Molecular diagnosis of Charcot–Marie–Tooth IA disease and hereditary neuropathy with liability to pressure palsies by quantifying CMT1A-REP sequences: consequences of recombinations at variant sites on chromosome 17p11.2–12

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The most frequent form of Charcot–Marie–Tooth disease (CMT1A; OMIM118220) is the result of a duplication on chromosome 17 in p11.2–p12. This region contains PMP22, a gene expressed in peripheral myelin. The mutation results from an unequal crossing-over involving repeated sequences, CMT1A-REP, located on both sides of the duplicated region. The reciprocal product of this recombination is a deletion of the same region, which is associated with hereditary neuropathy with liability to pressure palsies (HNPP; OMIM162500). Proximal and distal CMT1A-REP sequences can be distinguished by the presence of a variant EcoRI site. We quantified the number of these repeat sequences in 36 CMT1A and 40 HNPP patients. CMT1A-REP sequences are involved in almost all of the mutations. The majority of recombination breakpoints occur distally from the variant EcoRI site. However, a few have a breakpoint proximal to this site, which creates the risk of misinterpretation with respect to a duplicated/deleted status.

Indexing terms: heritable disorders • genetic analysis • restriction fragment length polymorphism

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Received December 14, 1995; accepted February 22, 1996.

With an estimated prevalence of 1 in 2500 [1] for Charcot–Marie–Tooth (CMT) disease in general—~70% being of the slow nerve conduction type 1 [2], of which ~70% are caused by duplications [3]—the overall prevalence of the CMT1A duplication should be ~1 in 5000.9 Some of these patients are carriers of de novo mutations, which makes the underlying mechanism of this mutation an important mutagenic event in peripheral hereditary motor and sensory neuropathy.

CMT1A was first mapped near the chromosome 17 centromeric region [4] and later localized to band 17p11.2–12 [5] by linkage studies. A 1.5-Mb duplication in this region [6, 7] was found to be associated with the disease. This region contains PMP22, the gene for peripheral myelin protein 22 [8–11]; this gene is believed to play a role in Schwann cell proliferation and differentiation and to have a structural function [12]. The presence of three copies of the PMP22 gene in CMT1A suggests an overproduction of the protein, thus causing the CMT1A pathology. Unequal crossing-over at meiosis is thought to be the mechanism for this duplication [7], which is mediated by repeated sequences of >17 kb known as CMT1A-REP flanking the duplicated region [13]. The reciprocal recombination product of this duplication is a deletion of the same length that provokes hereditary neuropathy with liability to pressure palsies (HNPP) [14]. CMT1A-REP sequences can be distinguished by EcoRI digestion, which produces a proximal 7.8-kb fragment and a distal 6.0-kb fragment [13]. Duplication and deletion generate different numbers of copies of these fragments [14], making the study of differences of hybridization intensity between the

9 Nonstandard abbreviations: CMT1A, Charcot-Marie-Tooth disease, type 1A; HNPP, hereditary neuropathy with liability to pressure palsies; and RFLP, restriction fragment length polymorphism.
fragments an attractive approach for diagnoses of these disorders [15, 16].

**Materials and Methods**

**PATIENTS**

Material was obtained from patients for diagnostic purposes or as material destined to contribute to research studies of the French Collaborative CMT Group. In both cases procedures were performed in agreement with the ethical standards of the respective hospitals. Peripheral blood samples were collected from 43 CMT1 and 55 HNPP clinically documented unrelated patients.

DNA PREPARATION, HYBRIDIZATION, AND QUANTIFICATION

Leukocytes were isolated from uncoagulated peripheral blood by osmotic lysis of erythrocytes. Lymphocytes were lysed in a solution of 75 mmol/L NaCl, 24 mmol/L EDTA, pH 8.0, 10 mOL/L sodium dodecyl sulfate, and 0.05 g/L Proteinase-K (Boehringer Mannheim, Mannheim, Germany) by incubation for 2 h at 50°C. DNA was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1 by vol), precipitated by adding 1/30 volume of 3 mol/L sodium acetate (pH 5.5) and 1 volume of isopropanol, washed in 70% ethanol, and carefully dissolved overnight in 10 mmOL/L Tris-Cl, pH 7.4, containing 0.1 mmol/L EDTA. _MspI_ digests were hybridized with the following probes, which detect restriction fragment length polymorphisms (RFLPs) inside the duplicated region in CMT1A [17]: _pVAW409R3a_ (D17S122), _pEW401HE_ (D17S61), and _pVAW412R3HEc_ (D17S125).

