Estimation of the size of the chromosome 17p11.2 duplication in Charcot-Marie-Tooth neuropathy type Ia (CMT1a)

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Abstract

We have previously shown a duplication in 17p11.2 with probe pVAW409R3 (D17S122) in 12 families with hereditary motor and sensory neuropathy type I (HMSN I) or Charcot-Marie-Tooth disease type 1 (CMT1). In this study we aimed to estimate the size of the duplication using additional polymorphic DNA markers located in 17p11.2-p12. Two other 17p11.2 markers, pVAW412R3 (D17S125) and pEW401 (D17S61), were found to be duplicated in all HMSN I patients tested. Furthermore, all HMSN I patients showed the same duplication junction fragment with probe pVAW409R3. On the genetic map the duplicated markers span a minimal distance of 10 cM while on the physical map they are present in the same NcoI restriction fragment of 1150 kb. The discrepancy between the genetic and physical map distances suggests that the 17p11.2 region is extremely prone to recombinational events. The high recombination rate may be a contributing factor to the genetic instability of this chromosomal region.

The hereditary motor and sensory neuropathies (HMSN) are a clinically heterogeneous group of peripheral neuropathies comprising seven distinct subgroups. The common features of HMSN are slow progressing weakness and atrophy mainly of the distal limb muscles. The most common form of HMSN is HMSN type I or Charcot-Marie-Tooth disease type 1 (CMT1). Recently the prevalence of HMSN I has been estimated to be 8/100 000 in children aged from 2 to 15 years in southwest Sweden and 15-3/100 000 in all cases in northern Spain. Clinically, HMSN I is characterised by pes cavus, reduced or absent deep tendon reflexes, and hypertrophic nerves. The first symptoms of the disease become apparent during late childhood or early adolescence and vary considerably among patients from nearly no symptoms to severe weakness, atrophy, and foot deformities. However, all HMSN I patients have largely reduced nerve conduction velocities (NCV) and segmental de- and remyelination with onion bulb formation on peripheral nerve biopsy.

Although both sporadic patients and patients with recessive inheritance have been reported, the majority of HMSN I patients belong to families in which the disease segregates as a single autosomal dominant gene. Genetic linkage studies suggested that at least three chromosomal loci may be responsible for autosomal dominant HMSN I. The linkage of HMSN I with the Duffy blood group localised on chromosome 1 has been observed in only a few families. Furthermore, in only one of these families was conclusive linkage evidence obtained for a disease locus, designated CMT1b and located in q22-q23, close to the FcYRII gene.

Recently, close linkage was detected in a large number of HMSN I families with two DNA markers pEW301 (D17S58) and pA10-41 (D17S71) located in the pericentromeric region of chromosome 17p, indicating that chromosome 17 contains a major locus for HMSN I. The chromosome 17 disease locus designated CMT1a was further localised distal to pEW301 in bands 17p11.2-p12. In addition, two HMSN I families have been reported showing no linkage to the FcYRII gene or to pEW301 and pA10-41, suggesting that at least one other chromosomal locus may cause HMSN I.

We recently showed total segregation of HMSN I with a duplication in chromosome 17p11.2 in 12 families. In one family both the disease and the duplication appeared de novo in one HMSN I patient indicating that the 17p11.2 duplication was most likely the disease causing mutation. The duplication was detected by two non-overlapping subclones, pVAW409R1 and pVAW409R3, of the same phage clone, representing the anonymous locus D17S122 located in 17p11.2. In order to obtain an estimate of the size of the duplication in HMSN I patients we tested additional DNA markers located in 17p11.2-p12 for their ability to detect the duplication using both standard agarose gel and pulsed field gel electrophoresis. Our results indicate that the HMSN I duplication comprises at least 1150 kb.
Material and methods

PROBES
Plasmid probes pA10-41 (D17S71) and pEW301 (D17S38) were provided by J Vance (Duke University, Durham, NC) and plasmid probes pVAW409R3 (D17S125), pEW401 (D17S61), pVAW410R1 (D17S123), and pEW503 (D17S67) by D Barker (University of Utah, Salt Lake City, UT). Probe SF85 (D21S46) was kindly provided by J Korenberg (Cedars-Sinai Medical Center, Los Angeles, CA). The restriction fragment length polymorphisms (RFLPs) recognised by these probes have been described in detail elsewhere.13 For the probes duplicated in HMSN I patients we used single copy fragments in the Southern blot hybridisation: a 1-4 kb EcoRI-BamHI fragment of probe pVAW409R3, a 2-4 kb EcoRI-BglI fragment of probe pVAW412R3, and a 1-8 kb EcoRI-HincII fragment of pEW401.

