Am. J. Hum. Genet. 62:1023–1033, 1998

Human Meiotic Recombination Products Revealed by Sequencing a Hotspot for Homologous Strand Exchange in Multiple HNPP Deletion Patients

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Summary

The HNPP (hereditary neuropathy with liability to pressure palsies) deletion and CMT1A (Charcot-Marie-Tooth disease type 1A) duplication are the reciprocal products of homologous recombination events between misaligned flanking CMT1A-REP repeats on chromosome 17p11.2-p12. A 1.7-kb hotspot for homologous recombination was previously identified wherein the relative risk of an exchange event is 50 times higher than in the surrounding 98.7% identical sequence shared by the CMT1A-REPs. To refine the region of exchange further, we designed a PCR strategy to amplify the recombinant CMT1A-REP from HNPP patients as well as the proximal and distal CMT1A-REPs from control individuals. By comparing the sequences across recombinant CMT1A-REPs to that of the proximal and distal CMT1A-REPs, the exchange was mapped to a 557-bp region within the previously identified 1.7-kb hotspot in 21 of 23 unrelated HNPP deletion patients. Two patients had recombined sequences suggesting an exchange event closer to the *mariner*-like element previously identified near the hotspot. Five individuals also had interspersed patches of proximal or distal repeat specific DNA sequence indicating potential gene conversion during the exchange of genetic material. Our studies provide a direct observation of human meiotic recombination products. These results are consistent with the hypothesis that minimum efficient processing segments, which have been characterized in *Escherichia coli*, yeast, and cultured mammalian cells, may be required for efficient homologous meiotic recombination in humans.

Received November 4, 1997; accepted for publication March 17, 1998; electronically published April 17, 1998.

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0002-9297/98/6205-0006$02.00

Introduction

Hereditary neuropathy with liability to pressure palsies (HNPP; OMIM 162500) and Charcot-Marie-Tooth disease type 1A (CMT1A; OMIM 118220) are two autosomal dominant peripheral neuropathies caused, in the majority of cases, by DNA rearrangements that apparently result from an unequal crossing-over event on chromosome 17p11.2-p12 (Pentao et al. 1992; Chance et al. 1994; Reiter et al. 1996*a;* Lupski 1997). HNPP patients suffer from acute recurrent transient palsies and sensory dysfunction as a direct result of compression to the peripheral nerve (Chance et al. 1993; reviewed in Murakami et al. 1996). This disorder is usually mild and somewhat underdiagnosed. However, in principle, new-mutation sporadic cases should be as common as the more debilitating CMT1A (Nelis et al. 1996), to which HNPP is related at the molecular level. Approximately 82% of familial and 86% of sporadic cases of HNPP are caused by the 1.5-Mb HNPP deletion on chromosome 17p11.2-p12 (Nelis et al. 1996), whereas a 1.5-Mb duplication in 17p11.2-p12 is found in the majority of patients with CMT1 (Hoogendijk et al. 1992; Wise et al. 1993; Nelis et al. 1996). The 1.5-Mb HNPP deletion and the 1.5-Mb CMT1A duplication are apparently the reciprocal recombination products of crossovers between misaligned proximal and distal CMT1A-REP repeats (Chance et al. 1994; Reiter et al. 1996*a;* Timmerman et al. 1997). The vast majority of these unequal crossing-over events appear to occur between homologous chromosomes during meiosis (Raeymaekers et al. 1991; Palau et al. 1993).

A number of mechanisms have been proposed to explain how the CMT1A duplication results in the CMT1 disease phenotype, including (1) gene dosage, (2) gene interruption, and (3) a position effect. However, the collective evidence supports the gene dosage model in which an altered copy number of the peripheral myelin protein 22 gene (*PMP22*) located between the CMT1A-REPs

causes the disease phenotype in both CMT1A and HNPP (reviewed in Reiter et al. 1996*b;* Lupski 1997).

We elsewhere identified a 1.7-kb hotspot for homologous recombination between misaligned CMT1A-REPs wherein the relative risk of a crossover within the hotspot is 53:1 over the surrounding homologous sequence (Reiter et al. 1996*a*). This hotspot for homologous recombination, detected by the identification of novel junction fragments from the recombinant CMT1A-REPs in both HNPP deletion and CMT1A duplication patients, is embedded within a 24-kb region of 98.7% sequence identity shared by the CMT1A-REPs (Reiter et al. 1997). We have previously hypothesized that the hotspot may be initiated by a double-strand break at a *mariner* insect transposon-like element, called "MITE," located near the site of frequent exchanges (Reiter et al. 1996*a*).

What distinguishes the hotspot within the CMT1A-REPs from previously described hotspots in the human genome is that >78% of 541 patients with CMT1 or HNPP, representing 420 independent recombination events, have undergone a crossover in the hotspot directly resulting in the disease phenotype (Kiyosawa et al. 1995; Lopes et al. 1996; Reiter et al. 1996*a;* Timmerman et al. 1997; Yamamoto et al. 1997). Such a large collection of patients with the same small recombinant junction provides a rare opportunity to examine homologous recombination at the DNA sequence level in a significant number of independent molecular events in the human genome. To this end, the site of exchange between misaligned CMT1A-REP elements was refined further by sequencing the recombinant CMT1A-REP across the hotspot region in 23 unrelated HNPP deletion patients. Recombinant junctions associated with various genetic disorders have been cloned and sequenced from individual patients (Lehrman et al. 1987; Keyeux et al. 1989; Yen et al. 1990; Metzenberg et al. 1991; Rüdiger et al. 1991), and a PCR strategy has recently been employed to examine a small number of inversion mutations associated with the Hunter syndrome (Lagerstedt et al. 1997). However, this is one of the first times in which a noncloning strategy has been used to examine such a large number of recombination events at the same locus. By comparing these sequences to the consensus sequences for the proximal and distal CMT1A-REPs, the region of exchange in 21 of 23 HNPP deletion patients was mapped to a 557-bp region within the 1.7-kb hotspot defined elsewhere (Reiter et al. 1996*a;* Timmerman et al. 1997). The recombination products observed are similar to the products of meiotic recombination recovered in other model organisms. Our observations provide some evidence supporting minimum sequence identity requirements for efficient homologous recombination in humans, as has been described in bacterial, yeast, and mammalian cells.

