tions in the genes for metachromatic leukodystrophy and Hurler disease in a small geographic location, Lower Galilee. They suggested that a high mutation rate and selective advantage of the carriers were responsible for the multiple mutations, and they proposed that a similar event may have occurred in the Réunion population. Perhaps there are multiple founders despite the evidence of a single common ancestor in the Réunion families, or, less likely, perhaps there is in the *CANP3* gene a mutation-rate increase due to exogenous (differential exposure to mutagens) or endogenous (unequal distribution of mutator genes) factors. Finally, perhaps there are unknown environmental or genetic factors that influence the manifestations of mutations in the gene in the Réunion population. The search for possible modifying genetic or environmental factors should continue, since it could disclose both the mechanism of action of mutated *CANP3* in LGMD2A and, possibly, factors of therapeutic importance (Beckmann 1996). Currently, we are offering this Amish population carrier testing and genetic counseling, on the basis of both the R769Q mutation analysis and monogenic autosomal recessive inheritance for LGMD2A.

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A C2055T Transition in Exon 8 of the ATP7A Gene Is Associated with Exon Skipping in an Occipital Horn Syndrome Family

To the Editor:

Mutations associated with abnormal splicing account for 10%–20% of all gene mutations (Krawczak et al. 1992). Mutations leading to abnormal splicing usually are located within the donor or acceptor splice sites. However, exonic consensus sequences that are implied in the splicing process have been described outside the splice sites (Ligtenberg et al. 1990; Steingrimsdottir et al. 1992). Direct study of the genomic DNA may not easily reveal the splicing mutations. However, reverse transcription (RT) followed by PCR analysis is likely to detect such mutations.

In the ATP7A gene, which encodes a copper-transporting P-type ATPase (Chelly et al. 1993; Mercer et al. 1993; Vulpe et al. 1993), splicing mutations are frequent in patients with Menkes disease (Das et al. 1994; Kaler et al. 1994, 1995; Tümer et al. 1997) and also are described in patients with occipital horn syndrome (OHS) (Kaler et al. 1994; Das et al. 1995). OHS, previously known as "X-linked cutis laxa" (MIM 304150), is a connective-tissue disorder characterized by skin laxity, hyperextensible joints, skeletal anomalies, including occipital exostoses, and inconstant mild mental retardation (Lazoff et al. 1975; Tsukahara et al. 1994). Here, we report an unusual splice mutation, which was detected by RT-PCR, in the ATP7A gene of an OHS family.

The proband (V-6 in fig. 1) was born at term, following a long delivery. At birth, he had good eye contact but was hypotonic. All developmental milestones were retarded. Sitting was acquired at 11 mo of age and walking at 18 mo of age, with backward moving during the first weeks, and speech was very delayed. He kept his mouth open while speaking and had a very poor lexical stock. He never learned to read, to write, or to calculate, and he was hypoactive. Strabism was observed at 5 mo of age. Ehlers Danlos syndrome was suggested within the first week after birth, owing to the combination of long length, pectus excavatum, loose skin, and joint laxity. Right and left inguinal hernias were observed from 4 mo of age and needed repeated surgical interventions. Recurrent urinary bacterial infections revealed bladder diverticulae at 15 mo of age, and repeated antibiotic treatments were needed in the following years. In addition, recurrent attacks of abdominal pain, with vomiting, abdominal distension, and ascitis, occurred from 12 years of age. A laparoscopy showed that the hepatic artery appeared sinuous. Portal hyperpressure was excluded, and blood clearance of alpha-1-antitrypsin was normal. Menkes disease had been suspected repeatedly and was confirmed by low blood ceruloplasmin and high urine copper levels, at 12 years of age. Skin biopsies at 5 years of age revealed so-called fragmented collagen fibers and a relative excess of elastic fibers. Radiocopper retention in the patient's fibroblasts (Tønnesen and Horn 1989) was determined to be 3.7 mCi ^{64}Cu/mg protein/24 h. This value is comparable to that observed in one Menkes disease patient (4.3 mCi ^{64}Cu/mg protein/24 h) and is much greater than the normal adult values (1.4–2.7 mCi ^{64}Cu/mg protein/24 h) studied in the same laboratory.

