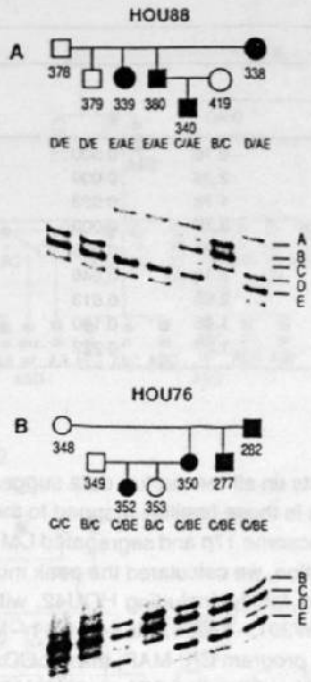

Figure 2. Detection of Three Alleles with the Marker RM11-GT in CMT Patients

$(GT)_n$ genotypes obtained by PCR analysis were scored as described in the legend to Figure 1. The genotypes are indicated below the pedigrees, with the slash indicating the pair of alleles segregating with CMT1A in each nuclear family. Shadow bands that differ from the primary bands in size by multiples of 2 bases are invariably seen with dinucleotide repeat polymorphisms; however, even without special precautions it is possible to read the genotypes unambiguously (Weber, 1990). (A) represents a nuclear family where CMT1A patients 88-338 and 88-340 exhibit three $(GT)_n$ alleles. The patients 88-339 and 88-380 are partially informative with respect to the number of $(GT)_n$ alleles, but the higher intensity of allele E in each of these patients suggests a double dose for this allele. (B) shows inheritance of three alleles in CMT patients from a nuclear family of Ashkenazic Jewish descent, in contrast to the other families, which are of French-Acadian descent.

A $(GT)_n$ Polymorphism at the D17S122 Locus Demonstrates a Duplication Associated with CMT1A

We screened CMT1A-linked 17p DNA probes for the presence of simple sequence repeats such as $(GT)_n$, which are known to be highly polymorphic and can be rapidly analyzed by the polymerase chain reaction (PCR) (Weber and May, 1989; Litt and Luty, 1989). $(GT)_n$ sequences were identified in several probes, one of which, RM11-GT, was identified from VAW409R1 located at the D17S122 locus (Wright et al., 1990). This marker maps to 17p11.2–p12 and is also closely linked to CMT1A (Vance et al., 1991).

The five large French-Acadian pedigrees segregating CMT1A and the two small kindreds of French-Acadian (HOU88) and Ashkenazic Jewish (HOU76) descent were genotyped for RM11-GT. Genotype data from two nuclear CMT1A families within HOU88 and HOU76 are shown in Figure 2. These data demonstrate a striking observation: six of eight CMT1A individuals show three $(GT)_n$ alleles (e.g., individuals 88-340, 76-352), but all unaffected individuals are either homozygous or heterozygous for $(GT)_n$ alleles. In certain matings, only two $(GT)_n$ alleles were segregating and thus only two $(GT)_n$ alleles could be detected in the affected child. However, careful examination of the autoradiograph often revealed that one of the two $(GT)_n$ alleles was present in two copies (e.g., 88-339, 88-380 in Figure 2A). These data indicate that CMT1A patients of French-Acadian (Figure 2A) and Ashkenazic Jewish (Figure 2B) descent have three copies of the D17S122 locus, suggesting a duplication of this locus in CMT1A patients.

Genotypes for RM11-GT for all seven CMT1A pedigrees are shown in Figure 1 and demonstrate that three RM11-GT alleles are present only in affected individuals and are never observed in 53 unaffected offspring and 31 unaffected spouses. The transmission of this duplication is also highly specific. By considering all completely informative RM11-GT matings, such as ABC × DE, we observed 45 cases of transmission of the duplicated allele from affected parents to affected offspring and 18 cases where the affected parent transmitted a single allele to their normal offspring. In these matings, none of the unaffected offspring received the duplicated DNA segment and none of the affected offspring received a single allele from the affected parent. Thus, in 63 fully informative meioses the duplication was faithfully transmitted to the affected offspring and without recombination with the normal chromosome (LOD score, $\hat{Z} = 18.96$ at $\hat{\theta} = 0.0$).