Subjects and Methods

Subjects

Peripheral blood samples were obtained from HNPP deletion patients and normal control individuals under institutional review board–approved informed consent in Houston, Texas, and in Antwerpen, Belgium. The patients used in this study were previously tested for the presence of the novel 7.8-kb junction fragment indicative of an exchange event in the CMT1A-REP 1.7-kb hotspot region (Reiter et al. 1996*a;* Timmerman et al. 1997).

PCR Amplification of CMT1A-REP Sequences

Genomic DNA was extracted from the peripheral blood by standard methods (Pentao et al. 1992). DNA was normalized to a concentration of 200 ng/μl for digestion. Two micrograms of DNA were digested for 5–6 h at 37°C in the following reaction: 16 units of *Sac*I (Boehringer Mannheim), 10 units of *Eco*RI (Boehringer Mannheim) in reaction buffer A (Boehringer Mannheim) with double-distilled water (ddH_2O) up to a total volume of 40-μl. Reactions were incubated in hydrophobic polyvinylidene fluoride (PVDF) columns (Millipore). After digestion, 60 μl of ddH_2O were added to each reaction, and the PVDF columns were spun for 30 s at full speed in a bench-top microfuge to clear the reaction of enzymatic proteins. The eluate was precipitated with 10 μl of 3 M NaAc plus two volumes of 100% ethanol (EtOH) and spun for 30 min at 14,000 rpm. Pellets were washed with 70% EtOH, dried via speed vac, and resuspended in 25 μl of ddH_2O.

PCR primers U6393 (5′-GAA AAC TGG GTA GGC TGA CAA G-3′) and L1693 (5′-CCT ATT GTC TTT TTA TTG AGC C-3′) were designed to anneal to both the proximal and distal CMT1A-REPs and to flank the *Eco*RI site unique to the distal CMT1A-REP and the *Sac*I site unique to the proximal CMT1A-REP. To ensure that the primers did not anneal to the template prior to complete disassociation of the DNA strands, PCR reactions were prepared in bulk and aliquoted into 96 well plates using Ampliwax beads (Perkin Elmer) to separate upper and lower reaction mixtures. The lower reaction (12.5 μl) consisted of 1 μl each of 1 pmol/μl primers, 0.4 μl of 10 mM dNTP mix, 2.5 μl of 10 × *Taq* Extender Buffer, and 7.6 μl of ddH_20. The upper reaction (37.5 μl) consisted of 2-μl restriction enzyme digested genomic DNA, 2.5 μl of 10 × *Taq* Extender Buffer, 1.6 μl of 1 U/μl *Taq* Extender (Perkin Elmer), 1.6 μl of 1 U/μl Amplitaq, and 32.3 μl of ddH_20. PCR conditions were as follows: 94°C, 5 min; 10 cycles of touchdown PCR at 94°C, 30 s; 69°C (−1°C per cycle), 2 min; 72°C, 6 min; 30 cycles at 94°C, 30 s; 59°C, 2 min; 72°C, 7 min; and a final extension of 72°C, 7 min. Five microliters of each 50-μl PCR reaction were subjected to electrophoresis in

a 0.8% agarose gel to gauge the efficiency of the PCR reaction.

DNA Sequencing

The CMT1A-REP hotspot region was sequenced in individual HNPP deletion patients and controls using dye terminator sequencing technology (Applied Biosystems division of Perkin Elmer). Ten sequencing primers were constructed, based on previously published sequence data across the hotspot (Reiter et al. 1996*a*), in order to obtain double-strand coverage across a 2.0-kb region of the 4.7-kb PCR product. Chromatograms were aligned, edited, and compared to controls using the sequence alignment program Sequencher (Gene Codes). Analysis of the 557-bp hotspot was performed using the BCM Search-Launcher command line interface (Smith et al. 1996).

Results

PCR Amplification of the CMT1A-REPs

On the basis of the published sequences of genomic clones pLR6.0 (GenBank HSU41165 [http://www2.ncbi.nlm.nih.gov/irx/cgi-bin/birx_doc?genbank+63719]) and pLR7.8 (GenBank HSU41166 [http://www2.ncbi.nlm.nih.gov/irx/cgi-bin/birx_doc?genbank+63720]), there is a total of 41 individual nucleotide differences between the proximal and distal CMT1A-REPs in the 3.2-kb region between the *Eco*RI site unique to the distal REP and the *Sac*I site unique to the proximal REP (Reiter et al. 1996*a*). These nucleotides represent 38 different sites across the 3.2-kb hotspot region that could be used to classify a recombinant CMT1A-REP sequence as either proximal matching or distal matching at that site. A strategy was designed for amplifying only the recombinant CMT1A-REP from individual HNPP deletion patients, as well as the proximal and distal REPs from control individuals. We used PCR primers that anneal to both CMT1A-REPs but flank the *Eco*RI site unique to the distal CMT1A-REP and the *Sac*I site unique to the proximal CMT1A-REP (fig. 1*A*). When genomic DNA from HNPP deletion patients and control individuals is digested prior to PCR with *Eco*RI, *Sac*I or a combination of *Eco*RI and *Sac*I, the primers will amplify products originating from the proximal (fig. 1*A*, I), distal (fig. 1*A*, II), or recombinant (fig. 1*A*, IV) CMT1A-REPs, respectively. A product is not expected from the recombinant CMT1A-REP of CMT1A duplication patients, because this template contains both restriction sites (fig. 1*A*, III).