A physical examination when the patient was 25 years of age showed tallness (181.50 cm, +1.2 SD), narrow shoulders, marked pectus excavatum and dorsal kyphosis, flat feet, loose wrists and finger joints, a weak abdominal wall, soft pinnae, and loose and hyperelastic skin. The hair was kinky, with numerous, although moderate, pili torti. All of the teeth had gray enamel, and the inferior incisors had particular spicules. The face was long and narrow, with moderate hypotelorism and an arched narrow palate. The eyelids were puffy. The patient had poor muscular development, moderate mental deficiency, and low tendon reflexes, and his occipitofrontal circumference (OFC) was at −2 SD. Skeletal x-rays showed bone undercalcification, mild occipital exostoses, thickening of muscle insertion zones on the long bones, and irregular shapes for the cubitus and the radius, with a distortion of the proximal radius end and with an enlargement of the distal tibial end. The patient died suddenly at 27 years of age, after a brief crisis of acute epigastric pain. An autopsy showed a perforated gastric ulcer and peritonitis, a thick bladder wall with a unique residual diverticulum, persistent thymus, and poorly developed brain-cortex gyri.

All the observed features were in accordance with published data from studies of other males with moderate or mild Menkes disease and especially of those with OHS, except for the abdominal pain crises and the spicules of the incisors, which to our knowledge have not been described before. On the other hand, our patient did not have chronic diarrhea, hypotension, a heart murmur, syncopal attacks, ataxia, antimongoloid palpebral fissures, or a hooked nose, which have been described in some other males affected by OHS, and he was not born prematurely.

The patient's father had a low biacromial diameter. Both parents were tall and healthy and had normal OFC. The patient's mother had a long face, large pinnae, and loose skin, which could be interpreted as symptoms of a possible carrier state. The five affected male relatives (III-1, III-6, III-8, III-13, and IV-7) all were hypoactive and had marked hypotonia, progressive motor handicap, and variable mental deficiency. These patients died at the ages of 6 years (patient III-1), 18 years (patient III-6), 20 mo (patient III-8), 3 years (patient III-13), and 4 years (patient IV-7).

One microgram of total RNA, extracted (after in-

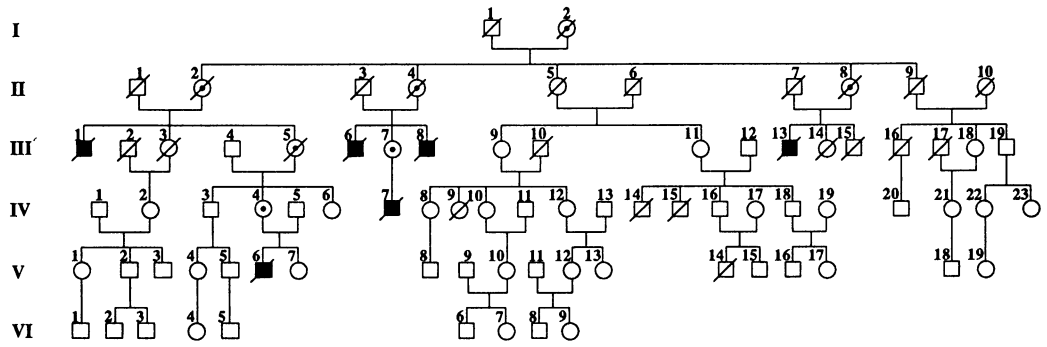

Figure 1 Pedigree of the OHS family

formed consent was obtained) from the patient's lymphoblast cell line, was amplified by RT-PCR, by use of six pairs of overlapping primers (table 1) covering the open reading frame of the ATP7A cDNA (Vulpe et al. 1993). PCR products were analyzed by electrophoresis on a 2% agarose gel. This gel was visualized after ethidium-bromide staining, and the PCR products were extracted from the gel by use of the Gel Extraction Kit (Qiagen). In order to produce single-stranded sequences, a fraction of the purified fragments was amplified by use of a primer ratio of 1:50. Sequencing of the amplified products was performed with Sequenase version 2.0 (United States Biochemical) and ^{33}P-dCTP.