The probes were radiolabeled by using [32P]dCTP and the random primer method [18]. When necessary, band intensities were compared with the intensities for fragments revealed with probe _SF85_ or _pVAW411R2_ as controls [probe _SF85_ detects a locus on chromosome 21 (D21S48), and _pVAW411R2_ detects a locus on chromosome 17 (D17S124) outside the CMT1A duplicated region]. _EcoRI_ digests were hybridized with the 1.8-kb _EcoRI_ fragment from cosmid clone _c20G2_ [13], which detects a 7.8-kb proximal and a 6.0-kb distal CMT1A-REP sequence. This 1.8-kb _EcoRI_ fragment is also known as probe _PNE102_ [16]. For some patients (Table 1), a _BamHI_ digest was hybridized with a full-length cDNA probe for PMP22 (pCD25).

Hybridization densities on autoradiographs were measured and compared by use of scanning apparatus and dosing software (Image Master™, Pharmacia Biotech, Uppsala, Sweden). All probes except cosmid clone _c20G2_ (obtained from J.R. Lupski, Houston, TX) were made available by the European CMT Consortium management (C. Van Broeckhoven, University of Antwerp, Belgium).

**RESULTS**

RFLP probes _pVAW409R3a_, _pEW401HE_, and _pVAW412HEc_ hybridize to loci in the duplicated (CMT1A) or deleted (HNPP) region and are routinely used for diagnostic purposes in this region. Dosage differences between alleles, or rarely the detection of three alleles (probe _pVAW409R3a_), indicate the presence of an extra allele in DNA of CMT1A patients. From our collection of CMT1 patients, selected according to clinical CMT1 inclusion criteria [3], 35 were heterozygous and found to have one or more duplicated RFLP probes localized in the duplication. For one completely homozygous uninformative patient and for partially informative patients, the presence of the duplication was detected by comparing fragment densities with those of external loci D17S124 and D21S48; thus, the total number of CMT1A patients with duplicated regions was 36, 84% of the 43 studied.

Fragment densities were also systematically compared for all HNPP patients to establish the presence of the deletion associated with this pathology: 40 of the 55 clinically selected patients did show a deletion (73%). In one HNPP and in one CMT1A patient, a discrepancy between the results obtained with the centromeric _pVAW409R3a_ probe and the distal _pEW401HE_ and _pVAW412R3HEc_ probes suggested a partial deletion and a partial duplication (Table 1: patients LY273 and LY326, respectively). Probing with pCD25, a full-length cDNA clone of PMP22, demonstrated a completely deleted PMP22 allele for the HNPP patient and a third PMP22 copy for the CMT1A patient. The 1.8-kb _EcoRI_ fragment of cosmid clone _c20G2_ detects a centromeric 7.8-kb fragment and a distal 6.0-kb _EcoRI_ fragment of the CMT1A-REP sequence. Quantitative ratios of 2:3 and 2:1 for these fragments are associated with CMT1A and HNPP pathologies, respectively [14].

Of our patients, 67 showed concordance between results obtained with the RFLP probes from the duplicated/deleted region and the CMT1A-REP probe, and 9 revealed unusual ratios among their CMT1A-REP fragments (Fig. 1). Two patients did not show dosage differences (Table 1: LY273 and LY506), and seven others had inverted ratios (LY074, LY268, LY326, LY419, LY455, LY534, and LY540). No false positives were noted among 45 controls.