Genomic DNA from 17 North American and 23 European HNPP deletion patients was digested with *Sac*I and *Eco*RI prior to PCR amplification. Similarly, genomic DNA from six control individuals was digested with either *Eco*RI to amplify the distal CMT1A-REP or *Sac*I to amplify the proximal CMT1A-REP. Touchdown/Long PCR was performed and the products were analyzed on an agarose gel for the presence of the expected 4.7-kb product. As anticipated, no PCR product was detected in reactions containing *Sac*I/*Eco*RI digested genomic DNA from control individuals who have no recombinant CMT1A-REP or from CMT1A duplication patients who have a recombinant CMT1A-REP that contains both an *Eco*RI and *Sac*I site (fig. 1*B*). A PCR product was detected in 28 of 41 HNPP deletion patients tested. The DNA samples that did not produce a PCR product may have been degraded by nucleases in the DNA preparation, may have encountered difficulties in the long PCR, or may have been from individuals with polymorphisms in the primer binding sites.

Sequence across the Region of Exchange

Ten sequencing primers were designed to anneal to both proximal and distal CMT1A-REPs. The individual chromatograms from previously published clones pLR6.0 (distal CMT1A-REP) and pLR7.8 (proximal CMT1A-REP) were compared with chromatograms from sequencing reads of the proximal and distal CMT1A-REPs of control individuals at particular base differences between the proximal and distal CMT1A-REPs. Three sites that differ between the two genomic clones do not appear to be variable in the sequences across the proximal and distal CMT1A-REPs of control individuals. In addition, there are four sites that differ between the proximal and distal CMT1A-REPs in control individuals but not in the published clones. Consensus sequences for the proximal and distal CMT1A-REPs were generated on the basis of the sequences from the PCR products of six control individuals (available from our website at http://www.bcm.tmc.edu/molgen/lupski). The differences used to map the site of exchange in HNPP deletion patients are summarized in figure 2*A*.

Two sites, the fifth and eighth base difference to the left of the *Nsi*I site in figure 2*A*, appeared polymorphic among the six control individuals and were not used to determine the region of exchange. It is important to note, however, that in some individuals, the presence of a G instead of a T in the distal CMT1A-REP at the polymorphic base located five sites to the left of the *Nsi*I recognition sequence in figure 2*A* results in an uninterrupted 456-bp stretch of sequence identity shared by the proximal and distal CMT1A-REPs. Two other sites, the third and fourth base differences to the left of the *Nsi*I site in figure 2*A*, were somewhat variable among the 23 HNPP deletion patients examined but not among the six control individuals. These bases also were not used in the mapping of crossover events. Although there are 17 differences between the proximal and distal CMT1A-