RT-PCR with the Mnk3/Mnk4 primers amplifying the cDNA from the exon 5–to–exon 13 region (Vulpe et al. 1993; Dierick et al. 1995) showed an abnormal pattern in the patient's lane, consisting of four distinct bands (fig. 2). The products in the first and third bands (p1 and p3, respectively) comigrated with the two normal products (in bands c1 and c3, respectively), as was observed in the lane of the normal control. The other two bands observed in the patient's lane (p2 and p4) were absent in the lane of the normal control. Sequencing of the patient's normal-sized top band (p1) revealed a $C_{2055}\rightarrow T$ change in exon 8. In addition, this transition was associated with two alternatively spliced abnormal products—one (p2 band) with only exon 8 skipped and the other (p4 band) with three contiguous exons, 8, 9, and 10, simultaneously skipped. The smallest RT-PCR product observed in the control lane (c3 band) revealed exon 10 splicing; this same exon also was spliced in the RT-PCR product of the p3 band in the patient's lane. Alternative splicing of exon 10 of the ATP7A gene was observed previously in several tissues, including lymphoblasts, from normal individuals (Dierick et al. 1995). Finally, in the patient's DNA, the acceptor and donor splice sites corresponding to exons 7–9 were sequenced and appeared to be normal, by comparison with the 10-bp intronic splice sites in normal individuals described in previous studies (Dierick et al. 1995; Tümer et al. 1995). The same Mnk3/Mnk4 RT-PCR pattern was detected in the patient's mother (IV-4) (data not shown), who was found to be heterozygous for the C2055T mutation in exon 8, as was obligate carrier III-7. The patient's healthy maternal uncle (IV-3) did not have the mutation. In this family, the mutation cosegregates with the disease, which suggests that this mutation probably is responsible for the disease.

The C2055T transition is associated with both normal processing of ATP7A mRNA and exon skipping. This mutation results in a serine^{637}-to-leucine change in the patient's normally spliced transcript. This conservative amino acid change concerns a polypeptide region that is located between the last metal binding site and the first transmembrane domain of the ATP7A copper-transporting ATPase and does not appear to adversely affect the transport function of the protein. The alternatively spliced transcript missing the exon 10 would produce a protein lacking the first two transmembrane domains. The role of this partially deleted protein, in the regulation of copper transport, remains unknown (Dierick et al. 1995; Kaler et al. 1995). The skipping of exon 8 leads to a shift in the reading frame and generates a premature stop codon in exon 9 (nt 2194), whereas the absence of exons 8–10 do not alter the reading frame. The protein derived from the transcript lacking exon 8 is deleted from all the transmembrane domains, as well as the transduction, the phosphorylation, and the ATP-binding domains (Vulpe et al. 1993). This truncated protein is expected to be nonfunctional. The transcript lacking contiguous exons 8, 9, and 10 would produce a protein that may be nonfunctional, owing to deletion of the first four transmembrane domains that are conserved in heavy metal–transporting P-type ATPases (Petrukhin et al. 1994).

Splicing mutations in the ATP7A gene were found previously in three OHS patients (Kaler et al. 1994; Das et al. 1995). In each case, the mutation was located in the donor or acceptor splice sites that are adjacent to the skipped exon. The normally and the abnormally processed ATP7A mRNA were produced simultaneously, and a small amount of functional protein was produced. In addition, a deletion in the regulatory region of the ATP7A gene was found in one patient with OHS, and no detectable reduction of the expression of the ATP7A mRNA was observed, by use of northern blot analysis (Levinson et al. 1996).