AGAROSE GEL ELECTROPHORESIS
Genomic DNA was isolated from total blood using a standard phenol-chloroform extraction procedure and digested with the respective restriction enzyme according to the manufacturer's specifications. The DNA fragments were separated in a 0.7% agarose gel in 1× Tris-acetate buffer.

Fresh human lymphocytes or Epstein-Barr virus transformed lymphoblasts embedded in agarose plugs were lysed and the DNA digested with the rare cutting restriction enzymes SacII or NotI.14 The DNA fragments were separated by pulsed field gel electrophoresis in 1% agarose gels in 0.5× Tris borate buffer at 15°C. Separation of 90 to 1000 kb fragments was obtained after a 40 hour run at 180 V and pulse times of 45 seconds using a LKB 2015 Pulsaphor. Fragments between 1 and 2 Mb were separated using the appropriate presettings of the Bio-Rad CHEF MAPPER XA.

Figure 1 Pedigrees of the HMSN I families studied. The numbers under the symbols refer to the motor median NCV. Persons with a sural nerve biopsy are marked by the letters NB, and persons available for DNA analysis with an asterisk.
SOUTHERN BLOT HYBRIDISATION
After agarose gel electrophoresis the DNA fragments were transferred to a nylon membrane (Hybond N+, Amersham, England) and hybridised with radiolabelled probes according to the method of Church and Gilbert.20 Plasmid DNA was isolated using a modified alkaline extraction procedure.21 Insert fragments or single copy fragments were recovered from standard agarose gels after digestion22 and were labelled with [$\alpha$-32P] dCTP by random priming (Bethesda Research Laboratories).

LINKAGE ANALYSIS
Two point and multipoint lod scores were calculated with the programs MLINK and LINKMAP, respectively, of the computer program package LINKAGE, version 5.04.23,24 A frequency of 0.0001 was used for the HMSN I gene and for the duplication. In each calculation we assumed equal male and female recombination rates. The allele and haplotype frequencies of the different RFLPs were as previously published.15,18,25 Recombination distances between the chromosome 17 markers were converted to genetic distances using Haldane's mapping functions.26

Results
Of the 12 HMSN I families in our initial report on the duplication in 17p11.2, seven were analysed in our laboratory. All HMSN I families were selected according to rigorous criteria with respect to autosomal dominant inheritance as well as clinical and electrophysiological diagnosis of the disease. Linkage of the family with chromosome 17 was not a criterion for selection. One family, CMT A, is an extended five generation Belgian HMSN I family which has been described previously in great detail.27 Family CMT A is linked to chromosome 17 and was used to sublocalise the CMT1a locus to bands 17p11.2-p12.19 The remaining six families live in Belgium and The Netherlands and were referred by different clinicians after a 'call for patients' in several European scientific journals. The pedigrees of the families are shown in fig 1. Owing to their small size none of the families had previously been analysed for linkage with chromosome 17 or chromosome 1 DNA markers. Furthermore, since HMSN I shows an age dependent penetrance, we did not include unaffected subjects younger than 20 years in our genetic analysis unless their NCV measurements were normal.

We have previously shown that the duplication was only detected in HMSN I patients on Southern blot hybridisation with the polymorphic probe pVAW409R3.17 In heterozygous HMSN I patients the duplication is detected easily since one of the MspI alleles shows double density compared to the other. In this study, we used a similar approach to select for other markers duplicated in the HMSN I families. The polymorphic markers tested are located in 17p11.2-p12 and their position on the chromosome 17 genetic map is shown in fig 2. In the analysis we also included the marker pA10-41, one of the initial two chromosome 17 DNA markers linked to CMT1a.3 Probe pA10-41 is tightly linked to pEW301 at a distance of 3 cM but its exact position on the genetic map relative to pEW301 is not known yet.26 Apart from the probe pVAW409R3, two other probes, pVAW412R3 and pEW401, were found to be duplicated in all HMSN I patients. Fig 3 shows the autoradiograph of a

Figure 2 Chromosomal and genetic localisation of the chromosome 17 markers used in the DNA analysis. Genetic distances and marker order are as published.16,25