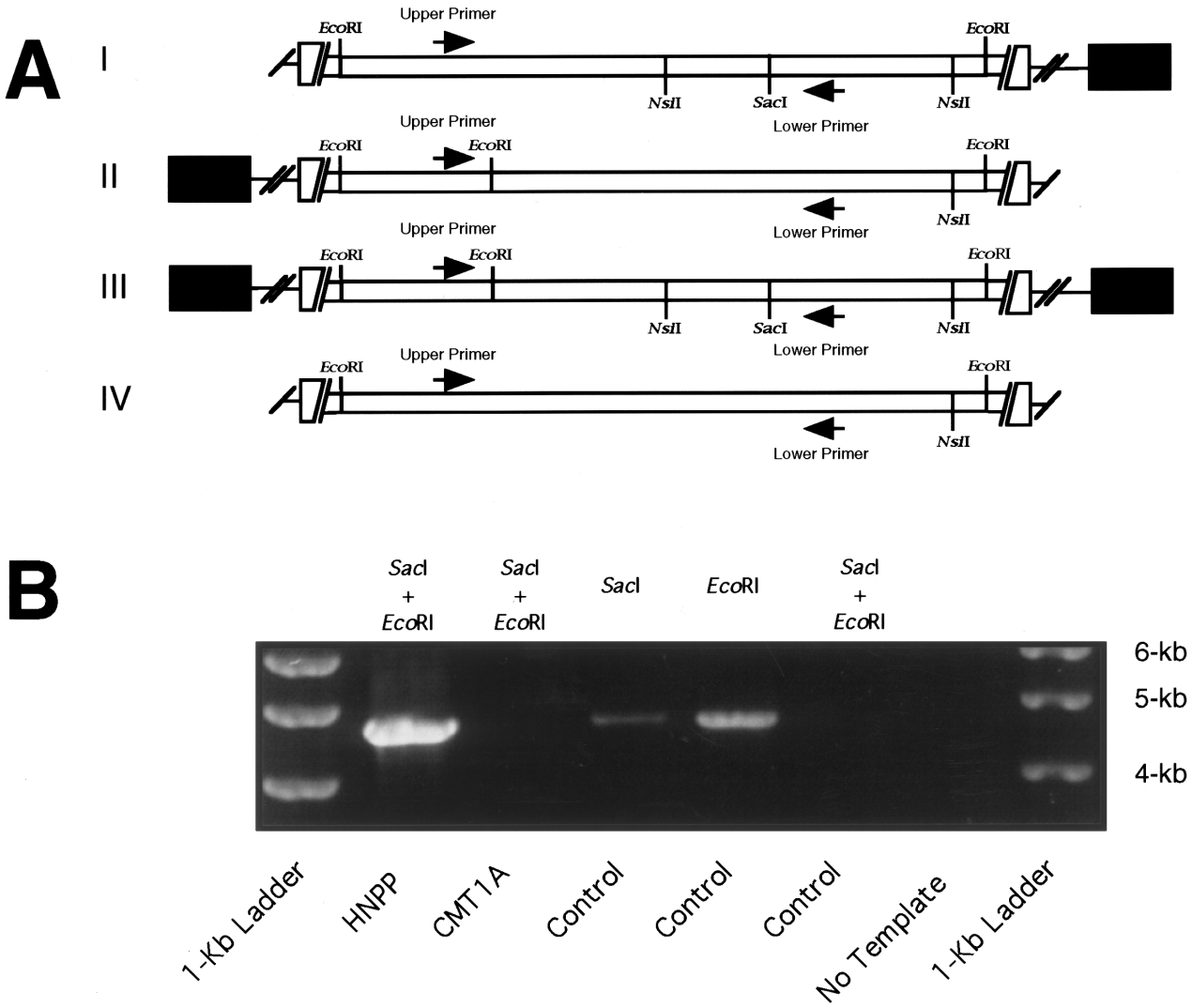

Figure 1 PCR strategy for amplifying recombination hotspot sequences from the recombinant, proximal, and distal CMT1A-REPs. *A,* Left-side filled boxes, Proximal CMT1A-REP; right-side filled boxes, distal CMT1A-REP. Open rectangles, 7.8-kb region that encompasses the 3.2- and 1.7-kb (*Eco*RI/*Sac*I and *Eco*RI/*Nsi*I) hotspots for homologous recombination previously described elsewhere (Reiter et al. 1996*a*; Timmerman et al. 1997). Roman numerals, 7.8-kb region from the (I) proximal, (II) distal, (III) recombinant CMT1A duplication, and (IV) recombinant HNPP deletion CMT1A-REP. The locations of *Eco*RI, *Sac*I, and *Nsi*I sites are indicated. The PCR primers used in this study lie just outside the *Eco*RI site unique to the distal CMT1A-REP (upper primer U6393) and the *Sac*I site unique to the proximal CMT1A-REP (lower primer L1693). Although these primers will anneal to any of the CMT1A-REP templates illustrated, a PCR product will be obtained only from the HNPP deletion recombinant CMT1A-REP if the DNA is digested with *Eco*RI + *Sac*I prior to amplification, since this recombinant CMT1A-REP does not contain either restriction site within the 7.8-kb region (IV). Similarly, the proximal or distal CMT1A-REP can be amplified by predigestion of the genomic DNA with either *Eco*RI (to exclude the distal CMT1A-REP) or *Sac*I (to exclude the proximal CMT1A-REP). *B,* Genomic DNA, which was digested with either *Eco*RI, *Sac*I, or a combination of *Eco*RI + *Sac*I prior to long PCR amplification. The expected 4.7-kb PCR recombinant CMT1A-REP product was obtained in PCR containing *Sac*I + *Eco*RI digested genomic DNA only from HNPP deletion patients. A 4.7-kb PCR product from the proximal or distal CMT1A-REP was observed in reactions containing genomic DNA from control individuals digested with *Eco*RI or *Sac*I, respectively. No PCR products were detected when the template for PCR was *Sac*I + *Eco*RI digested genomic DNA from CMT1A duplication patients, control individuals, or a negative control containing all PCR reagents except template DNA.

REPs, based on consensus sequences from control individuals across the region sequenced, only 16 of these differences were used to map the site of exchange in HNPP deletion patients, since the sequence information at one site was uninformative (fig. 2*A*, uppercase letters).

The same sequencing primers used to sequence the proximal and distal CMT1A-REPs from control individuals were used to sequence across the 3.2-kb *Eco*RI/*Sac*I hotspot region of exchange in PCR products originating from the recombinant CMT1A-REPs of HNPP

