The early-onset torsion dystonia gene (*DYT1*) encodes an ATP-binding protein

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Early-onset torsion dystonia is a movement disorder, characterized by twisting muscle contractures, that begins in childhood. Symptoms are believed to result from altered neuronal communication in the basal ganglia. This study identifies the *DYT1* gene on human chromosome 9q34 as being responsible for this dominant disease. Almost all cases of early-onset dystonia have a unique 3-bp deletion that appears to have arisen independently in different ethnic populations. This deletion results in loss of one of a pair of glutamic-acid residues in a conserved region of a novel ATP-binding protein, termed torsinA. This protein has homologues in nematode, rat, mouse and humans, with some resemblance to the family of heat-shock proteins and Clp proteases.

Movement disorders constitute a group of human neurological diseases in which aberrant neurotransmission in the basal ganglia is associated with uncontrollable body movements, such as chorea in Huntington disease, tremor and rigidity in Parkinson disease and twisting contractions in torsion dystonia. Dystonic symptoms can be secondary to a number of neurological conditions, and to drug or traumatic injury to the brain, but primary or torsion dystonia is distinguished by lack of other neurological involvement[1,2], a strong hereditary predisposition and, in contrast to these other two neurodegenerative diseases, the absence of any distinct neuropathology.

Early-onset dystonia (DYT1) represents the most severe and most common form of hereditary dystonia. Symptoms usually begin in an arm or leg at approximately twelve years (this can range, however, between 4 and 44 years) and spread to other limbs within about five years[4,5]. The clinical spectrum of early-onset dystonia is similar in all ethnic populations, with highest prevalence in the Ashkenazic-Jewish (AJ) population[6–8], due to a founder mutation[9–11]. Early-onset dystonia follows an autosomal dominant mode of inheritance with 30–40% penetrance[12,10]. The responsible gene in Jewish and non-Jewish families has been mapped to human chromosome 9q34 (refs 13–15). Haplotype analysis of the founder mutation in AJ families placed the *DYT1* gene in a 1–2-cM interval centromeric to the *ASS* locus on chromosome 9 (ref. 9), with highest lod scores obtained with adjacent markers *D9S62a/b* and *D9S63* (ref. 11). A YAC contig was constructed across a 600-kb interval including these two markers, and the most likely position of the gene in this region was refined to a 150-kb interval by extended haplotype analysis in affected AJ individuals using a set of eleven polymorphic markers spanning this region[16].

In this study, the *DYT1* gene was identified by i) construction of a cosmid contig spanning the target region, ii) exon trapping and identification of four cDNAs encoded in this region and iii) mutational screening by single-strand conformation polymorphism (SSCP) and sequence analysis of cDNA and genomic DNA from affected individuals and controls. A 3-bp deletion in the coding sequence of one of these genes was found in all affected and obligate carrier individuals with chromosome 9–linked primary dystonia, regardless of ethnic background and surrounding haplotype. The deduced protein, termed torsinA, contains an ATP-binding domain and a putative N-terminal leader sequence. It has high homology to three additional mammalian genes and a nematode gene in the databases and distant similarity to the heat-shock protein/Clp protease family.

Cosmid contig and transcript map

To facilitate identification of candidate genes, a cosmid contig was constructed across the 150-kb target region on chromosome 9q34 between polymorphic markers *D9S2161* and *D9S63* (ref. 16). Gridded arrays of cosmids from two chromosome 9–specific libraries were screened initially with four YACs (8H12, 183D9, 251H9 and 22A4) spanning this region. A positive subset of cosmids was screened sequentially with end sequences from cosmids, starting on the centromeric side with the end of cosmid LL09NC0150H11, which hybridized to YAC 8H12, and, at the telomeric end, with 37F5LA and 18D5LA, which hybridized to the other three YACs (Fig. 1). A cosmid contig with more than threefold redundancy was generated across the genomic region, and a restriction map was constructed with a representative subset of eleven overlapping cosmids with the restriction enzymes *Eco*RI, *Xho*I and *Not*I. Fragments were aligned by size and hybridization patterns using markers *D9S2161* and *D9S63*, cosmid end clones, cloned exons and oligonucleotides from unique regions. The estimated length of the contig between the defining markers *D9S2161* and *D9S63* is 150 kb.

Genes in this region were identified by exon amplification, which allows cloning of exons by virtue of flanking splice sites in genomic DNA during cellular processing of RNA expressed by splicing

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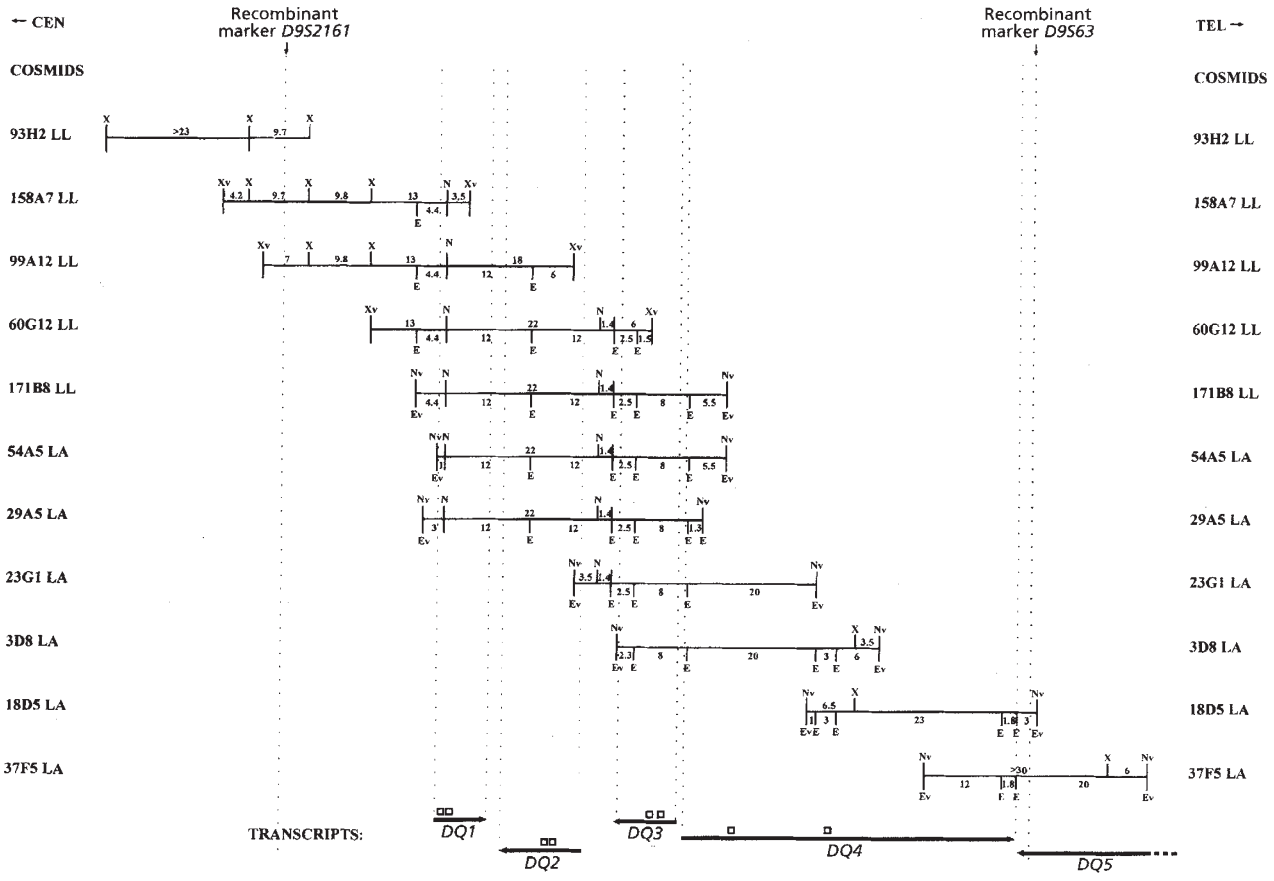