Dosage Differences at an MspI RFLP Detected by Probe VAW409R3 at the D17S122 Locus Confirm the CMT1A-Specific Duplication

The demonstration of three copies of D17S122 in CMT1A patients by $(GT)_n$ allele analysis led us to examine the dosage of polymorphic MspI alleles at this locus. Two MspI restriction fragment length polymorphisms (RFLPs) are detected by the marker D17S122 (Wright et al., 1989; Vance et al. 1991) by Southern blot analysis using 11 kb (VAW409R1) and 2.1 kb EcoRI (VAW409R3) subclones of phage VAW409 as standard two- and three-allele RFLPs, respectively. Dosage differences that followed Mendelian inheritance were observed in CMT1A patients using the probe VAW409R3, as shown in Figure 3.

The MspI genotypes in a nuclear family of pedigree HOU85 are shown in Figure 3A. The unaffected father (85-301) has genotype BB, and his unaffected daughters (85-326 and 85-312) have genotype AB. The affected mother (85-302) and her affected sons (85-303 and 85-304) also have genotype AB, but inspection of the autoradiograph shows clear dosage differences between the two alleles such that 85-302, 85-303, and 85-304 have genotypes AAB, ABB, and ABB, respectively. The VAW409R3 genotypes in Figure 3A also show that the CMT1A chromosome harbors both an A and a B allele and that the AB combination segregates in a Mendelian fashion.

Comparative Southern analysis of eight unrelated CMT1A patients (Figure 3C) and control individuals (Figure 3D) with the probe VAW409R3 is also shown. The most

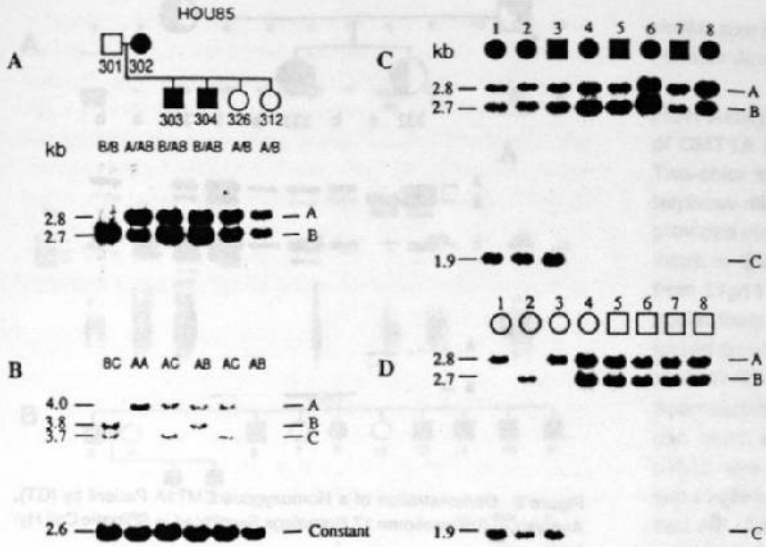

Figure 3. Southern Blot Analysis Demonstrates Dosage Differences of Polymorphic Alleles in CMT1A Patients

(A) Southern analysis of MspI-digested genomic DNA from a nuclear family (HOU85) with the probe VAW409R3 (D17S122). Southern analysis was conducted on 5 μg of genomic DNA as described (Patel et al., 1990a). Squares and circles represent males and females, respectively. Note the difference in the relative intensity of alleles A and B in CMT patients (85-302, 85-303, 85-304) versus unaffected individuals (85-301, 85-312, 85-326).

(B) Southern analysis with a probe from outside the duplication region. The Southern blot from (A) was rehybridized with the control probe 10-5, representing the myosin heavy chain locus in 17p13 (Schwartz et al., 1986; Nakamura et al., 1988). No difference in the intensity of the polymorphic alleles was noted.