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**Table 1. Number of RFLP alleles from CMT1A and HNPP patients with unexpected ratios of CMT1A-REP alleles.**

<table>
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<td>LY074</td>
<td>CMT1A</td>
<td>3</td>
<td>—</td>
<td>—</td>
<td>3</td>
<td>1.89</td>
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<td>HNPP</td>
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<td>—</td>
<td>1</td>
<td>1</td>
<td>0.41</td>
<td>1:2</td>
</tr>
<tr>
<td>LY273</td>
<td>HNPP</td>
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<td>2</td>
<td>1</td>
<td>2.06</td>
<td>2:2</td>
<td></td>
</tr>
<tr>
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<td>CMT1A</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>1.47</td>
<td>3:2</td>
<td></td>
</tr>
<tr>
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<td>HNPP</td>
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<td>—</td>
<td>1</td>
<td>1</td>
<td>0.42</td>
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</tr>
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<td>CMT1A</td>
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<td>—</td>
<td>3</td>
<td>1.49</td>
<td>3:2</td>
<td></td>
</tr>
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<td>HNPP</td>
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<td>—</td>
<td>1</td>
<td>1</td>
<td>1.02</td>
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</tr>
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<td>CMT1A</td>
<td>3</td>
<td>—</td>
<td>—</td>
<td>3</td>
<td>1.45</td>
<td>3:2</td>
</tr>
<tr>
<td>LY540</td>
<td>CMT1A</td>
<td>3</td>
<td>—</td>
<td>—</td>
<td>3</td>
<td>1.49</td>
<td>3:2</td>
</tr>
</tbody>
</table>

* RFLP probes from the duplicated/deleted region are ordered according to their chromosomal localization from centromere to telomere (see also Fig. 2a).

The exact order of the clustered probes _pEW401_ and _pVAW412_ is unknown.

* Experimentally obtained ratios between the 7.8- and 6.0-kb fragments after hybridization with the 1.8 kg _EcoRI_ fragment of cosmids clone _c20G2_.

* Deduced rounded-off ratios.

* Underexposed film.
DNA (5 μg) from patients and controls was digested with EcoRI. After Southern transfer, the digest was hybridized with the 1.8-kb EcoRI fragment from cosmid clone c20G2. This probe detects fragments of 7.8 kb and 6.0 kb, respectively, in the proximal and distal CMT1A-REP sequences flanking the duplicated/deleted region in CMT1A/HNPP. A normal control, a CMT1A control, and an HNPP control were included. Lanes LY268, LY273, LY419, and LY506 show samples from HNPP patients; LY326, LY455, and LY540 show samples from CMT1A patients, whose fragments give unexpected hybridization intensity ratios.

**Discussion**

Patel et al. [8] proposed unequal crossing-over at meiosis as the mechanism generating the duplication in CMT1A. This event is thought to be triggered by the presence of repeated sequences, known as CMT1A-REP, flanking the duplicated region [13]. The discovery of a deletion covering the same region and associated with HNPP [19] suggests that both pathologies result from the same mutational event and are the reciprocal products of this recombination [14]. These repeat sequences are at least 17 kb long [13] and have a 7.8-kb EcoRI fragment in the proximal sequence and a 6.0-kb EcoRI fragment in the distal sequence. Controls show an equal hybridization density signal when Southern transfer materials are hybridized to a 1.8-kb EcoRI fragment of cosmid clone c20G2. For CMT1A and HNPP patients, the differences in density observed seem to be characteristic of the pathology.

On the basis of these observations, Chance et al. proposed a model for an unequal recombination event that involves strand breaks in the CMT1A-REP sequences [14]. This model implicates a crossing-over distally from the 6.0-kb EcoRI fragment on the first chromosome (Fig. 2a) or at a site in the 6.0-kb sequence [14]. Such a cross-over generates a CMT1A chromosome with two copies of this 6.0-kb fragment and an HNPP chromosome lacking this fragment. As a result, CMT1A patients have a 2:3 ratio of their 7.8- and 6.0-kb fragments, and HNPP patients have a 2:1 ratio. This is probably the site of recombination in the majority of the CMT1A/HNPP mutations, given the 88% concordance between the RFLP results and the dosage differences in our patients. Similar results were found in a French study [15] for a different collection of subjects, in which all 27 deleted HNPP patients (100%) had a 2:1 ratio of the 7.8- and 6.0-kb fragments. The 12% of our results that were unexpected (discordant) might be explained by the occurrence of recombinations involving other sequences.