Fig. 1 Cosmid contig and transcript map across the *DYT1* target region. Horizontal lines depict cosmids; vertical lines denote restriction sites (X, *Xho*I; E, *Eco*RI; N, *Not*I; Nv,Xv,Ev are vector ends); and numbers indicate size of fragments in kilobases. The dotted vertical lines indicate the 5′ and 3′ ends of each cDNA and the position of the recombinant markers *D9S2161* and *D9S63*. Dark horizontal arrows at the bottom represent transcripts, with the direction of the arrow indicating the direction of transcription. The boxes above the cDNAs point out some of the trapped exons. The cosmids used to construct the map are listed on both sides. The cosmid names followed by 'LL' are from the Lawrence Livermore chromosome 9–specific library, and designated names are all preceded by 'LL09NC01'; those followed by 'LA' are from the Los Alamos chromosome 9–specific library. 'CEN' stands for centromere and 'TEL' for telomere; they indicate the orientation of this map with respect to chromosome 9q.

vectors[17,18]. More than 60 putative exons were trapped from cosmids in the critical region, yielding 28 independent sequences. These exon clones were then used to screen human cDNA libraries from different human adult and fetal tissues, which had been generated by priming with oligo dT or random primers. Five cDNAs were represented in overlapping clones: *DQ1* from fetal brain, adult frontal cortex and adult liver; *DQ2* from adult substantia nigra, hippocampus and frontal cortex; *DQ3* from adult frontal cortex; *DQ4* from adult frontal cortex and fetal brain; and *DQ5* from adult occipital cortex, substantia nigra and frontal cortex. All but three of the 28 unique putative exons were accounted for by these cDNAs. Hybridization of these three to northern blots from a number of adult human tissues revealed no corresponding message species, and these may represent the result of cryptic splice junctions or be low-abundance messages.

The five cDNAs were extended in both directions by 5′- and 3′-RACE[19] and sequenced in multiple clones. Transcripts were then aligned across the cosmid contig by hybridizing Southern blots of restriction-digested cosmid DNA with 5′ and 3′ cDNA ends, exon clones and oligonucleotides corresponding to cDNA sequence (Fig. 1). Estimated genomic regions covered by these genes are 8 kb for *DQ1*, 13 kb for *DQ2*, 10 kb for *DQ3*, 52 kb for *DQ4* and more than 40 kb for *DQ5*. Because only the 3′ untranslated region of cDNA *DQ5* overlapped the critical interval, this gene was excluded from the study. Given the extensive exon trapping carried out in this region, and the fact that two to ten exons were identified for each cDNA, these transcripts presumably account for most, if not all, genes in the critical region. However, several large regions (more than 10 kb each), one centromeric to *DQ1* and two within the first two introns of *DQ4*, could contain other genes, particularly genes with one or no intron that would be missed by exon amplification.

Sequence of cDNAs

DQ1 and *DQ2* are highly homologous cDNAs, with 72% identity at the nucleotide level and 69% amino-acid identity in the predicted protein sequence. The genes are in opposite orientations, with their 3′ ends less than 12 kb apart in the genome (Fig. 1). The cDNA for *DQ2* consists of 2,072 bp, with a predicted open reading frame of 998 bp from nucleotide 43 to 1041 (Fig. 2). The sequence around the putative ATG translation start site contains the critical –3 purine residue, but none of the other features of the Kozac[20] consensus sequence. The 3′ untranslated portion is 1,031 bp long and contains two poly A^+ addition sites—one, ATTTAAA, at nucleotide 1390 and the other, AATAAA, at nucleotide 2054—with poly A tails present about 20 bp down from each of them in several of the cDNA clones. The *DQ2* cDNA appears to be essentially full length, based on the sizes of corresponding transcripts. Northern blots of adult and fetal human