(C) Southern analysis of MspI-digested genomic DNA from eight unrelated CMT1A patients with the probe VAW409R3 (D17S122). Note the presence of three polymorphic alleles in lanes 1–3. This genotype clearly illustrates the duplication, but was observed in only 3 of 131 CMT1A patients. Lanes 4–8 show individuals who had two polymorphic alleles and in whom a duplication could be discerned by noting the difference in the relative intensity of one allele when compared to that of the other allele. This Southern blot was rehybridized with a control VNTR probe, YNH24 from chromosome 2 (Nakamura et al., 1987), and showed no difference in the intensity of the polymorphic alleles (data not shown).

(D) Southern analysis of MspI-digested genomic DNA from eight control individuals with the probe VAW409R3. Note the lack of dosage difference between alleles in all individuals.

common examples of informative CMT1A individuals are shown in lanes 4–6 (genotype ABB) and lanes 7 and 8 (genotype AAB). The presence of an extra allele can be noted in individuals of AAB and ABB genotypes by comparing the ratio of the hybridization signal for one allele to the other. Lanes 1–3 in Figure 3C represent CMT1A individuals who were fully informative for the RFLP and demonstrated three polymorphic alleles resulting in a genotype ABC. Three copies of the allele could also be noted in affected individuals of genotype AAA or BBB when the signal from a control probe was used for normalization (data not shown).

To confirm this observation, 103 CMT1A patients from seven families (Figure 1) as well as 26 other unrelated patients were examined by Southern blot analysis with VAW409R3. Dosage of alleles was determined by visual examination and densitometry of autoradiographs or by quantitation of total radioactivity in each allele using a Betascope analyzer (Sullivan et al., 1987). Dosage was determined only in individuals who were heterozygous for the RFLP since the results were most reproducible and reliable in such cases. Seventy-six CMT1A patients were heterozygous for this RFLP and were conclusively demonstrated to have three copies of the D17S122 locus. In contrast, none of 63 controls (27 unaffected at-risk individuals with normal NCV and 36 controls with no family history of CMT) who were heterozygous for this marker showed dosage differences for this RFLP, suggesting that the genotype with dosage differences was specific to CMT1A patients ($\chi^2 = 48.72$; $P < 10^{-5}$). Similar dosage differences were observed with the marker VAW409R1 (data not shown).

Demonstration of Two $(GT)_n$ Alleles in MspI Fragments Showing Dosage Differences

We next demonstrated that the MspI alleles present in two copies by dosage differences in CMT1A patients contain two $(GT)_n$ alleles, using preparative gel separation of the polymorphic alleles (Bedford and van Helden, 1990). MspI alleles revealed by VAW409R1 (D17S122) showing dosage differences in CMT1A patients, and from which the marker RM11-GT was derived, were separated on agarose gels and used as templates for PCR amplification of RM11-GT. The analysis required affected individuals to have three distinguishable $(GT)_n$ alleles and that these individuals be heterozygous for the MspI RFLP.

Figure 4A displays representative data from a nuclear family within kindred HOU42. The unfractionated genomic DNA from these individuals as well as their separated MspI allelic fractions were genotyped for RM11-GT. Figure 4B indicates that in each instance, a patient with a polymorphic allele of double intensity had two $(GT)_n$ alleles, whereas a single $(GT)_n$ allele was evident in the other polymorphic allele showing normal intensity in the patients and in all unaffected individuals.

Homozygosity for the Duplication Mutation in a Severely Affected Individual

A severe clinical phenotype has been previously reported in an individual who was the product of a consanguineous mating between first cousins affected with CMT and hypothesized to represent homozygous expression of a dominant gene for CMT (Killian and Kloepfer, 1979). A small nuclear family within pedigree HOU42 (Figure 1) demonstrated a mating between two affected individuals. One of