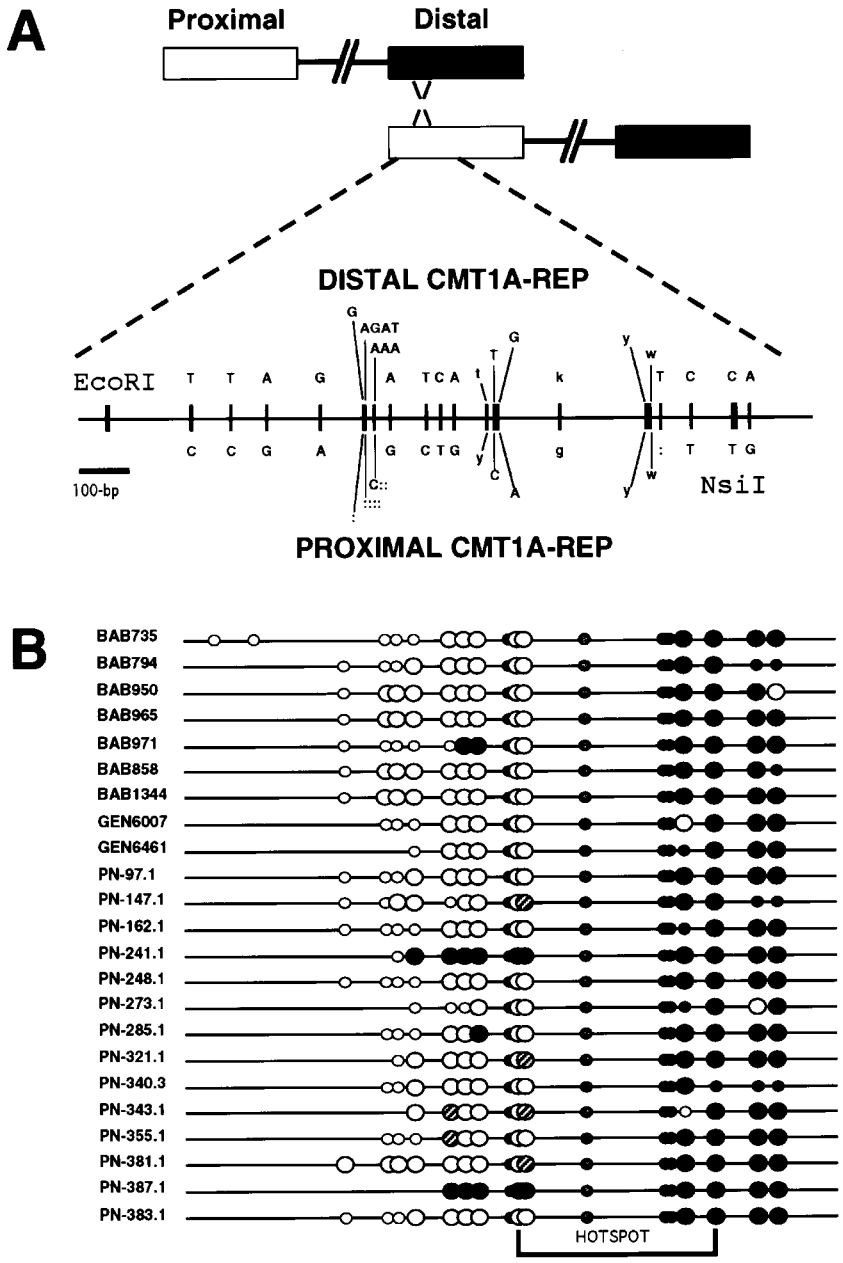

Figure 2 Sequence differences between the proximal and distal CMT1A-REPs across the 1.7-kb homologous recombination hotspot region. *A,* Sequences of the proximal and distal CMT1A-REPs from the PCR products of six control individuals were used to construct consensus sequences (consensus sequences and chromatograms are available from our website at http://www.bcm.tmc.edu/molgen/lupski). Seven sites across this region differ from previously published cloned DNA sequences (Reiter et al. 1996*a*) and may have resulted from cloning artifacts caused by DNA methylation and point mutations during propagation of human DNA sequences in the bacterial host, or they may represent cloned DNA from individuals with rare polymorphic alleles. The four sites in lower case are variable among control and/or HNPP deletion individuals and could not be assigned to either of the CMT1A-REPs. A total of 17 sites (capital letters) differ between the proximal and distal CMT1A-REPs within the hotspot. Sixteen of these sites were used to map the region of exchange in HNPP deletion patients. *B,* Mapping the region of exchange in 23 unrelated HNPP deletion patients. Each row represents sequence information from individual unrelated HNPP deletion patients of North American (beginning with BAB or GEN, rows 1–9) or European (numbers beginning with PN, rows 10–23) descent. The differences between the proximal and distal CMT1A-REPs described in panel *A* are represented by circles indicating single-strand (small circle) or double-strand (large circle) DNA sequence coverage at this site. Blackened circles denote matches to the distal CMT1A-REP; unblackened circles denote matches to the proximal CMT1A-REP; striped circles denote sites that could not be assigned to proximal or distal CMT1A-REPs; and small shaded circles denote polymorphic sites. Twenty-one of 23 unrelated HNPP deletion patients underwent a transition from proximal matching to distal matching sequence somewhere within the 557-bp region (brackets). Two patients (PN-241.1 and PN-387.1) seem to have undergone an exchange event closer to the *Eco*RI site unique to the distal CMT1A-REP (*left*). Four patients exhibited points or lengths of conversion that may be indicative of heteroduplex repair on either side of the hotspot (BAB950, BAB971, PN-273.1, and PN-285.1).

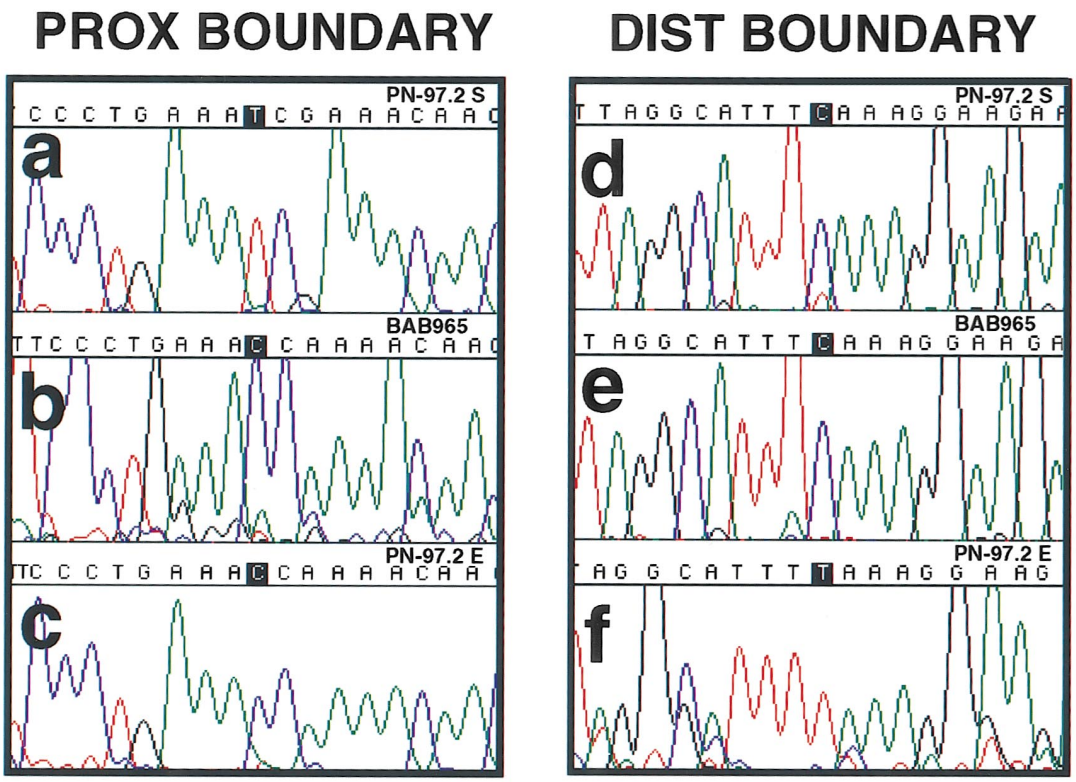

Figure 3 DNA sequencing reads from controls and HNPP deletion individuals at the boundaries of the 557-bp region of exchange. The three panels on the left side (*a*, *b*, and *c*) are the chromatograms from dye terminator sequencing reads from the proximal boundary of the hotspot. The panels on the right side (*d*, *e*, and *f*) are the chromatograms from the distal boundary of the hotspot. Numbers in the upper right corner indicate patient and control sample identification numbers. Genomic DNA from control individual PN-97.2 was digested with either *Eco*RI to sequence the proximal CMT1A-REP (*c* and *f*) or *Sac*I to sequence the distal CMT1A-REP (*a*, *d*). Patient BAB965 has the sequence pattern indicative of an exchange event in the 557-bp hotspot. This pattern, which was detected in 21 of 23 unrelated HNPP deletion patients, consists of a C at the proximal boundary (*b*) that matches the proximal CMT1A-REP (*c*) and a C at the distal boundary (*e*) that matches the distal CMT1A-REP (*d*).