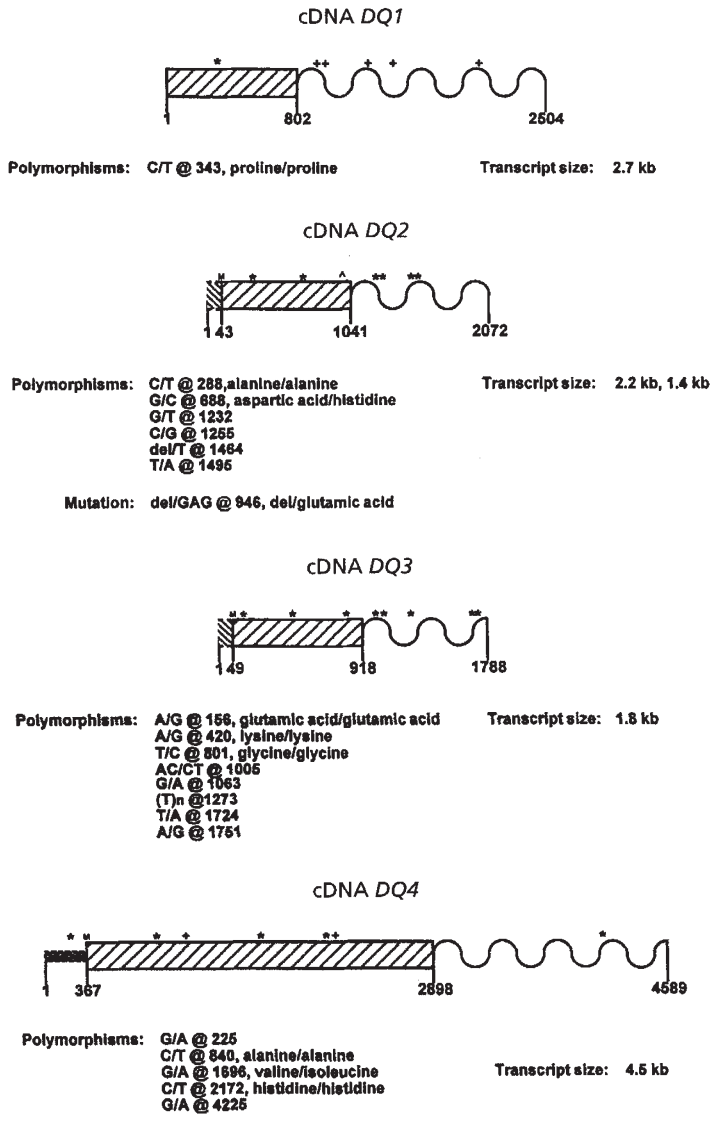

Fig. 2 Sequence variations in cDNAs in the *DYT1* region. Diagrams are scaled representations of each of the four cDNA transcripts in the critical region. The striped black box indicates 5′ untranslated sequence, the striped white box indicates deduced open reading frame and the wavy line indicates 3′ untranslated sequences. The dashed lines at the 5′ end of the open reading frame of cDNAs *DQ2* and *DQ3* indicate that no stop codon 5′ to the first predicted methionine (M) has been found. The numbers flanking the open reading frame box indicate the beginning and end of the cDNAs and the nucleotide position of the predicted start and stop codons. Regions generating SSCP shifts are indicated above the transcript diagram: + marks those for which nucleotide changes have not yet been determined; * marks the location of known nucleotide changes corresponding to SSCP shifts; ^ marks the GAG deletion in cDNA *DQ2*. Nucleotide changes and resulting amino-acid conversions are given below each cDNA. Transcript sizes were estimated by northern-blot analysis.

RNA showed two ubiquitous messages of about 1.8 kb and 2.2 kb (Fig. 3). Only the 2.2-kb message hybridized to sequences 3′ to the first poly A^+ addition site, indicating that the larger species may represent utilization of the second poly A^+ addition site (data not shown). In fetal brain, lung and kidney, as well as in adult brain, heart and pancreas, another message species of about 5 kb was present in low abundance. The 1.8-kb and 2.2-kb messages, and no novel species, were seen in tissues from AJ individuals bearing the founder mutation, including adult lymphoblasts, fibroblasts and cerebellum at autopsy, as well as fetal brain, muscle, spleen, intestines, eye, lung and liver. The open reading frame of cDNA *DQ2* predicts a 6.81 pI polypeptide of 332 amino acids with a calculated molecular weight of 37,813 D, termed torsinA (Fig. 4).

cDNA *DQ1* is 2,504 bp, with an open reading frame of 802 bp (Fig. 2). Based on the strong similarities between cDNAs *DQ1* and *DQ2*, the methionine start site for the *DQ1* message is probably not in the existing *DQ1* clone. The 3′ untranslated portion is 1,702 bp long, with a poly A^+ addition site, AATAAA, at position 2483 and a poly A tail starting at 2505. Northern-blot analysis revealed a ubiquitously expressed message of about 2.8 kb, present at low levels in adult brain but not detectable in fetal brain (data not shown). The open reading frame of the existing clone encodes 289 amino acids, suggesting that the corresponding protein, which we have named torsinB, has a molecular weight greater than 32,000 D (Fig. 4). cDNA sequences for the other transcripts in the target region will be described in other papers: *DQ3* (Ozelius *et al.*, in preparation), *DQ4* (Hewett *et al.*, in preparation) and *DQ5* (Cox *et al.*, in preparation).

Mutational analysis

All four cDNAs in the critical region were screened for heterozygous mutations in affected individuals with torsion dystonia. Gross alterations in sequence were excluded by Southern-blot analysis using genomic DNA from 30 dystonia patients with different ethnic backgrounds hybridized to these cDNAs (data not shown). Transcripts from the critical region were then screened for sequence variations by reverse transcriptase—polymerase chain reaction (RT-PCR) by use of lymphoblast RNA from fourteen affected individuals from different families, representing twelve unique haplotypes in an extended region surrounding the *DYT1* gene (*D9S62a* to *ASS*[16]), as well as in two control individuals, one AJ

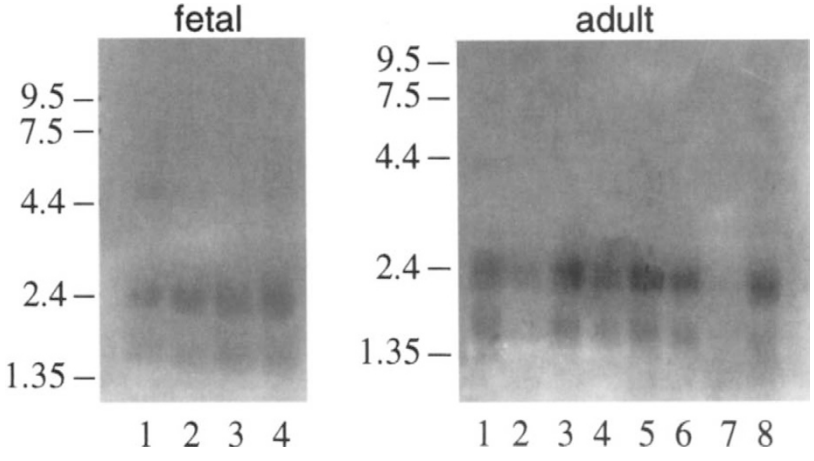

Fig. 3 Northern-blot analysis of *DQ2* transcript. Northern blots of human RNA in order from left to right (fetal): 1) brain, 2) lung, 3) liver and 4) kidney; (adult): 1) heart, 2) brain, 3) placenta, 4) lung, 5) liver, 6) skeletal muscle, 7) kidney and 8) pancreas. Blots hybridized to PCR probe corresponding to nucleotides 149–1307 of cDNA *DQ2*. Marker sizes are indicated by bars.