Figure 3 Autoradiograph of a MspI Southern blot of seven patients each belonging to one of the HMSN I families (fig 1) and of one normal control. The Southern blot was consecutively hybridised with the single copy fragments of the probes pVAW409R3, pVAW412R3, and pEW401. Numbers 1, 2, and 3 of pVAW409R3 correspond to the polymorphic MspI alleles 2.8 kb, 2.7 kb and 1.9 kb. Numbers 1 and 2 of pVAW412R3 and pEW401 correspond to the polymorphic MspI alleles 10.5 kb, 5.4 kb and 5.5 kb, 4.4 kb respectively. The 4.5 kb signal of the chromosome 21 probe SF85 was used as a reference in the densitometric scanning of the autoradiograph.
Southern blot of MspI digested DNA of one patient selected from each of the seven HMSN I families and hybridised sequentially with probes pVAW409R3, pVAW412R3, and pEW401. A diallelic MspI RFLP has been described for the latter two probes. The HMSN I patient selected from each family is heterozygous for the pVAW409R3 RFLP. Six of the seven selected patients are heterozygous for the pEW401 RFLP and clearly show the density difference between the polymorphic MspI alleles, indicating that probe pEW401 is also duplicated in these patients. In four families, CMT H, CMT N, CMT O, and CMT 36, the selected patient as well as all other patients in the family were homozygous for the pVAW412R3 RFLP. In family CMT 36 all patients were also homozygous for the pEW401 RFLP. In these four families the presence of the duplication in probes pVAW412R3 and pEW401 was confirmed by densitometric scanning of the autoradiograph. The results for family CMT 36 are illustrated in fig 4. Thus, in all HMSN I patients belonging to the seven families analysed, the duplication comprises all three of the markers, pVAW409R3, pVAW412R3, and pEW401. Further, none of the markers pEW301 and pEW401R1 are not involved in the duplication they may be considered to be flanking markers of the duplication on the centromeric and telomeric side respectively.

The two point lod scores calculated for linkage of HMSN I with the markers pEW301, pA10–41, pVAW409R3, and pEW503 located outside the duplication are summarised in the table. The differences between the present and published lod scores of the markers pEW301 and pA10–41 in family CMT A are the result of changes in the affected status of some family members. Three subjects (IV.9, V.8, and V.9) with an unknown disease status and one clinically asymptomatic child (V.13) had NCV measured and were diagnosed as affected. In addition, we found the duplication in three subjects, a mother and two daughters (II.9, IV.15, and IV.16) who were previously considered to be unaffected. Careful re-examination of the clinical records showed that none of them showed foot drop, steppage gait, or abnormal deep tendon reflexes. However, all three showed slight caving of the feet which may constitute minor expression of HMSN I. Since these subjects refused electrophysiological examination to confirm their disease status we classified them as unknown in the linkage analysis. Owing to the small size of the six nuclear HMSN I families and since only a limited number of subjects was available for DNA analysis, only a few of the families were informative for each marker. With probe pEW301 negative lod scores were obtained in families CMT 27 and CMT 36, resulting from two and one informative recombinants respectively. In family CMT 27 the two recombinants were also detected with probe pA10–41. In both families the recombinants occurred distal to pEW301 but only family CMT 27 was sufficiently informative to map the recombinants more precisely. The representative part of the pedigree of family CMT 27 is presented with detailed marker information in fig 5. Both recombinants happened in the germline of the affected mother (II.3) and recombinant chromosomes were passed on to one affected daughter (III.5) and to one unaffected daughter (III.7). The recombinants occurred between the markers pVAW409R3 and pEW401, markers that are duplicated in the affected mother (II.3) and her two affected daughters (III.4 and III.5). Marker pVAW412R3 was not informative in the affected mother. As a consequence of the recombination the haplotype of the pVAW409R3 MspI alleles in the duplication changed from (1,2) to (2,2) in the recombinant daughter (III.5). A similar change of the duplication haplotype was observed in family CMT A. In this case

<table>
<thead>
<tr>
<th>Allele</th>
<th>CMT36 IV.2</th>
<th>Normal Control</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>3.16</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>3.27</td>
<td>1</td>
</tr>
</tbody>
</table>

- **pVAW412R3**
  - Normal Control
  - Allele 1: SF85
  - Allele 2: SF85

- **pEW401**
  - Normal Control
  - Allele 1: SF85
  - Allele 2: SF85

Figure 4  Densitometric scanning of the hybridisation signals obtained with the probes pVAW412R3 and pEW401 relative to SF85 for the normal control and for the patient CMT36 IV.2. The autoradiographs in fig 3 were scanned at 600 nm in a computerised densitometer (Appraiser, Beckman Instruments Inc, CA). We computed the area under each signal curve and eliminated differences in DNA concentration by normalising each of the pVAW412R3 and pEW401 signals to the internal SF85 control signal. Finally, dosages were calculated by comparing the normalised signals obtained in patient CMT36 IV.2 to the signals in the normal control. For both probes pVAW412R3 and pEW401, the patient CMT36 IV.2 had three dosages of allele 2 indicating that CMT36 IV.2 is duplicated for both markers.
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Two point lod scores in the informative HMSN I families with 17p11.2-p12 DNA markers.