Exon 8 of the ATP7A gene seems to be particularly vulnerable to mutations. Indeed, mutations in the copper-transporting ATPase gene, resulting in the skipping

Table 1

Primers Used for PCR Amplification of ATP7A cDNA

Primer	Sequence (5′→3′)	Nucleotide Position[a]
Mnk1	GACTTCTCCGATTGTGTGAGC	21–41
Mnk2	CTTACTTCTGCCTTGCCAGC	1718–1737
Mnk3	CACCAGTTCAAGACAAGGAGG	1572–1592
Mnk4	CATGTGTTGCGCAGATAAGC	2845–2864
Mnk5	TATTGCACTAGGCCGATGG	2515–2533
Mnk6	TTCTGTCCTATCTGGCTCCG	4412–4431
Mnk7	CATTGAAGCAGCTGATGTGG	4120–4140
Mnk8	ATTCCTTGAATCCGGTAGGC	5734–5753
Mnk9	ATCTTGGCTCACTGCAACC	5712–5730
Mnk10	CCAAGAGGATGACTCCAAGG	6097–7016
Mnk11	CTCAAGCAGTTGAACACAGC	6951–6970
Mnk12	ATCCTTGGCAGAAATATGATC	8422–8442

[a] According to Vulpe et al. (1993).

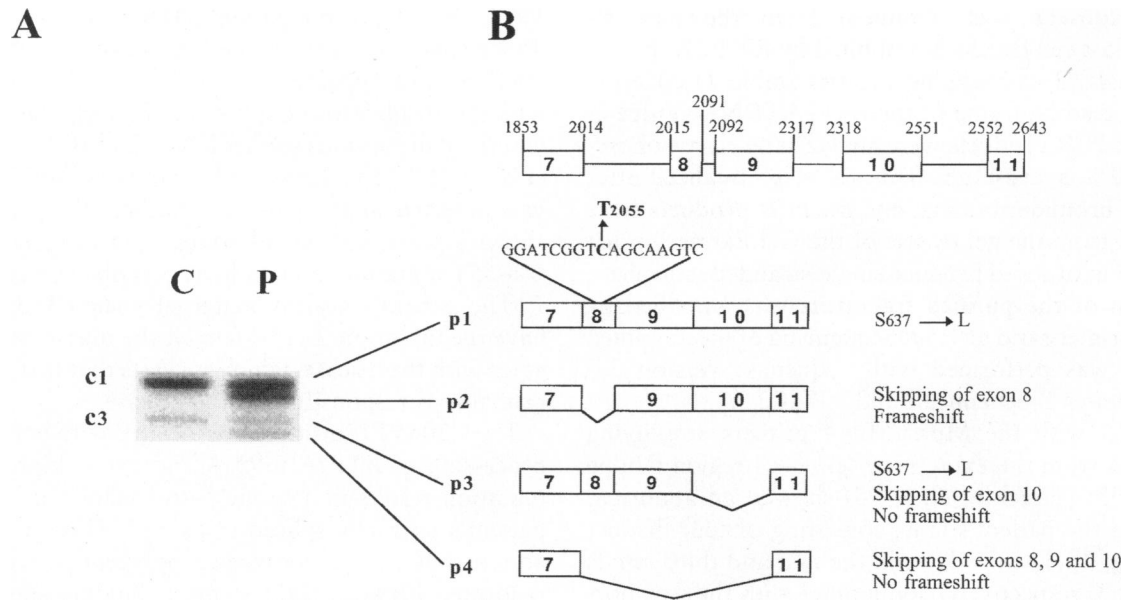

Figure 2 *A*, Autoradiogram of PCR amplification with the Mnk3/Mnk4 primers of the ATP7A cDNA. DNA from the normal control (C) and from the OHS patient (P) were amplified and then were separated on a 2% agarose gel, blotted on a nylon membrane, and hybridized with oligonucleotide 5′-CATCAGAGGCTCTTGCAAAG-3′ located in exon 11. *B* (*top*), Genomic organization in the middle region of the ATP7A locus (Dierick et al. 1995). Exons are shown as boxes, and the cDNA base numbers corresponding to the exonic boundaries are noted above the diagram and are in accordance with the numbering of the sequence reported by Vulpe et al. (1993). *B* (*bottom*), Splicing patterns in normal and mutant transcripts, in the OHS patient.