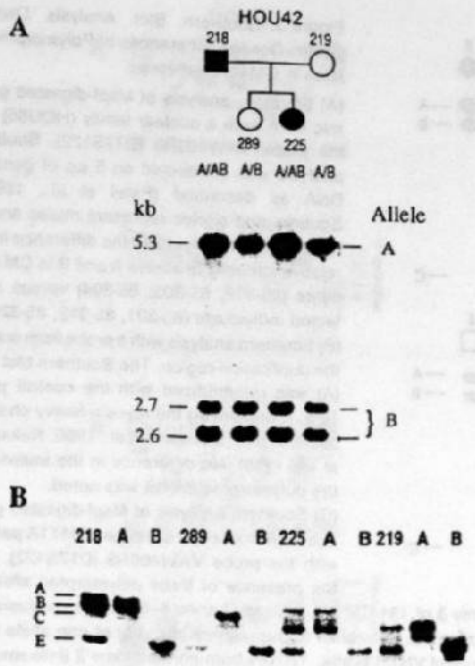

Figure 4. Demonstration of Two $(GT)_n$ Alleles in Polymorphic MspI Fragments at the D17S122 Locus Showing Double Dosage by Allele Separation

(A) A 5.3 kb MspI fragment from within VAW409R1 at the D17S122 locus was hybridized to a Southern blot of MspI-digested DNAs from a nuclear family from HOU42. The RFLP genotypes based on dosage of alleles are indicated at the top of the autoradiograph. Measurement of total counts in each band using the Betascope analyzer (Sullivan et al., 1987) confirmed this visually determined genotype. Note that the affected individuals 42-218 and 42-225 have two copies of the A allele and one copy of the B allele. Examination of Mendelian inheritance in this kindred indicated that the disease segregates with the alleles AB. (B) An agarose gel similar to that in (A) was prepared, and the regions corresponding to alleles A and B, respectively, were cut out and the allelic fractions genotyped for $(GT)_n$ alleles as described in Experimental Procedures. The products obtained with undigested DNA from each individual are shown in the lanes identified by the identification number of the individual, and those obtained from the corresponding A and B alleles are shown in the lanes marked A and B. Note that the A allele of individual 42-218 and 42-225, which is present in two copies, shows two $(GT)_n$ alleles while all other alleles are present in a single copy and show one $(GT)_n$ allele.

the two affected offsprings of this mating (42-333) demonstrated a severe clinical phenotype including early onset (<1 year) and markedly reduced motor NCV (~10 m/s vs. affected 20–40 m/s; unaffected >40 m/s). Examination of the segregation of 17p markers in HOU42 demonstrated that individual 42-333 had inherited two CMT1A chromosomes. The $(GT)_n$ alleles A and E segregate with CMT1A in the families of both the affected mother and affected father. The $(GT)_n$ genotype of individual 42-333 is AE and suggests that she inherited a CMT1A chromosome from each of her parents. Her sister, 42-332, has inherited one chromosome with the duplication genotype AE and has a less severe clinical phenotype.

For further confirmation, somatic cell hybrids retaining individual chromosome 17 homologs from patient 42-333, her affected mother (42-331), and her affected sister (42-332) were constructed. These hybrids were genotyped for RM11-GT, and the results are shown in Figure 5A. They confirm the following: first, patients 42-331 and 42-332 are heterozygous for the chromosome carrying the duplication; and second, patient 42-333 is homozygous for the duplication, and each chromosome 17 homolog contains two copies of the D17S122 locus. This nuclear family lends support to the hypothesis that the duplication is responsible for the clinical phenotype of CMT1A and that CMT1A is a semidominant mutation, since homozygosity for the duplication results in a more severe clinical phenotype.

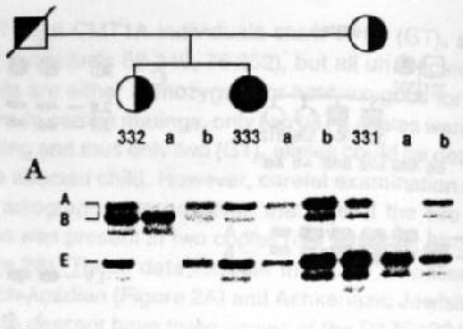

Figure 5. Demonstration of a Homozygous CMT1A Patient by $(GT)_n$ Analysis of Chromosome 17 Homologs Separated in Somatic Cell Hybrids