<table>
<thead>
<tr>
<th></th>
<th>Recombination fraction</th>
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<tbody>
<tr>
<td></td>
<td>0.00</td>
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<tr>
<td>pEW301</td>
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</tr>
<tr>
<td>CMT A</td>
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</tr>
<tr>
<td>CMT O</td>
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</tr>
<tr>
<td>CMT 27</td>
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<tr>
<td>CMT 33</td>
<td>1.00</td>
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<tr>
<td>CMT 36</td>
<td>-∞</td>
</tr>
<tr>
<td>Total</td>
<td>-∞</td>
</tr>
<tr>
<td>pA10-41</td>
<td></td>
</tr>
<tr>
<td>CMT A</td>
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</tr>
<tr>
<td>CMT N</td>
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</tr>
<tr>
<td>CMT 27</td>
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</tr>
<tr>
<td>pVAW41OR1</td>
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<tr>
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<tr>
<td>CMT 27</td>
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<td>CMT 33</td>
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<tr>
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</tr>
<tr>
<td>pEW503</td>
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<tr>
<td>CMT H</td>
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</tr>
<tr>
<td>CMT 27</td>
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<tr>
<td>CMT 36</td>
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</tr>
<tr>
<td>Total</td>
<td>-∞</td>
</tr>
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One informative recombinant occurred proximal to pEW503 between pEW401 and pVAW409R3, changing the haplotype of the pEW401 MspI alleles on the chromosome carrying the duplication (data not shown). Meiotic recombination events between these markers is to be expected since the probes pVAW409R3, pVAW412R3, and pEW401 are genetically separate markers. However, since informative meiotic recombinants will change the marker haplotype of the duplication, it is impossible to code for the duplicated alleles in linkage analysis. Instead, the duplication is scored for each subject as present or absent for affected or unaffected subjects respectively. The results for the multipoint linkage analysis of the seven HMSN I families with the markers pEW301, pVAW409R3, pVAW412R3, pEW401, pVAW410R1, and pEW503 are shown in fig. 6. The order and genetic distances between the markers is as in fig 2. The peak lod score of 34.04 at zero recombination with the duplicated markers reflects the maximal information content of the seven HMSN I families analysed. The regions centromeric of pEW301 and telomeric of pEW503 are excluded with odds of $10^{13}$ and $10^{10}$ respectively relative to the

Figures 5 and 6. Multipoint linkage analysis between the CMT1a gene and markers on chromosome 17p11.2-p12.
duplicated region, confirming our previous localization of the CMT1a gene.\(^{15}\)

On the genetic map the average recombination distance between \textit{pVAW409R3} and \textit{pEW401} and between \textit{pEW301} and \textit{pVAW410R1} is 10 cm and 16 cm, representing respectively the minimal and maximal genetic size of the CMT1a duplication. Since female recombination rates in this region of chromosome 17p are five times higher than male recombination rates,\(^{25}\) the major contribution to the genetic size of the duplication is derived from female recombinants. To obtain information on the actual physical distances between the markers we performed long range restriction mapping using pulsed field gel electrophoresis. The DNA of normal subjects was digested with five different rare cutting restriction enzymes, \textit{NotI}, \textit{MluI}, \textit{SacII}, \textit{BssHII}, and \textit{EagI}, and hybridised with all three probes involved in the CMT1a duplication, that is, \textit{pVAW409R3}, \textit{pVAW412R3}, and \textit{pEW401}. The smallest common fragment of 1-15 Mb was detected with the restriction enzyme \textit{NotI}. Next we hybridised DNA of HMSN I patients digested with the same five rare cutting restriction enzymes. An extra fragment was detected with probe \textit{pVAW409R3} in \textit{SacII} digested DNA of the HMSN I patients (fig 7). The five patients tested, although belonging to different families (CMT A, CMT N, CMT O, CMT 33, CMT 36), all showed the same \textit{SacII} fragment of approximately 450 kb. This 450 kb \textit{SacII} fragment most likely represents the proximal junction of the CMT1a duplication in these patients. Furthermore, the minimally affected mother and her two daughters in family CMT A (III.9, IV.15, and IV.16) all carried the \textit{SacII} junction fragment suggesting their disease status. Since no altered hybridisation pattern was detected with the probes \textit{pVAW409R3}, \textit{pVAW412R3}, and \textit{pEW401} on \textit{NotI} digested DNA, the physical size of the CMT1a duplication is at least 1-15 Mb (fig 7).