We should distinguish between recombinations that occur in the CMT1A-REP sequence but at a different site, and recombinations that probably involve sequences other than the CMT1A-REP sequence. First, a recombination event can occur in the repeat sequence but proximally to the 6.0-kb fragment (Fig. 2b) of the first chromosome. The result of such a cross-over is the production of a CMT1A chromosome with two copies of the 7.8-kb fragment and one copy of the 6.0-kb fragment. The reciprocal product generates an HNPP chromosome with only one 6.0-kb fragment. The involvement of these crossing-over sites gives inverse density ratios when compared with the first mechanism shown in Fig. 2a. Such a mechanism is probably responsible for the results found in patients LY074, LY268, LY419, LY455, LY534, and LY540 (8%). This would confirm the results of a recent study [20] of 30 CMT1A and 22 HNPP unrelated patients in which the majority (50 of 52) showed a gain (CMT1A) or loss (HNPP) of a 6.0-kb fragment, and 1 each showed a gain (CMT1A) or loss (HNPP) of a 7.8-kb fragment. The different products obtained in Fig. 2a and 2b could result from the same initiation process of recombination but might include branch migration and subsequent nicking of the heteroduplex structures at different sites. In any case, recombination in CMT1A-REP sequences is responsible for the majority of mutations in our CMT1A/HNPP patients (96%).

Second, recombination can probably occur in sequences other than the CMT1A-REP sequence. This mechanism is suggested when we compare the results of the RFLP and the repeat-probe in patient LY273, for whom RFLP probe analysis demonstrates a partial deletion. This deletion involves the PMP22 gene but not the more distally located pVAW412 and pEW401 loci. A model for such a recombination is proposed in Fig. 2c and involves the recombination of a sequence between the PMP22 gene and the pEW401/pVAW412 loci on the first chromosome and a sequence proximal to the PMP22 gene on the second chromosome. The result of such a mechanism is an unchanged, equal dosage ratio for the 7.8/6.0-kb fragments. Still other sequences can probably recombine to produce a normal 7.8/6.0-kb dosage ratio but show deleted alleles by the RFLP probes. For HNPP patient LY050, a recombination could have occurred in a sequence between the pEW401/pVAW412 loci and the distal CMT1A-REP sequence on the first chromosome and in a sequence between the proximal CMT1A-REP and the PMP22 gene on the second chromosome (Fig. 2d). Also, the LY326 result could be the product of a recombination between PMP22 and pEW401/pVAW412 on the first chromosome and a sequence proximal to the CMT1A-REP sequence carrying the 7.8-kb EcoRI fragment of the second chromosome (Fig. 2c). The latter series of proposed recombination sites can be confirmed by finding the reciprocal recombination products and by gathering additional sequence and physical mapping data from the regions supposedly involved.

The mutations in LY273 and LY326 involve a partial deletion and a partial duplication. Partial duplications have been described [21-24] and seem to be underestimated common products of recombinations mediated by as yet uncharacterized sequences in the 17p11.2-p12 region.

In conclusion, CMT1A-REP sequences are involved in almost all duplications and deletions in CMT1A and HNPP. This confirms a recent study of 518 CMT1A and 47 HNPP patients by pulsed-field gel electrophoresis and hybridization with the CMT1A-REP probe, in which all but one patient showed
junction fragments [25]. Here, using a 1.8-kb EcoRI fragment of the CMT1A-REP repeat sequence as a probe and subsequent comparison of EcoRI fragment hybridization densities, we found that a majority of these patients show a breakpoint distally to the 6.0-kb fragment of the first chromosome. In a few patients, this breakpoint is located proximally to the 6.0-kb fragment. One analysis gave a false negative with respect to a deletion, one partial deletion did not alter ratios of c20G2, and one partial duplication gave ratios expected to correspond to HNPP. The duplication/deletion status may be misinterpreted when the CMT1A-REP probe alone is used in combination with density comparisons of CMT1A-REP-specific EcoRI fragments, which makes this method less attractive for diagnostic purposes. The results also suggest the presence of minor variant sites on 17p11.2–12 at which recombination can occur to produce a duplicated (CMT1A) or a deleted (HNPP) phenotype.

We thank J.R. Lupski for providing the cosmid clone c20G2 and C. Bonnebouche and S. Gamon for technical assistance. Financial support was obtained from the Hospices Civils de Lyon, the Association Française contre les Myopathies, and the Groupe- ment de Recherches et d'Etudes sur les Génomes.

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