of exon 8, were described, in a previous study, in four Menkes disease patients (Das et al. 1994). In one patient, a nonsense mutation was detected in exon 8 and was associated with both the normal-sized transcript and the transcript deleted for exon 8. In the other patients, the mutation is located in the donor splice site or in adjacent exons. In each case, the mutation would be predicted to have a severe effect on both the structure and the function of the gene product (Das et al. 1994; Kaler et al. 1996). In addition, six splice-site mutations that were predicted to affect the splicing efficiency of exon 8, one missense mutation, and one nonsense mutation were detected in exon 8, in Menkes disease patients (Tümer et al. 1997). The missense mutation Ala629Pro was predicted to affect the structure of the protein. The nonsense mutation Ser637Ter was characterized by a C2055G change. In our OHS patient, the same nucleotide was mutated in thymine and led to a missense mutation. The mutations described by Tümer et al. (1997), except for the missense mutation, would lead to a premature termination of translation and consequently to the production of a truncated protein. The fact that the OHS phenotype but not the Menkes phenotype was observed in our patient could be explained by the presence of the normally processed mRNA and by the likely production of a functional ATP7A protein. Further identification of mutations in the ATP7A gene and analysis of the corresponding mRNA splicing would help in the understanding of molecular changes leading to OHS and to classic Menkes disease, respectively.

The base C_{2055} in the ATP7A gene is localized 41 bp distal to the 3′ splice site and, when mutated, leads to exon 8 skipping. A missense mutation in exon 20, which leads to exon 20 skipping, was reported in the ATP7A gene in a Menkes disease patient (Das et al. 1994). Both normal-sized and deleted RT-PCR products were detected. This mutation, which results in a glycine-to-arginine change, is located within the highly conserved ATP-binding site and probably affects the ATPase activity, as was suggested by the observation of the classic severe Menkes phenotype (Das et al. 1994). In the HEXB gene responsible for juvenile Sandhoff disease (Wakamatsu et al. 1992), a missense mutation leading to exon skipping also has been observed, and an alternative transcript using a cryptic acceptor splice site has been detected, in addition to the normal-sized transcript and the transcript deleted for the mutated exon. This mutation results in a proline-to-leucine substitution, and the protein derived from normal mRNA would be expected to be functional (Wakamatsu et al. 1992), as suggested by the expression of the juvenile and not the more severe, infantile form of the disease. These studies highly suggest that some exonic sequences are implied in the splicing process.

It has been proposed that some point mutations in an exon could alter the RNA secondary structure (Ligtenberg et al. 1990; Steingrimsdottir et al. 1992). These mutations could lead to aberrations in RNA splicing and, consequently, could lead to deletions of one or several adjacent exons (Fisher et al. 1993), as was observed in our patient's mutant transcripts. Another hypothesis, proposed by Dietz et al. (1993), is that some deleterious mutations could be recognized on the pre-mRNA, leading the mutated exon to be skipped so as to produce at least a partially functional protein.

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Nonreplication of Linkage Disequilibrium between the Dopamine D4 Receptor Locus and Tourette Syndrome

To the Editor:

Grice et al. (1996) recently reported linkage disequilibrium between the seven-repeat allele (DRD4*7R) of the exon 3 VNTR polymorphic site at the D4 dopamine receptor locus and expression of chronic multiple tics and Tourette syndrome (TS). The study cohort encompassed 64 family trios each of which consisted of an affected person and two parents, of whom at least one was heterozygous for the DRD4*7R. Fifty-two of the trios stemmed from four large TS kindreds; the remaining 12 were independent nuclear-family trios. Grice et al. pointed out that confirmation of their finding depends on either replication or the identification of a transmitted functional mutation.