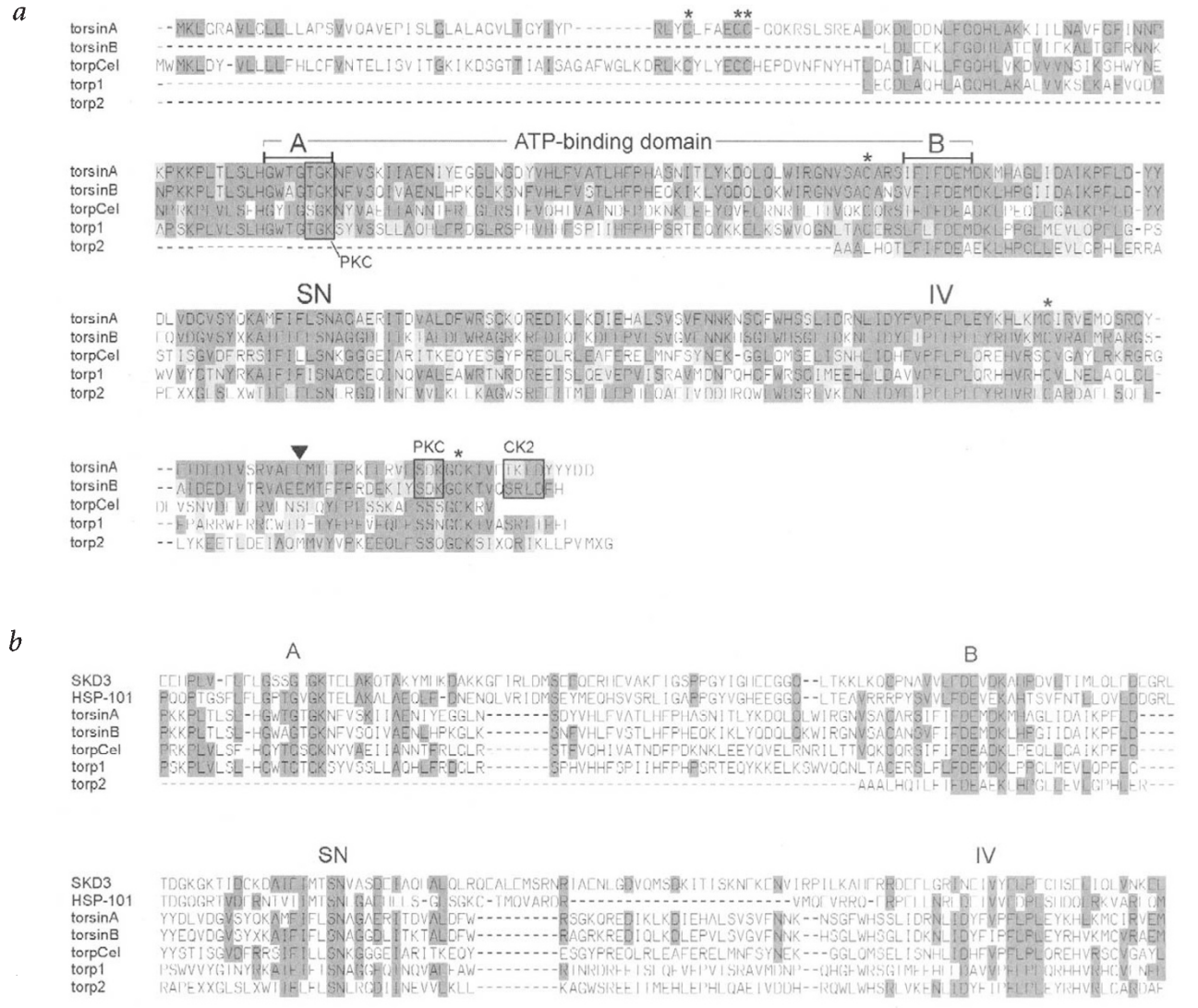

Fig. 4 *Comparison of predicted amino-acid sequences of torsin gene family members.* ***a****, Alignment of torsins and torps. TorsinA and torsinB are encoded by cDNAs DQ2 and DQ1, respectively. TorpCel is the predicted amino-acid sequence from a Caenorhabditis elegans genomic sequence. Torp-1 and torp-2 correspond to overlapping expressed sequence tag cDNAs from human and mouse, respectively. The solid triangle represents the site of the GAG (E) deletion in torsinA. Conserved cysteine residues are represented by asterisks. Darkly shaded residues are identical to a consensus sequence; lightly shaded residues are similar. Conserved possible phosphorylation sites for protein kinase C (PKC) and casein kinase 2 (CK2) are boxed.* ***b****, Comparison of torsins and torps with two representative members of the HSP100 family. SKD3, from mouse, is an HSP100 family member of class 2M; HSP101, from soybean, is a heat-shock protein of class 1B (ref. 22). Shaded residues are identical to a consensus sequence. The conserved motifs (A, B, SN and IV) are present in all seven proteins.*

and one non-Jewish. Four of these haplotypes were from families demonstrating linkage to chromosome 9. They included four non-Jewish families[15], two of which were French-Canadian and shared a common haplotype, and an AJ family with the founder mutation. These unique haplotypes were initially assumed to represent independent mutations in *DYT1*. Nested PCR was carried out in overlapping fragments of 150–300 bp spanning the cDNAs. Fragments were resolved by SSCP analysis, and all variant bands were sequenced in both directions. All transcripts showed a number of variations in both coding and noncoding regions (Fig. 2). Most of these were 'silent', involving single-base-pair substitutions in the 5′ and 3′ untranslated regions or in the third position of triplet codons. Only three of these changes affected the amino-acid sequence: i) valine^{174}→isoleucine in *DQ4*, ii) aspartic acid^{174}→histidine in *DQ2* and iii) deletion of a glutamic acid (GAG) in *DQ2*. All sequence variations in the coding regions were analysed in genomic DNA after the corresponding exon/intron structure in cosmid DNA had been determined. All these nucleotide changes except one—the GAG deletion in *DQ2*—were confirmed as polymorphisms by their presence in control samples. Surprisingly, the GAG deletion in *DQ2* was present in all six individuals representing the four confirmed DYT1-linked haplotypes.