The chromosomes 17 of patients 42-332 and 42-333, offspring of a mating between two affected individuals, and of their affected mother, 42-331, were isolated in somatic cell hybrids as described in Experimental Procedures. Positive clones from each fusion were screened for the identity of the chromosome(s) 17 retained by PCR analysis of the cell lysate with primers to a polymorphic marker within the gene for the β subunit of the muscle acetylcholine receptor locus in 17p. Lysates from clones retaining each of the two chromosome 17 homologs were analyzed for the $(GT)_n$ polymorphism at the D17S122 locus. The results of this analysis are shown in (A), where the numbered lanes refer to the products obtained from the respective patients' DNA and the letters a and b identify lanes showing amplification products from the corresponding pair of hybrids, each retaining a chromosome 17 homolog from the respective patient. (B) shows the amplification products obtained with primers from the acetylcholine receptor β subunit gene polymorphic locus in 17p outside the duplication region using DNA from patients 42-332 and 42-333 and the corresponding hybrids illustrating the successful separation of the chromosome 17 homologs. The disease segregates with the $(GT)_n$ alleles A and E in the families of both the mother and the father of patient 42-333, who is homozygous for the disease chromosome. The pedigree symbols reflect the scoring of the genotype with respect to the disease allele.

PFGE Analysis Identifies a Novel SacII Fragment in CMT1A Patients

To define this duplication more precisely and obtain an estimate of its size, we performed long-range restriction mapping using pulsed-field gel electrophoresis (PFGE) (Schwartz and Cantor, 1984). The restriction enzymes NotI, MluI, SacII, and NruI were used to digest DNA from

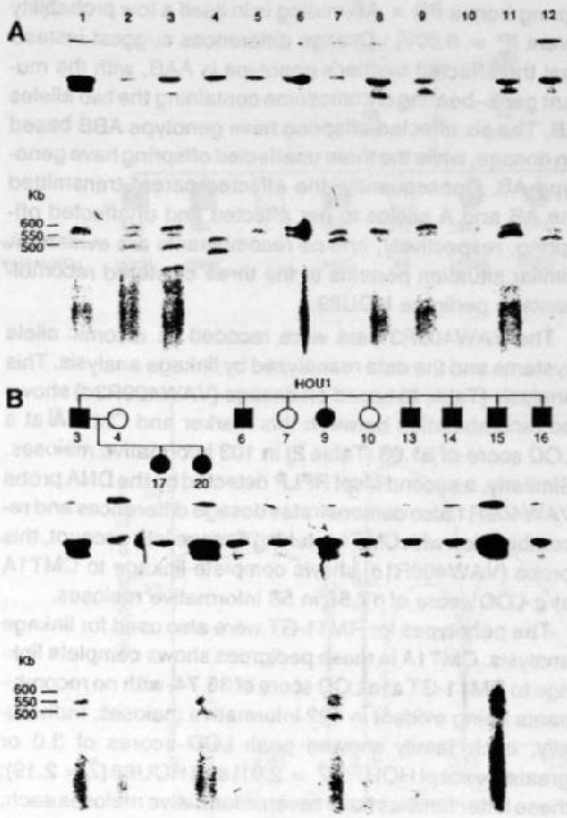

Figure 6. An Additional SacII Allele Is Identified in CMT1A Individuals by PFGE

(A) Lymphoblasts from five CMT1A patients (lanes 1–5) and seven unaffected control individuals (lanes 6–12) were used for preparation of plugs as described (Westerveld et al., 1971). Approximately one-fifth of each plug (4 μg of DNA) was digested with SacII and electrophoresed in a CHEFII-DR PFGE apparatus (Bio-Rad) for 24 hr in 0.5 × TBE buffer using pulse times of 50–90 s ramp at 200 V. The Southern blot was hybridized with the probe VAW409R3 as described (Patel et al., 1990a) with the exception that 0.5 mg/ml human placental DNA was used for preassociation of repeats in the probe. The patients used were individual 76-270, 76-272, 42-332, 42-333, and 42-286 in lanes 1 through 5, respectively. The additional PFGE fragment of approximately 350 kb in patient 76-272 is sometimes faintly visible in other lymphoblastoid cell lines and may represent a methylation artifact. It does not demonstrate Mendelian inheritance. Note that lane 4 shows the pattern for the homozygous patient 42-333.