deletion patients. Interpretable sequence information was obtained from 23 of these PCR products. Most nucleotide differences found in patients matched the differences between the proximal and distal CMT1A-REPs found in normal controls, but some base determinations on the proximal boundary of the exchange were indeterminate and could not be used to map the boundaries of the exchange event (fig. 2*B*, patients PN-147.1, PN-321.1, PN-343.1, PN-355.1, and PN-381.1). In addition, the crossover event in two patients (fig. 2*B*, PN-241.1 and PN-387.1) appeared to have occurred in a region close to the *Eco*RI site unique to the distal CMT1A-REP.

The region of exchange in 21 of 23 HNPP deletion patients was mapped to a 557-bp interval between a C unique to the proximal CMT1A-REP located 1,091 bp from the *Eco*RI site unique to the distal CMT1A-REP and a C unique to the distal CMT1A-REP located 118 bp from the *Nsi*I site unique to the proximal CMT1A-REP (fig. 2*B*). Automated dye terminator DNA sequencing data from patient BAB965, who underwent an exchange event in the common 557-bp region, are illustrated in figure 3*b* and *e*. Note that, at the proximal boundary of the hotspot (fig. 3, *left*), this patient's DNA sequence matches a sequencing read from the proximal CMT1A-REP of control individual PN-97.2 (fig. 3*c*) but not the distal CMT1A-REP (fig. 3*a*). Conversely, at the distal boundary of the hotspot (fig. 3, *right*), this patient's DNA sequence matches a sequencing read from the distal CMT1A-REP of control individual PN-97.2 (fig. 3*d*), but not the proximal CMT1A-REP (fig. 3*f*).

Regions of what appears to be gene conversion, perhaps resulting from heteroduplex repair, were identified in four patients on both sides of the 557-bp hotspot region (fig. 2*B*). These potential gene conversion events were observed as patches of distal matching sequence surrounded by proximal matching sequence (fig. 2*B*, BAB971 and PN-285.1), as well as by patches of prox-

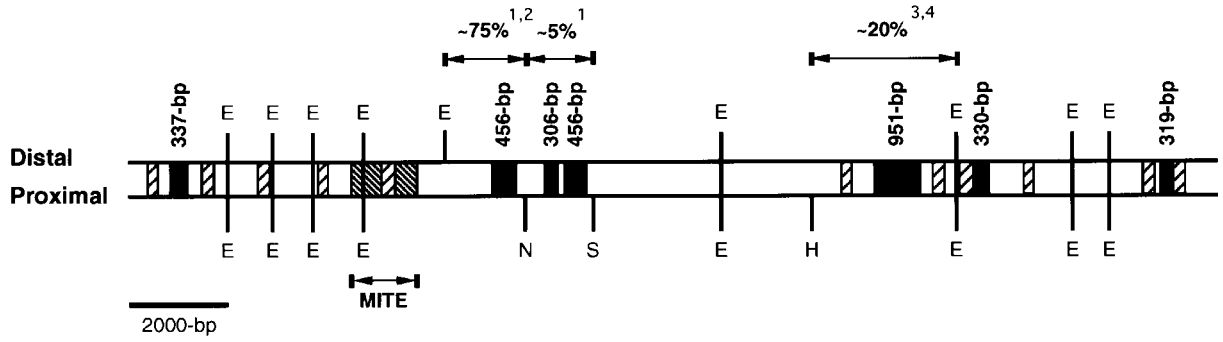

Figure 4 Localization of stretches of identity >200 bp shared by the proximal and distal CMT1A-REPs. The left side of the figure is the centromeric side, and the right side is the telomeric side of each CMT1A-REP. Uninterrupted stretches of sequence identity >200 bp were identified across the 24-kb CMT1A-REPs using previously published sequence information (GenBank accession HSU71217 and HUS71218) as well as sequence information from controls in this study. Thick hatched boxes indicate stretches of identity 200–300 bp; solid boxes indicate stretches >300 bp. Relevant restriction sites are represented by single letter designations: E = *Eco*RI; N = *Nsi*I; S = *Sac*I; and H = *Hin*dIII. The location of the MITE element in both CMT1A-REPs is indicated by a thin hatched box. The percentage of exchange events in particular regions indicated are from previously published studies by Reiter et al. (1996*a*), Timmerman et al. (1997), Lopes et al. (1996), and Kiyosawa et al. (1995)

imal matching sequence surrounded by distal matching sequence (fig. 2*B*, BAB950 and PN-273.1).

Search for Sequence Features That Promote Homologous Recombination

Double-strand breaks promote homologous recombination in all organisms in which they have been studied (Lichten and Goldman 1995). Sequence features known to promote double-strand breaks during meiosis include promoter regions and, in mammalian cells, large palindromes (Akgün et al. 1997). Multiple DNA sequence analysis searches for promoter regions, transcription factor binding sites, putative coding regions, and large palindromic sequences did not reveal any particular motifs within this 557 bp that could account for the generation of double-strand breaks.