In the present study we evaluated different genotypes at the D4 dopamine receptor locus of ≤102 TS index probands and their parents. Subgroups of the patients, encompassing children, adolescents, and young adults, have been described elsewhere (Hebebrand et al. 1993; Nöthen et al. 1994*a*, 1994*b*). By use of the transmission-disequilibrium test (TDT) (Spielman et al. 1993), the following polymorphisms at the DRD4 locus were investigated: (1) the DRD4*7 allele, against all other alleles at the multiallelic VNTR polymorphism in exon 3 (van Tol et al. 1992; Lichter et al. 1993), in 102 trios; (2) the 13-bp deletion in exon 1, with 2% frequency of the deletion allele in the German population (Nöthen et al. 1994*a*), in 102 trios; (3) the two-allele system in exon 1, encompassing an allele with a 12-bp duplication (Catalano et al. 1993), in 102 trios (we found an additional, third allele for the 12-bp duplication in exon 1, characterized by the threefold occurrence of the 12-bp unit); and (4) the two-allele system based on the arginine→glycine substitution at position 11 in exon 1, in 87 trios (Cichon et al. 1995). The variants tested in our study include all known variants of the DRD4 gene that alter the amino acid composition of the receptor and have a frequency of >1% in Caucasians. Genotypes were determined as described elsewhere (Lichter et al. 1993; Nöthen et al. 1994*a*; Cichon et al. 1995).

None of the two-sided TDTs for the investigated polymorphisms reached a P of <.05. Because of the attempt to replicate the findings of Grice et al.(1996), the transmission patterns of the DRD4*7R allele deserve special consideration. A total of 58 parents were heterozygous for the DRD4*7R allele. In these meioses the DRD4*7R allele was transmitted 32 times and was not transmitted 26 times. This reveals a P of .26, by use of the one-sided exact binomial TDT.

For power calculations, we took the findings of Grice et al. (1996) as given. These investigators had performed three TDTs based on inclusion and exclusion of inferred genotypes and two different diagnostic models in extended pedigrees; the range of transmitted DRD4*7R alleles from parents heterozygous for this allele was 68%–73%. On the basis of our 58 heterozygous parents, the exact randomized one-sided binomial TDT has 86%–98% power to detect transmission disequilibrium for a significance level of 5%.

The two-sided TDT for the Gly11Arg polymorphism had a P of .06 (not corrected for multiple testing) on the basis of the binomial test; only five parents were heterozygous, and in all five cases the wild-type allele (11Gly) was transmitted to the index proband.

The Gly11Arg variant is seemingly in linkage disequilibrium with the exon 3 VNTR polymorphism. The following haplotypes bearing the 11Arg allele were observed: 11Arg-DRD4*2R (n = 2), 11Arg-DRD4*6R (n = 1), and 11Arg-DRD4*7R (n = 2). None of the haplotypes contained the DRD4*4R allele, which is the most frequent (.69 of nontransmitted parental alleles) among the VNTR alleles. The probability of nonobservation of the haplotype 11Arg-DRD4*4R is low (.0033); however, on use of the two-sided TDT, the DRD4*4R allele in itself was not linked or associated with TS (P = .92). The two-sided TDT for the haplotype consisting of the DRD4*4R and the 11Gly alleles revealed a P of .68. In conclusion, although there seems to be linkage disequilibrium between Gly11Arg and the exon 3 VNTR polymorphism, no indication for transmission disequilibrium in TS was detected.

We conclude that in the families that we have studied there is no evidence that the DRD4*7R allele of the exon 3 VNTR is linked or associated with TS.