(B) PFGE plugs were prepared from lymphocytes isolated from the whole blood of related affected and unaffected individuals. They were digested with SacII and electrophoresed, and the resulting Southern blot was hybridized as described above. Note the Mendelian inheritance of the novel 500 kb SacII allele in affected individuals.

affected and control individuals to identify altered and/or novel fragments in CMT1A patients. Two SacII fragments of 600 kb and 550 kb, which are either polymorphic alleles or variants arising as a result of methylation differences, were seen in 16 control individuals using VAW409R3 as a probe (Figure 6A, lanes 6–12, and further data not shown). However, a novel 500 kb SacII fragment was seen in CMT1A patients of French-Acadian and Ashkenazic Jewish origin (Figure 6A), and this SacII fragment showed Mendelian inheritance (Figure 6B). These results suggest the presence of a large genomic DNA rearrangement of similar size in CMT1A patients of French-Acadian and Ashkenazic Jewish origin.

FISH Analysis Reveals a Duplication in Nuclei of CMT1A Patients

Two-color fluorescence in situ hybridization (FISH) in interphase nuclei (Lawrence et al., 1990; Trask et al., 1991) provided direct visualization of duplication of the VAW409 locus in CMT1A patients. VAW409 and a control probe from 17p11.2 (c1516) were hybridized in a blind study to nuclei from CMT1A patients 2-440 and 42-331 and unaffected controls 42-289 and 76-271. The hybridization sites of VAW409 and c1516 were labeled with red and green fluorochromes, respectively. Because DNA replication can result in double hybridization signals in interphase, c1516 was included to identify cells that contained only two single hybridization sites for this probe and, therefore, had not replicated the CMT1A region.

A total of three red VAW409 sites (two near one of the c1516 sites and one paired with the second c1516 site) were observed in the majority of these cells from the CMT1A patients (60% and 59% in 2-440 and 42-331, respectively) but in few cells from unaffected individuals (3% and 6% in 42-289 and 76-271, respectively). In contrast, only one VAW409 hybridization site was paired with each single c1516 site in the majority of cells from unaffected individuals (90% and 79% in 42-289 and 76-271, respectively). The nuclei from the homozygous patient 42-333 were similarly subjected to FISH analysis and demonstrated a total of four red VAW409 sites, two paired with each green c1516 site (Figure 7). Lymphoblasts from an additional three CMT1A patients and four control individuals, for a total of six patients including one from the Ashkenazic Jewish family and six controls, were included in a blind study to determine the relative number of hybridization sites of VAW409 and c1516. In each case, the presence of a duplicated region in CMT1A patients was confirmed. This study demonstrates that duplications can be readily detected in interphase nuclei using FISH.

Consequences of the Duplication on Linkage Analysis for CMT1A Gene Localization

Genetic mapping in the CEPH reference families (Dausset, 1986) localizes probe VAW409 between A10-41 and MYH2 at a distance of 1.3 cM from A10-41. In Table 2, LOD scores between CMT1A and polymorphisms detected by probe VAW409 are presented. When scored as disomic allelic systems, the recombination value between CMT1A and VAW409R3 is 7.3% and surprisingly higher than the other closely linked CMT1A markers. There were six recombinants with this probe, three each in families HOU85 (85-312, 85-320, 85-326) and HOU89 (89-342, 89-343, and 89-344). These recombinants were surprising since they were clustered and greater in number than the five previously detected with other 17p markers spanning 9.9 cM. The observation of dosage differences detected by VAW-409 clarified not only the occurrence of a DNA duplication, but also that failure to account for this duplication in linkage analyses produces false recombinants.