The minimal efficient processing segment (MEPS) required for homologous recombination in cultured mouse cells has been determined to be between 132 and 232 bp of perfect shared sequence identity between recombining homologues (Liskay et al. 1987; Waldman and Liskay 1988). A search for lengths of sequence identity >200 bp shared by the misaligned proximal and distal 24-kb CMT1A-REPs (GenBank accession HSU71217 and HSU71218) revealed 18 sites, 7 of which have >300 bp of identity, that may be potential regions of exchange (fig. 4). One of the three longest stretches of identity is a 456-bp region located within the 557-bp region of exchange mapped in 21 of 23 HNPP deletion patients. This is the closest stretch of >200 bp of sequence identity in proximity to the end of the MITE element previously implicated as the site of the double-strand break that stimulates recombination in this region (Reiter et al. 1996*a*). Exchange events in both CMT1A duplication and HNPP deletion patients have been documented in the other two long regions of identity (fig. 4) located between the *Nsi*I and *Sac*I sites unique to the proximal CMT1A-REP (456 and 306 bp) (Reiter et al. 1996*a;* Timmerman et al. 1997) and between the *Hin*dIII site unique to the proximal CMT1A-REP and a common *Eco*RI site (951 bp) (Kiyosawa et al. 1995; Lopes et al. 1996).

Discussion

A meiotic homologous recombination event between misaligned CMT1A-REPs is the molecular basis of both the 1.5-Mb HNPP deletion and 1.5-Mb CMT1A duplication (Pentao et al. 1992; Chance et al. 1994; Reiter et al. 1996*a*). We have previously described a hotspot for homologous recombination at this locus through the detection of novel junction fragments from the recombinant chromosomes of HNPP deletion and CMT1A duplication patients (Reiter et al. 1996*a;* Timmerman et al. 1997). In this article, we sequenced the recombinant CMT1A-REP from 23 unrelated HNPP deletion patients by digesting genomic DNA from HNPP patients with *Eco*RI and *Sac*I prior to PCR amplification of the hotspot region to allow specific amplification of the recombinant chromosome. Comparison of these sequences to the consensus sequences of proximal and distal CMT1A-REP of control individuals allowed us to map the site of exchange in 21 of 23 unrelated HNPP deletion patients to a 557-bp region of the 24,011-bp recombinant

CMT1A-REP. This region contains a 456-bp region of sequence identity that apparently exceeds the minimum length of sequence identity between two homologies required for efficient homologous recombination, as previously established in cultured mouse cells (Liskay and Stachelek 1986; Waldman and Liskay 1988).

Of the 541 unrelated CMT1A duplication and HNPP deletion patients examined by several groups (Kiyosawa et al. 1995; Lopes et al. 1996; Reiter et al. 1996*a;* Timmerman et al. 1997; Yamamoto et al. 1997), crossover events have only been detected in the three regions containing stretches of sequence identity >456 bp (fig. 4). The next longest stretch of sequence identity shared by the CMT1A-REPs is 337 bp, but this is not the site of frequent exchange events, even though it is also located near the MITE element. There are 11 other regions of >200-bp sequence identity shared by the proximal and distal CMT1A-REPs in which no apparent crossover exchanges have been identified. On the basis of these observations, we suggest that the MEPS for efficient meiotic homologous recombination in humans may be between 337 and 456 bp. However, meiotic homologous recombination products from other regions of the human genome will need to be assessed further to delineate the extent of perfect sequence identity required for efficient homologous recombination.

Five nucleotide differences exist between the proximal and distal CMT1A-REPs in this 557-bp hotspot region, but three of these positions are polymorphic among both unaffected and affected individuals. These polymorphic sites may be manifest as stretches of sequence identity shared by the proximal and distal CMT1A-REPs in some individuals in the population. These individuals may potentially be more prone to de novo unequal crossing-over events. The strategy described herein should also allow one to examine the unaltered sequence of the proximal, distal, and recombinant CMT1A-REPs from HNPP deletion families in which the deletion is a de novo event.

Two sites at the boundaries of the region exhibited some degree of ambiguity in the dye terminator DNA sequencing reads and therefore could not be used to refine the region of exchange further. However, the PCR sequencing strategy has allowed us to confirm the hotspot identified through the detection of novel junction fragments from the recombinant chromosome by Southern analysis (Reiter et al. 1996*a;* Timmerman et al. 1997) and also to refine the hotspot of recombination between misaligned CMT1A-REPs to $<\frac{1}{3}$ of the 1.7-kb hotspot region previously identified. It appears that in terms of defining the boundaries of the hotspot, we have reached the limits of resolution for the HNPP deletion, because nucleotide differences between flanking CMT1A-REPs are required for mapping the exchange event in the recombinant CMT1A-REP. It is interesting to note that this same 557-bp region is within the preferred 741-bp site of exchange in CMT1A duplication patients, as the unequal crossing-over model predicts (Lopes et al. 1998).

We previously identified a *mariner*-like element in the CMT1A-REPs located near the 1.7-kb hotspot region and proposed that this element may stimulate homologous recombination at the hotspot by providing a target site for double-strand breaks initiated by a *mariner* transposase expressed elsewhere in the human genome (Reiter et al. 1996*a*). Evidence from the study of *mariner* elements in insects suggests that the transposition events can indeed be initiated by *trans* acting *mariner* transposases (Hartl et al. 1997). In addition, in vitro experiments with the purified *mariner* transposase clearly suggest that no host factors are required for transposition (Lampe et al. 1996). Multiple almost identical *mariner* transposons have been detected in the genomes of organisms as diverse as hydras, flatworms, insects, and zebra fish, clearly establishing that these elements can undergo horizontal transfer and remain active for some time in the genomes of their new hosts (Robertson 1993, 1997; Ivics et al. 1996; Gueiros-Filho and Beverley 1997). The refined hotspot is located 1,774 bp away from the end of the MITE element at which we proposed that the double-strand breaks occur. There are previous examples in which a homologous recombination event initiated at a double-strand break is transposed to a distantly linked site. The occurrence of an exchange event remote from the double-strand break where recombination initiated is well known in RecBCD mediated recombination in *Escherichia coli* (reviewed in Stahl and Myers 1995) in which the event is transposed from the double-strand end to a χ sequence by exonuclease activity on both DNA strands. Because DNA is lost in this process, the formation of intact recombinant products in meiosis would require extensive new DNA synthesis. Another example is in yeast, in which an HO endonuclease cut site induces an event several kilobases away with no evidence of heteroduplex formation in the intervening region (Ray et al. 1988). It was suggested that the event was transposed by single-strand exonuclease activity. Extended exonucleolytic degradation of the 5′-ending strands of a double-strand break can reveal distant homologous lengths and allow either single-strand annealing or homologous recombination involving strand invasion (Sugawara and Haber 1992). Other mechanisms may exist as well. For example, a ligated double Holliday junction formed at the site of initiation may migrate (Sobell 1972) to regions where the MEPS are located. The processing of the initiating double-strand break may open the chromatin structure, making nearby sequences subject to enhanced levels of unrelated recombination events. A joint molecule formed by a RecA-like protein while searching for homologous se-