To pursue this finding, we examined the co-segregation of the GAG deletion with carrier status in all known chromosome 9–linked families, as well as in a large number of AJ and non-Jewish controls. This GAG deletion was analysed with PCR products generated from genomic DNA samples in three ways: SSCP, direct sequencing and/or digestion with *Bse*RI, which cuts 10 bp downstream of the normal GAGGAG sequence but does not cut the GAG-deletion sequence (Fig. 5). The association of the GAG deletion with carrier status in the chromosome 9–linked families was complete. All 261 affected and unaffected obligate gene carriers in 68 families linked to chromosome 9 were heterozygous for this deletion, including 64 AJ families carrying the founder haplotype and

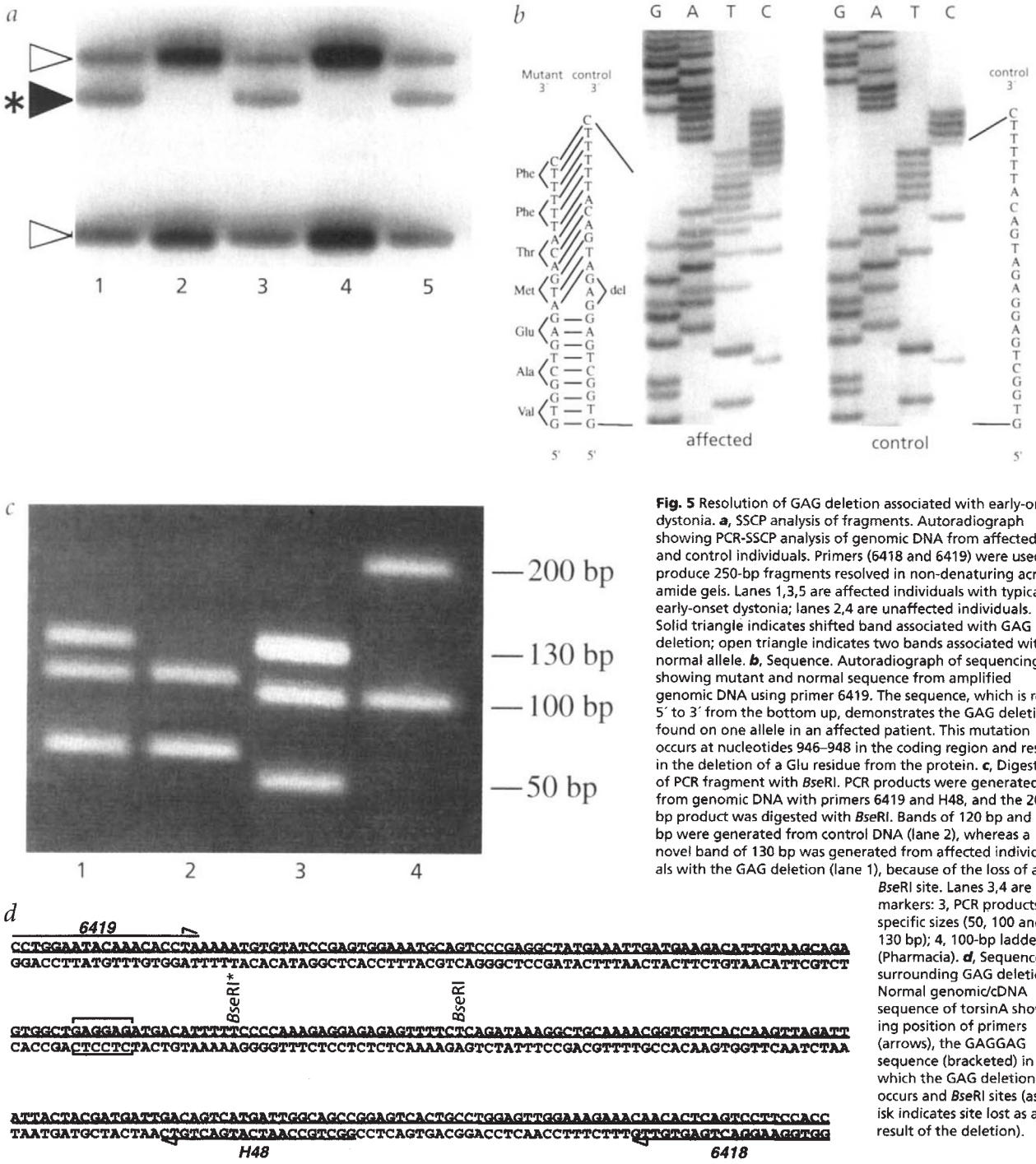

Fig. 5 Resolution of GAG deletion associated with early-onset dystonia. ***a***, SSCP analysis of fragments. Autoradiograph showing PCR-SSCP analysis of genomic DNA from affected and control individuals. Primers (6418 and 6419) were used to produce 250-bp fragments resolved in non-denaturing acrylamide gels. Lanes 1,3,5 are affected individuals with typical early-onset dystonia; lanes 2,4 are unaffected individuals. Solid triangle indicates shifted band associated with GAG deletion; open triangle indicates two bands associated with normal allele. ***b***, Sequence. Autoradiograph of sequencing gel showing mutant and normal sequence from amplified genomic DNA using primer 6419. The sequence, which is read 5′ to 3′ from the bottom up, demonstrates the GAG deletion found on one allele in an affected patient. This mutation occurs at nucleotides 946–948 in the coding region and results in the deletion of a Glu residue from the protein. **c**, Digestion of PCR fragment with *Bse*RI. PCR products were generated from genomic DNA with primers 6419 and H48, and the 200-bp product was digested with *Bse*RI. Bands of 120 bp and 70 bp were generated from control DNA (lane 2), whereas a novel band of 130 bp was generated from affected individuals with the GAG deletion (lane 1), because of the loss of a *Bse*RI site. Lanes 3,4 are markers: 3, PCR products of specific sizes (50, 100 and 130 bp); 4, 100-bp ladder (Pharmacia). ***d***, Sequence surrounding GAG deletion. Normal genomic/cDNA sequence of torsinA showing position of primers (arrows), the GAGGAG sequence (bracketed) in which the GAG deletion occurs and *Bse*RI sites (asterisk indicates site lost as a result of the deletion).

four non-Jewish families (Table 1). Strikingly, the deletion was not present in 260 AJ and 274 non-Jewish control chromosomes, and it was not observed in the homozygous state in any individual.

