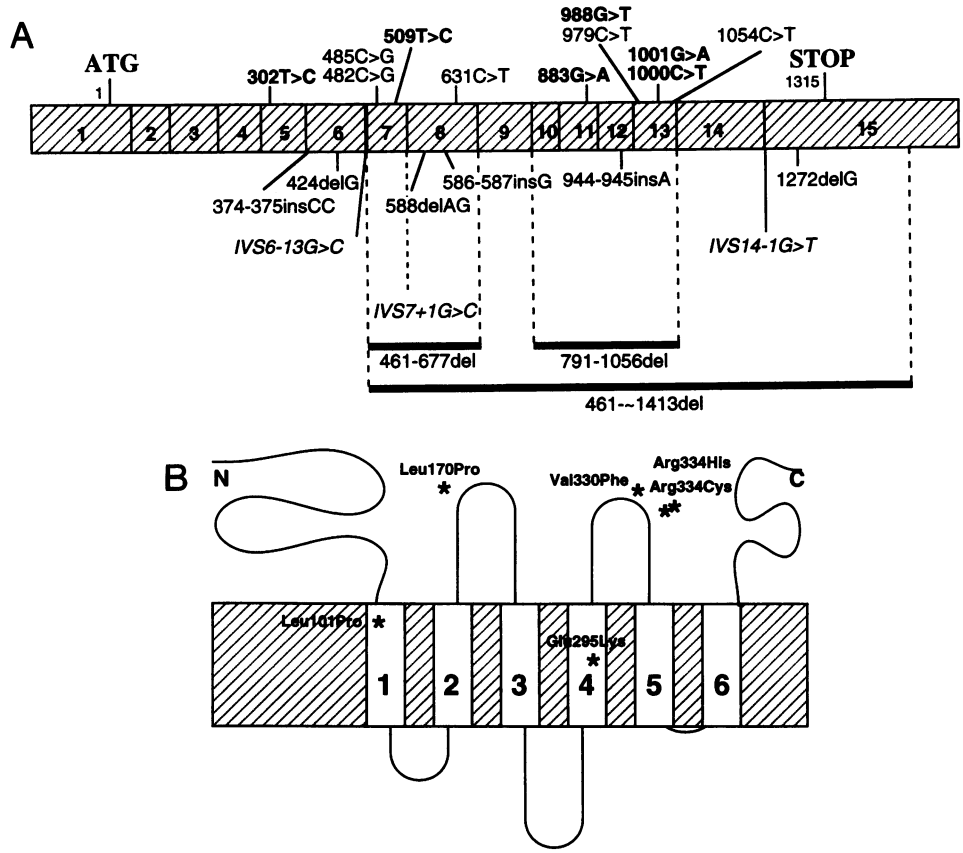

Figure 1 Schematic representation of locations of mutations in *CLN3*. *A*, Designation of mutations, according to Antonarakis's (1996) recommendations for a nomenclature system for human gene mutations (http://ariel.ucs.unimelb.edu.au:80/~cotton/antonara.htm). Mutations identified above the cDNA are point mutations in the ORF; those below are deletions, insertions, or point mutations in introns. Missense mutations are in boldface, and intronic mutations are in italics. The location of the four previously reported mutations (The International Batten Disease Consortium 1995) are indicated by dotted lines; three are large genomic deletions; the deleted nucleotides shown relate to the cDNA only. *B*, Location of the six missense mutations identified in this study, shown on the predicted structure of CLN3 protein (Janes et al. 1996). Amino acid numbering is according to recommendations for a nomenclature system for human gene mutations (http://ariel.ucs.unimelb.edu.au:80/~cotton/antonara.htm).

(The International Batten Disease Consortium 1995). Sequence analysis of the *CLN3* open-reading frame in the 46 patients with other mutations resulted in the characterization of 19 new mutations. The predicted effects of two mutations (intronic and splice site) were confirmed by RT-PCR analysis. Further analysis will be necessary to determine the cellular consequences of the mutations.

The mutations in 32/46 patients were delineated in this study; mutations on both chromosomes were identified in 31/46. Therefore, the disease-causing mutations in 92% (173/188) of the patients in our resource were defined, making a total of 23 disease-causing mutations reported, to date, in *CLN3*. Mutations were not found in 14 patients with classical JNCL (13 of these patients were heterozygous for the 1.02-kb deletion, and 1 does not have the 1.02-kb deletion on either chromosome). All coding regions have been sequenced in these patients, and the results suggest that mutations must lie in the promoter region or elsewhere in an intron. Analysis of these regions is underway.