This important phenomenon is illustrated in Figure 8A

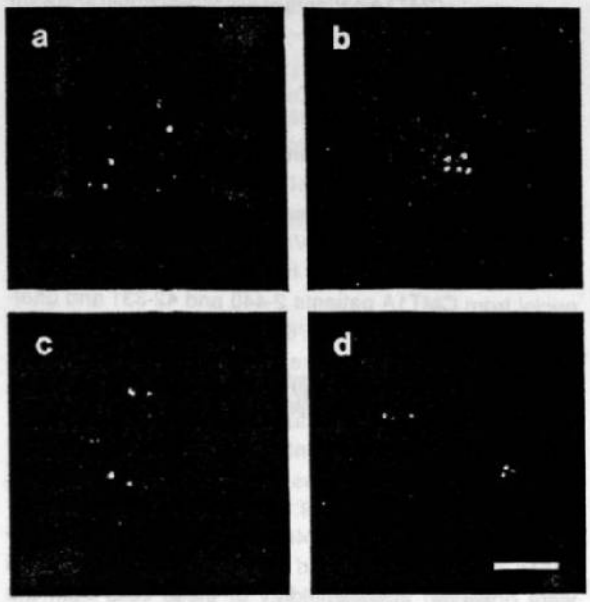

Figure 7. FISH Analysis of Interphase Nuclei from CMT1A and Normal Individuals with VAW409 and c1516

Four lymphoblastoid cell lines were analyzed in a blind study by FISH as described (Trask et al., 1991). Interphase nuclei preparations were hybridized simultaneously with biotinylated probes VAW409R1 and VAW409R3 and digoxigenin-labeled cosmid c1516, which maps to 17p11.2. The hybridization sites of VAW409 and c1516 were labeled with Texas red and fluorescein, respectively, and viewed together through a double band-pass filter. The hybridization pattern of c1516 was used as an internal assay for the replication status of the proximal 17p region. The nuclei shown are representative of the predominant hybridization pattern observed in each sample in terms of the relative number of red and green sites. The difference in the hybridization pattern of patient and control samples was not due to differences in hybridization efficiency; the fraction of nuclei lacking a VAW409 signal paired with one or both c1516 sites was similar in all cell lines (9%–17%). (a) and (b) represent CMT1A patients 2-440 and 42-331, respectively; (c) represents a normal control, 42-289; (d) represents the homozygous CMT1A patient 42-333. Bar = 5 μm.

with the VAW409R3 MspI RFLP data from a nuclear family from HOU85. If dosage differences are ignored, the affected mother has genotype AB with the A chromosome carrying the CMT1A mutant gene; the unaffected father is BB. Since all nine offspring are AB but six are affected and three are unaffected, the unaffected individuals are recombinant. (Note that the segregation of nine AB offspring from a BB × AB mating is in itself a low probability event [P = 0.002].) Dosage differences suggest instead that the affected mother's genotype is AAB, with the mutant gene–bearing chromosome containing the two alleles AB. The six affected offspring have genotype ABB based on dosage, while the three unaffected offspring have genotype AB. Consequently, the affected parent transmitted the AB and A alleles to her affected and unaffected offspring, respectively, and no recombinants are evident. A similar situation pertains to the three clustered recombinants in pedigree HOU89.

The VAW409R3 data were recoded as trisomic allele systems and the data reanalyzed by linkage analysis. This analysis (Table 2) based on dosage (VAW409R3d) shows no recombination between this marker and CMT1A at a LOD score of 31.08 (Table 2) in 103 informative meioses. Similarly, a second MspI RFLP detected by the DNA probe VAW409R1 also demonstrates dosage differences and recombination with CMT1A; taking dosage into account, this probe (VAW409R1d) shows complete linkage to CMT1A at a LOD score of 17.56 in 58 informative meioses.

The genotypes for RM11-GT were also used for linkage analysis. CMT1A in these pedigrees shows complete linkage to RM11-GT at a LOD score of 36.74, with no recombinants being evident in 122 informative meioses. Individually, each family showed peak LOD scores of 3.0 or greater, except HOU76 ($\hat{Z}$ = 2.01) and HOU88 ($\hat{Z}$ = 2.19); these latter families have seven informative meioses each. Thus, taking dosage differences into account at VAW-409R1, VAW409R3, and RM11-GT, locus D17S122 shows complete linkage to CMT1A.