To further assess the role of this deletion in torsion dystonia, we typed an additional 155 individuals with varying clinical manifestations from 131 families of different ethnic backgrounds that were too small for linkage analysis (Table 1). These included individuals from 19 families with typical early-onset dystonia, 74% (14/19) of whom carried the GAG deletion. Typical cases that do not carry the GAG-deletion may have other, as yet unidentified, mutations in *DQ2*. Only 10% (4/38) of families with an uncertain diagnosis of early-onset dystonia (see Methods for clinical criteria) carried the GAG-deletion, whereas none of the 76 families classified as having atypical dystonia possessed the GAG deletion. Collectively, these observations provide compelling evidence that the GAG deletion is responsible for the vast majority of cases of typical early-onset dystonia in both AJ and non-Jewish individuals.

The identification of a single mutation (GAG deletion) on affected chromosomes responsible for almost all cases of typical early-onset dystonia is remarkable. Two possible explanations may account for this surprising finding: i) all these cases may be ancestrally related, representing a common founder mutation that predates the introduction of this mutation into the AJ population; ii) the same mutation has arisen independently, and is the only change (or perhaps one of a few) that results in the early-onset dystonia phenotype. To distinguish between these possibilities, we identi-

Table 1 • Genotype of GAG deletion in candidate cDNA

Categories	Families	Genotype in individuals +/+[a]	+/–	–/–
Controls:				
AJ	130	130	0	0
NJ	137	137	0	0
Affected and obligate carriers[b] in 9-linked families:				
AJ founder haplotype	64	0	173	0
NJ	4	0	88	0
Affecteds of unknown linkage[c].				
typical[d]	19	5	36	0
uncertain	38	34	4	0
atypical	76	76	0	0

[a]+: GAGGAG. –: GAG (del). [b]Lod score > +2 for 9q34 markers[15]. [c]Families too small for linkage analysis. [d]See clinical criteria in Methods for definitions. [e]From 14 families.

fied three new single-base-pair polymorphisms in a 5-kb region surrounding the GAG deletion in *DQ2* and carried out allelic analysis with them. In affected individuals carrying the four confirmed DYT1-linked haplotypes, two different patterns of alleles were observed: 1, 1, 2 on three of the disease-bearing chromosomes and 1, 2, 1 on one of the disease-bearing chromosomes. This finding clearly supports the conclusion that the same mutation (GAG deletion) has arisen more than once.

Discussion

Positional cloning was used to identify *DYT1*, which is responsible for the early-onset form of primary dystonia, on human chromosome 9q34. Mutational analysis revealed a 3-bp deletion in the coding portion of a transcript, which was the only non-polymorphic change identified on disease-bearing chromosomes. This mutation was uniquely associated with typical cases of early-onset dystonia, and appears to have arisen independently on different haplotypes in a number of ethnic groups. Thus, apparently only one variation—or one of a few variations—in the encoded protein can give rise to this phenotype. The deletion results in loss of one of a pair of glutamic-acid residues near the carboxy terminus of a novel protein, termed torsinA. These glutamic acids and flanking cysteine residues are conserved in an adjacent, homologous human gene, encoding a protein termed torsinB, and in related mouse and rat sequences. TorsinA and B are ATP-binding proteins with a distant relationship to the HSP100/Clp family of proteins[22].

Insights into the protein relationships and possible function of torsinA were revealed by searches of GenBank protein databases. A closely related deduced protein sequence is encoded by cosmids from *Caenorhabditis elegans*, here termed torsin-related protein in *C. elegans* (torpCel). Identified expressed sequence tags also corresponded to human, mouse and rat torsinA and torsinB, as well as to two other related proteins, torp1 and torp2. Fig. 4*a* shows an amino-acid alignment of these predicted proteins. The glutamic-acid pair, bearing the deletion in affected individuals, is conserved in all human, rat and mouse torsinA and torsinB transcripts, suggesting that it is part of a functional domain. Although the glutamic-acid pair is absent in the torps, the neighboring residues are fairly well conserved, including the cysteine residues that flank this region. A phylogeny analysis suggests that torsinA and torsinB are most closely related to each other (about 70% amino-acid sequence identity), and that they and the torps are equally distant (about 50% identity).

The torsins and torps bear four short domains—A, B, SN and IV—with some similarity to conserved domains in the HSP100/Clp family of proteins[22,23]. The torsin family is comparable to two representative members of the HSP100 protein family (Fig. 4*b*): HSP101, a heat-shock protein from soybeans in the HSP100 subfamily 1B, and SKD3, a ubiquitous mouse protein in the subfamily 2M[22,23]. The most robust feature is a conserved ATP/GTP-binding sequence comprising two motifs: the nucleotide-binding site 'A' (GxTGxGKT/S), followed approximately 60 amino acids later by the Mg^{++}-binding site 'B' (ShhhFDEhEKxH), where 'x' indicates variable residues and 'h' indicates hydrophobic residues[25,26]. In a conserved stretch of 140 amino acids that include the nucleotide-binding domain, torsin family members are 25–30% identical to HSP100 family proteins. Key residues of the HSP100 consensus site IV (ref. 22) and another site (SN; Fig. 4*b*) are also conserved, but consensus site V is absent from the torsins. Members of the HSP100/Clp family have chaperone functions or proteolytic activity, which can confer thermotolerance, allow correct folding and localization of proteins, and regulate protein signalling[24]. HSP100/Clp proteins are distinguished by two features: they bind ATP and/or have ATPase activity, and they occur in oligomeric complexes with one or more additional protein species. Mutations in the carboxy region of two of these proteins can block binding to companion proteins and function in a dominant manner[27,28]. The discrete glutamic-acid deletion in the carboxy end of torsinA would be consistent with a dominant negative effect mediated by disruption of the activity of a multimeric complex.

Few other clues from the deduced protein sequences of the torsins and torps yield insight into their function. The 41 amino acids at the putative N-terminal of torsinA consist of two 20 amino-acid hydrophobic domains. The first of these begins with two basic amino acids and ends with a polar and an acidic amino acid; it fulfils the criteria of a leader sequence for a transmembrane or membrane-translocated protein[29]. Several possible phosphorylation sites are conserved in both torsinA and torsinB—two for protein kinase C and one for casein kinase II—as well as a number of putative N-myristylation sites (Prosite analysis). Six cysteine residues are conserved with other torsin family members (Fig. 4*a*).