Eleven of the newly identified mutations can be detected by use of a restriction-digestion test (table 2). These tests can now be used, in conjunction with the 1.02-kb deletion assay, to confirm JNCL in newly diagnosed families and will allow both easier presymptomatic testing of siblings and carrier testing.

A founder effect is associated with the 1.02-kb deletion. All chromosomes carrying this deletion have, for alleles at markers *D16S299-D16S298*, either haplotype "*56*" or a related haplotype (Mitchison et al. 1993, 1994, 1995; Lerner et al. 1994). Of the 19 novel mutations identified in this study, the majority are "private," occurring in only one family. However, five mutations occur in more than one family (table 2), and all families with the same mutation have identical or related haplotypes. This suggests the existence of smaller founder effects, two of which (c.424delG/haplotype"44" and

c.1000C→T/haplotype"66") are concentrated in the Dutch population and three of which (c.944-945insA/haplotype"63," c.1000G→A/haplotype"46," and c.1054C→T/haplotype"54") are more widespread.

The majority (163/174) of patients with JNCL in whom mutations have been identified have, on both chromosomes, mutations that are predicted to give rise to truncated proteins. All of these patients have the classical JNCL phenotype. The 11 remaining patients are heterozygous for the 1.02-kb deletion and a missense mutation. Eight are heterozygous for missense mutations affecting either residue 330 or residue 334: seven of these have classical JNCL, whereas one (patient L204Pa) has an atypical phenotype. Since patient L204Pa is 15 years old, it is still too early in the disease course to predict final disease severity. The three remaining patients are heterozygous for three different missense mutations, and all have an atypical phenotype. The first of these patients, L39Pa, has nonclassical JNCL, diagnosed on the basis of the fact that the patient remained conversant until death at age 25 years (see Patients and Methods). The other two patients, L46Pb and L285Pa, are now >20 years of age and are therefore late in the course of their disease. Both have a phenotype that is dominated by visual failure, which suggests that the spectrum of clinical variability of JNCL might be greater than previously recognized. This raises the possibility that undiscovered missense mutations in *CLN3* might account for a subset of patients with retinopathy only and preserved CNS function, some of whom may not be diagnosed with JNCL.

Intrafamilial phenotypic variability was observed in two families (families BB and L46). In family BB, three siblings who were all compound heterozygotes for a splice-site mutation and the 1.02-kb deletion presented, at the same age, with visual failure (at age 5 years) and regression (at age 12 years). However, different ages at onset of seizures (ages 12, 13, and 17 years) and of preterminal disease (ages 15, 21, and 28 years) were observed. In family L46, of two siblings who were compound heterozygotes for the 1.02-kb deletion and the missense mutation (Leu170Pro), the eldest child had classical JNCL (and died at age 22 years), whereas the younger sibling, L46Pb, now 22 years old, has visual failure, a nearly normal IQ, and easily controlled seizures. Intrafamilial variation such as this has been observed in many diseases—such as type I Gaucher disease (Amaral et al. 1994), cystic fibrosis (Dean et al. 1990), and phenylketonuria (Tyfield et al. 1995)—but its cause is still unexplained in most cases. Both genetic and environmental factors may play a role.

Homologues of the CLN3 protein have now been identified in other species, suggesting that the protein has a function that is basic to eukaryotes. All six missense mutations in *CLN3* affect residues that are conserved between the human form and its homologues in *Saccharomyces cerevisiae* (*YHC3*) (GenBank accession number Z49334), dog (GenBank accession number L76281), and mouse (GenBank accession number U47106) (Lee et al. 1996). Five of the six residues are conserved in *Caenorhabditis elegans* (GenBank accession number Z77656). A structural model for the Batten disease protein has recently been proposed (Janes et al. 1996). The location of the missense mutations identified in the present study are shown in figure 1*B*. Two are located in predicted transmembrane segments, and four are located on predicted extracellular loops on one face of the protein only. This suggests that one face of the CLN3 protein is of particular functional importance. Except for one possible exception (patient L204Pa), patients with missense mutations affecting amino acid residues 330 and 334 (fig. 1*B*) all have the classical JNCL phenotype, whereas patients with the other three missense mutations all have an atypical clinical course. This indicates that amino acid residues 330 and 334 must be critical for normal function of the CLN3 protein. The identification of such residues will greatly facilitate the determination of the important structural and functional domains of the CLN3 protein.

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