### Discussion

We initially observed the CMT1a duplication in HMSN I patients with probe \textit{pVAW409R3} located in 17p11.2.\(^{37}\) Here, we showed that the CMT1a duplication is also detectable with two other 17p11.2 probes, \textit{pVAW412R3} and \textit{pEW401}, but not with probes \textit{pEW301} and \textit{pVAW410R1} The latter two map centromeric and telomeric respectively to the three duplicated markers. The average genetic distance between the outer two duplicated probes, \textit{pVAW409R3} and \textit{pEW401}, is 10 cm.\(^{25}\) However, all three duplicated markers are located in the same \textit{NotI} fragment in both normal subjects and HMSN I patients, suggesting that the physical size of the CMT1a duplication is at least 1-15 Mb. The large discrepancy between the genetic and physical distance of the duplicated markers suggests that the 17p11.2 region must be extremely prone to meiotic recombination. Significant higher recombination rates in females have already been reported.\(^{25}\)

In two of the HMSN I families analysed we observed informative recombinants occurring in the duplication resulting in a switch of the haplotype of one of the duplicated markers. Consequently, the high recombination frequency in 17p11.2 has major implications for the analysis of linkage disequilibrium between the duplication and the haplotype of the duplicated markers. In our initial report on the CMT1a duplication we were unable to detect significant allelic association of the duplication with a particular haplotype of probe \textit{pVAW409R3}, and in one HMSN I family the disease was the result of a de novo duplication following an unequal crossing over event at meiosis. We therefore concluded that in each HMSN I family the duplication was most likely the product of an independent mutation implying a high mutation rate in HMSN I. Our present observations, however, do not exclude the possibility that the absence of allelic association is in fact the consequence of the unusual high recombination rate in the 17p11.2 region. Indeed, it cannot yet be excluded that in most HMSN I families the duplication is the product of a mutation in the same ancestral chromosome but that frequent recombination in 17p11.2 has changed the haplotype of the duplicated chromosome during its transmission over the following generations. Thus, it will be necessary to examine a
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significant number of clinically sporadic patients, as well as their parents, for de novo duplications in 17p11.2 before conclusions can be drawn about the frequency of new mutations in HMSN I. Secondly, although the duplication mutation is present in an HMSN I family, the high recombination rate in 17p11.2 may lead to negative linkage results with markers flanking the duplication, especially in smaller families or in families loaded with female patients. On the basis of these negative results some families may have been previously excluded from chromosome 17. Therefore, these families should be re-examined carefully for the CMT1a duplication before the existence of other disease loci in HMSN I than the ones on chromosomes 17 and 1 can be postulated.

At present the exact size of the CMT1a duplication is not known. All patients examined so far have duplications of all three markers, pVAW409R3, pVAW412R3, and pEW401, on Southern blot hybridisation. In addition, analysis by pulsed field gel electrophoresis showed in five patients, each belonging to a different family, a similar sized SacII duplication junction fragment with probe pVAW409R3. These results suggest that the CMT1a duplication in HMSN I patients may be very homogeneous in size and location. However, several more families need to be examined to sustain this preliminary observation, and more detailed pulsed field mapping of the duplicated region is needed to determine the junctions and hence the exact size of the CMT1a duplication in HMSN I patients. Nevertheless, the detection of the SacII junction fragment with probe pVAW409R3 provides a firmer basis for the DNA diagnosis of HMSN I patients, especially if the patient's DNA is homozygous for one or more of the MsgI RFLP's detected by the duplicated probes.

Note
At a meeting of the CMT consortium group organised by the Muscular Dystrophy Association on 14 June 1991 in Tucson, Arizona, USA, Dr J Lupski and Dr P Patel (Baylor College, Houston, USA) presented the finding of a duplication in HMSN I patients with probes pVAW409R3 and pVAW409R1. The duplication was shown using different techniques including dosage differences at RFLP alleles and pulsed field gel electrophoresis. Their results, which are similar to ours (present study), were published during the revision of this manuscript.

This work was funded by the Flemish Biotechnology Programme, the National Fund for Scientific Research (CWB), the Institute for Scientific Research in Industry and Agriculture (VT, EN), the Association Française contre les Myopathies, and Innogenetics Inc, Belgium. The authors are grateful to L Sandkuijl for critical discussion of the linkage results and to V O Van Hoof and L G Lepoutre for help with the densitometric scanning of the autoradiographs.