The finding of the same 3-bp mutation in the heterozygous state in most cases of typical early-onset dystonia is surprising, yet robust. There are only a few examples of the same recurrent mutation causing a dominantly inherited condition. These include loss of a positively charged arginine in the fourth transmembrane helix of the a_1 subunit of the L-type voltage-sensitive calcium channel, which is the only type of mutation found to cause hypokalaemic periodic paralysis[30,31]; a glycine to arginine substitution in the transmembrane domain of the fibroblast growth factor receptor–3 (FGFR3), seen in almost all cases of achondroplasia[32]; common missense mutations, seen in hypertrophic cardiomyopathy[33]; and CAG expansions in the coding regions of a number of genes causing neurodegenerative diseases[34,35]. In all these cases, it appears that the same mutations occur repeatedly as independent events, whereas other mutations in the same gene cause a different syndrome, have no phenotype or are incompatible with life.

Seven genes causing dystonia have been mapped on human chromosomes: dopa-responsive dominant on 14q21–22 (refs 36,37) and recessive on 11p11.5 (refs 38,39), paroxysmal on 2q (refs 40,41), a late-onset focal on 18p (ref. 42), a mixed phenotype on 8 (ref. 21), the *DYT1* gene on chromosome 9q34 (ref. 13) and the gene for dystonia-parkinsonism (Lubag) on Xq13.1 (refs 43,44). Only two other dystonia genes have been identified, and both implicate decreased dopaminergic transmission in dystonia. Dopa-responsive dystonia can be caused by disruption of tyrosine hydroxylase, the rate-limiting enzyme in dopa synthesis[38,39] and by haploinsufficiency of GTP cyclohydrolase I, needed for synthe-

sis of the tyrosine hydroxylase co-factor, biopterin[45]. The only genetic rodent models reported to date—the dystonic (*dt*) rat[46], the mouse mutant, dystonia musculorum (*dMd*)[47] and hamsters with paroxysmal dystonia (*dt52*)[48–50]—do not match the genetic or neurobiological features of human dystonias. Identification of the *DYT1* gene will allow assessment of the role of this torsin family and interacting proteins in human dystonia, and generation of authentic genetic animal models of torsion dystonia.

Although there is no distinctive neuropathology in primary dystonia[6,51], this disorder is believed to result from imbalance of neural transmission in the basal ganglia, as cases of secondary dystonia reveal lesions in the caudate nucleus, putamen and globus pallidus, as well as in the thalamus and rostral brain[52–54]. Several lines of evidence suggest that altered dopaminergic input to the basal ganglia may be implicated in dystonia. For example, drugs that block dopaminergic transmission via the D2 receptor can elicit acute or tardive dystonic symptoms[55,56], and abnormally low levels of dopaminergic metabolites have been noted in the cerebrospinal fluid of some dystonic patients[57–59]. Probably the most compelling evidence is that dopa-responsive dystonia, which is similar to early-onset torsion dystonia in age at onset and clinical phenotype, results from a deficit in dopa synthesis and is cured by dopa administration. It follows that early-onset dystonia, which does not respond to dopa, may be caused by a defect in release rather than in synthesis of dopamine.

Identification of the *DYT1* gene is the first step in understanding the molecular aetiology of early-onset dystonia and designing rational therapies. The ease of detection of the GAG deletion, which appears to underlie most cases of early-onset dystonia, will support differential DNA diagnosis in all ethnic groups, even in apparently sporadic cases. Identification of gene carriers before the onset of symptoms should allow evaluation of genetic and environmental factors that trigger the symptoms. Modifying genetic factors could include polymorphic variations in torsinA or mutations in associated proteins, while environmental factors could include sensory overload to the part of the body subserved by susceptible neurons, such as that caused by overuse or trauma[60]; high body temperature; or exposure to toxic agents. Understanding the function of this class of novel ATP-binding proteins should provide a unique window into developmental patterning and plasticity of neuronal circuitry in the basal ganglia and help to elucidate neuronal mechanisms underlying loss of movement control in primary and secondary dystonias, as well as other movement disorders.

Methods

Clinical criteria and patient samples. Individuals and families were ascertained from a database of patients diagnosed and treated by members of the Movement Disorders Group at Columbia Presbyterian Medical Center and the Movement Disorders Division at Mount Sinai Medical Center (New York), and through advertisements in the newsletters of the Dystonia Medical Research Foundation. The criteria for the diagnosis of primary torsion dystonia and the method of evaluation were the same as described previously[4]. All subjects gave informed consent before their participation in the study.

Two groups of individuals with primary dystonia were considered. The first group consisted of known *DYT1* gene carriers from four non-Jewish families previously demonstrating linkage to chromosome 9q34 (ref. 15). These four families were chosen from the seven previously described families on the basis of individual family lod scores of at least +2 at 9q34 markers. We also included in this group Ashkenazic individuals who carried the founder haplotype of 9q34 alleles[4]. The second group of individuals had primary dystonia, but their linkage status was unknown; that is, non-Jewish and non–Ashkenazic-Jewish individuals from small families and Ashkenazim who did not have the founder haplotype. The latter group was further divided into three clinical subgroups, based on previous studies delineating the *DYT1* phenotype[4,15,61]: i) typical or probable early-onset (DYT1) phenotype (under 28 years) limb dystonia with spread to at least one other limb but not to cranial muscles; ii) atypical or unlikely DYT1 phenotype (focal or segmental cervical-cranial dystonia of any age at onset, or writer's cramp beginning after age 44 years); and iii) uncertain DYT1 phenotype (dystonia not fitting into either of these other categories, such as isolated writer's cramp beginning before age 45, cervical or cranial-onset dystonia spreading down to limbs or onset in limbs and spread up to cranial muscles. Patients with symptoms typical of early-onset dystonia were also categorized as 'uncertain' if they had confounding neurological abnormalities.

The four 'uncertain' cases that carried the GAG deletion are described below. One patient had a clinical phenotype typical for DYT1, but was classified as 'uncertain' because she had had polio as a child, possibly confounding the classification. Another carrier also had a phenotype typical for DYT1 but was classified as 'uncertain' because of concurrent head tremor and a family history of head and arm tremor. The remaining two carriers had features of typical early-onset dystonia; one had early limb onset and spread to other limbs and also to cranial muscles, and the other had early onset in an arm and spread to the neck.

In families of unknown linkage status with several afflicted members, a single classification was assigned to all affecteds within the family. If families had members with both uncertain and typical phenotypes, a classification of 'typical' was assigned; if families had members with both uncertain and atypical phenotypes, a classification of 'atypical' was assigned; and if families had members with both atypical and typical phenotypes, a classification of 'uncertain' was assigned.