quences might traverse substantial lengths of DNA before encountering the required MEPS. A similar model is described by Vulic´ et al. (1997) to explain the control of genetic exchange in enterobacteria. Finally, it is possible that some as-yet-undefined feature within the CMT1A/HNPP hotspot causes both the initiating double-strand break and the resolution event.

Shen and Huang (1986) found that minimal lengths of sequence identity between two homologies, MEPS, were required for homologous recombination events to occur efficiently in *E. coli*. In yeast, mitotic homologous recombination events that result in unequal crossovers require a MEPS unit of 250 bp (Jinks-Robertson et al. 1993). Experiments in mouse L cells have established that the MEPS may be as little as 132 bp, but that stretches of identity <232 bp provide poor substrates for homologous recombination and that the recombination rate increases linearly as the length of identity increases (Liskay et al. 1987; Waldman and Liskay 1988).

A search for stretches of identity within CMT1A-REPs >200 bp revealed several sites across the 24-kb CMT1A-REP that, according to previous studies in cultured mouse cells, could be sites of increased recombination. Interestingly, the three longest stretches of identity, which are presumably the most efficiently processed substrates (Liskay et al. 1987), are located within regions previously determined to be hotspots for homologous recombination (Kiyosawa et al. 1995; Lopes et al. 1996; Reiter et al. 1996*a;* Timmerman et al. 1997). One segment that has a potential length of 456 bp, depending on the alleles present at the polymorphic sites illustrated in figure 2*A*, is located within the 557-bp region defined in this study, where 21 of 23 HNPP deletion patients showed an exchange event. This is the closest stretch of identity >200 bp in length in proximity to the end of the MITE element. We have hypothesized that the double-strand breaks that initiate homologous recombination between CMT1A-REPs occur at the boundaries of the MITE element (Reiter et al. 1996*a*). Another two stretches of identity >200 bp are located between the *Nsi*I and *Sac*I sites unique to the proximal CMT1A-REP, telomeric to the primary hotspot in the CMT1A-REPs, where ~5% of 123 CMT1A duplication and HNPP deletion patients examined in our previous study underwent an exchange event (Reiter et al. 1996*a*). The longest stretch of sequence identity shared by the CMT1A-REPs is a 951-bp region bound by a *Hin*dIII site unique to the proximal CMT1A-REP and an *Eco*RI site common to both CMT1A-REPs where ~20% of the CMT1A duplication and HNPP deletion patients examined in two independent studies (fig. 4) showed an exchange event representing a potential secondary hotspot (Kiyosawa et al. 1995; Lopes et al. 1996). Although exchange events have not yet been mapped at the sequence level in these two other hotspots, we would anticipate that the boundaries of the exchange may flank a stretch of identity between 337 and $\geqslant$456 bp. This prediction is based on observations from this study of the primary hotspot for homologous recombination between misaligned CMT1A-REPs. It seems plausible that the closest length of identity to the site of the double-strand break would be the preferred region of exchange (Sugawara and Haber 1992). The two individuals in whom the apparent exchange point is to the left of the hotspot and in a region without a significant length of identity shared by the CMT1A-REPs may represent lengths of gene conversion contiguous with a crossover within the 456-bp stretch of identity nearby.

In summary, we describe a detailed examination of a large number of independent recombination events at a single locus in the human genome. This examination has enabled us to refine the region of exchange for the CMT1A-REP homologous recombination hotspot to a 557-bp interval by direct DNA sequence analysis. Several individuals examined had interspersed patches of proximal and distal CMT1A-REP sequences, providing evidence for gene conversion associated with crossing over during human meiosis. A 456-bp stretch of sequence identity shared by the proximal and distal CMT1A-REPs is located within the interval mapped. This preferred region of exchange may potentially help to define the boundaries for a MEPS in human meiotic recombination. Other stretches of shared identity >200 bp are also in previously documented regions of frequent unequal crossing over between CMT1A-REPs. This is perhaps one of the first times that a correlation has been made between sites of frequent unequal crossing over events and a potential minimal processing segment for efficient meiotic homologous recombination to occur in humans. Similar findings of potential MEPS and gene conversion events were recently obtained through in-depth sequence analysis of the junction accompanying an inversion in the *IDS* locus responsible for a high frequency of patients with the Hunter syndrome (Lagerstedt et al. 1997). Further experiments will be required to define the human MEPS functionally and to determine whether DNA transposons such as the estimated 1,000 *mariner*-like elements in the human genome are involved in initiating double-strand breaks generating homologous recombination hotspots.

Acknowledgments

We thank Els De Vriendt of the Van Broeckhoven laboratory and Jestina Mason in the Child Health Resource Center for their expert technical assistance. We also thank Drs. John Wilson and Susan Rosenberg for their critical review. Special thanks to Vincent Timmerman, Gishlè Smeyers, and the members of the Van Broeckhoven laboratory for their generous hospitality. L.T.R. was partly supported by a travel award from

the Boehringer Ingelheim Fonds to perform experiments in Europe. The research was supported by grants from National Institute of Neurological Disorders and Strokes (RO1NS2742) and Muscular Dystrophy Association of America grants to J.R.L., as well as by grants from the Fund for Scientific Research–Flanders, Belgium and by a Special Research Fund of the University of Antwerp, Belgium, to C.V.B. EV is a research assistant of the F.W.O.

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