We also sought to include individuals with a wide range of ethnic and geographic ancestry. This study was approved by the human subjects review boards of both institutions participating in the study.

DNA isolation, lymphoblast lines and Southern blots. Venous blood samples were obtained from participating individuals. DNA was extracted from whole blood[62] or from lymphoblast lines established from blood lymphocytes by EBV transformation[63]. CEPH pedigree DNA was obtained from CEPH (Centre d'Étude du Polymorphisms Humain, Paris). For Southern blots, genomic DNA was digested with *Pst*I, *Hin*dIII and *Eco*RI (NEB) according to the manufacturer's instructions. Digested DNA was resolved on 1% agarose gels at 70 V for 16 h. Southern blotting was performed by standard techniques, and the filters were hybridized to cDNAs (see below).

Isolation of RNA, northern blots, RT-PCR. Cytoplasmic RNA was isolated from lymphoblasts, and fibroblasts were established from patients and controls[64]. Total RNA was extracted from human adult and fetal tissue obtained at autopsy by the guanidinium-thiocyante method[65]. Tissues were obtained from both control and *DYT1*-carrier individuals, and included brain cortex, cerebellum, hippocampus, lung, liver, muscle, placenta, spleen, thyroid, intestines and eye. A northern blot was prepared from this RNA according to standard procedures[64]. In addition, northern-blot filters containing 2 μg of poly (A^+) RNA from eight adult human tissues and four fetal human tissues were used (Clontech). First-strand cDNA synthesis was performed on lymphoblast RNA samples with oligo-dT and random primers with Superscript II RT (Gibco)[66]. The reactions were carried out at 42 °C for 90 min, followed by gene-specific PCR amplification to generate the various cDNAs in the critical *DYT1* region from patients and controls.

Cosmid contig. Cosmids were isolated from two human chromosome 9–specific libraries: the Lawrence Livermore library, which was constructed in Lawrist 16 with DNA from a chromosome 9–only somatic cell hybrid[67], and the Los Alamos library, which was constructed in sCos (Stratagene) from flow-sorted metaphase human chromosomes[68]. Cosmid colony grid filters were stamped and prepared for hybridization as described[69,70]. Filters were screened with gel-purified YAC DNA from a 400-kb critical region[16] and more than 800 positive colonies were picked, gridded and stamped for hybridization. A cosmid walk was initiated from both ends of the critical region, starting with the end clone of cosmid LL09NC0150H11 and several *D9S63*-positive cosmids (18D5LA, 37H5LA). End sequences of hybridizing cosmids were used to generate PCR primers to continue the walk by re-screening grids. The resulting set of about 60 cosmids was digested with *Eco*RI, and fragments were resolved by agarose-gel electrophoresis to distinguish similar and novel regions. An overlapping subset of eleven cosmids was then aligned by digestion with *Eco*RI, *Xho*I and *Not*I in a series of single and double digests. Fragments were resolved by electrophoresis in 1%

agarose gels, transferred to Southern blots and hybridized to cosmid ends, exons and unique sequence in the region, generated by PCR or with synthetic 20-bp oligonucleotides.

Hybridization. Probes (gel-purified YAC DNA, cosmid ends, exon clones, PCR products and cDNAs) were labelled by random priming[71] with [α^{32}P]dATP (3,000 Ci/mmol; NEN). Oligonucleotides were end-labelled with T4 polynucleotide kinase (NEB) using [γ^{32}P]dATP (6,000 Ci/mmol; NEN). Probes were pre-annealed with CotI DNA, human placental DNA and vector DNA as necessary to saturate repeat sequences[16].

Hybridizations were performed in Church-Gilbert buffer at 55 °C overnight. Northern filters were hybridized in 5× SSPE, 50% formamide, 5× Denhardt's solution, 0.5% SDS and 300 µg/ml salmon sperm DNA overnight at 42 °C. Filters were washed and exposed to autoradiographic film as described[16,70].

Exon amplification, cDNA library screening and extension. Exon amplification was performed on cosmids spanning the region using vectors pSPL1 (ref. 17) and pSPL3-IV (ref. 18). RT-PCR products were digested with *Bst*X1 to eliminate vector-only products[18]. Cloned exon fragments were used to screen human fetal and adult cDNA libraries by colony hybridization[64]. Libraries were prepared in λZAP by Stratagene and included adult human striatum, hippocampus, substantia nigra, caudate putamen, brain stem, heart, spleen and liver, and human fetal brain and retina. The sequences generated from these cDNA clones were aligned and edited with the Sequencher program (Gene Codes). cDNA contigs were extended in two ways—by re-screening libraries with PCR fragments generated from the ends of the contig and by using 5′- and 3′-RACE and MARATHON PCR systems[19,72] as modified by Clontech.

Sequencing. Dideoxy sequencing was performed with the Sequitherm Long Read Cycle Sequencing Kit (Epicenter Technologies), either with infrared-labelled vector primers for the LICOR sequencing machine or with specific primers labelled with α33P-dATP (2,000 Ci/mmol; NEN) for standard sequencing. Direct sequencing of PCR products was done by means of an enzymatic cleanup process with exonuclease I and shrimp alkaline phosphatase (USB) for 15 min at 37 °C and 15 min at 85 °C, followed by sequencing with Sequenase (USB). The LICOR sequence was read with the BaseImagIR software package (LICOR), which includes data collection and image analysis software. The α^{33}P-dATP gels were transferred to 3 MM Whatman, dried and exposed to autoradiographic film overnight, and then read and entered manually into the GCG programs (Wisconsin Package Version 9.0, Genetics Computer Group).

SSCP analysis. DNA sequences were screened for mutations by PCR of 100–300-bp fragments, followed by SSCP analysis, using first-strand cDNAs synthesized from patient and control lymphoblast RNA and genomic DNA. PCR reactions were performed as described[9] in a total reaction volume of 10 µl. SSCP analysis of the PCR amplification products was carried out as described[73,74]. All fragments with altered migration were sequenced directly and evaluated in families to check inheritance, and in controls to determine whether they represented normal polymorphisms. When single-base-pair changes altered restriction sites, restriction digestion of PCR products was used to replace SSCP analysis. In these cases, standard PCR was performed in a 25-µl final volume without radioactivity. The PCR products were digested according to the manufacturer's instructions (NEB) and visualized by staining with ethidium bromide after electrophoresis in 2–3.5% agarose gels.

Accession numbers: *DQ1*=torsinB, AF007872; *DQ2*=torsinA, AF007871.

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