Multipoint linkage analysis of CMT1A using the map A10-41—(1.3 cM)—RM11-GT—(11.7 cM)—MYH2 was then performed using the program LINKAGE to calculate confidence limits on the location of CMT1A. The peak multipoint LOD score was 34.5; the CMT1A locus had the maximum likelihood position at RM11-GT, between A10-41 and MYH2. All other intervals were excluded with odds of 10^{12}:1 or greater. The approximate 95% confidence limits on the CMT1A location defined a 3 cM interval containing the probe RM11-GT. A more extensive analysis using the markers LEW301—YNM67-R5—A10-41—RM11-GT—MYH2 and the program CRI-MAP verified the placement of CMT1A at locus RM11-GT and between the probes A10-41 and MYH2 with odds exceeding 1000:1.

Table 2. LOD Scores between DNA Markers within the Duplication Mutation and CMT1A

	Recombination Value							
Marker	0.00	0.05	0.10	0.20	0.30	0.40	$\hat{\theta}$	$\hat{Z}$
409R3	−∞	6.75	6.76	5.60	3.89	1.98	0.073	6.86
409R3d	31.08	28.26	25.35	19.23	12.67	5.70	0.000	31.08
409R1	−∞	5.34	4.81	3.42	1.99	0.72	0.027	5.46
409R1d	17.56	15.86	14.21	10.55	6.86	3.10	0.000	17.56
RM11-GT	36.74	31.76	29.96	22.67	14.85	6.47	0.000	36.74

LOD scores at the D17S122 marker locus. 409R1 and 409R3 refer to the MspI RFLPs scored without dosage between alleles, the suffix "d" refers to scoring of VAW409 MspI RFLPs with dosage, and RM11-GT refers to the $(GT)_n$ repeat polymorphism. The RM11-GT linkage analysis also includes HOU76 and HOU88.

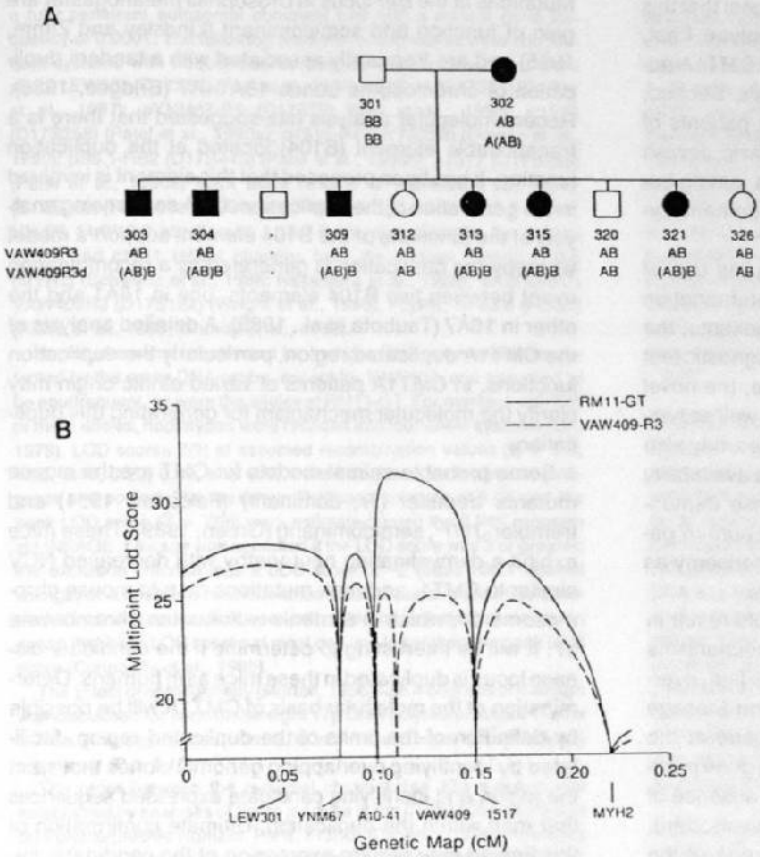

Figure 8. Consequences of Duplication Mutation on Linkage Analysis

(A) A nuclear family of pedigree HOU85 showing the misclassification of the VAW409R3 MspI RFLP. Shown below the pedigree symbol in descending order are the identification number of the individual and the VAW409R3 genotype scored without and with consideration of dosage of the alleles, respectively. Segregation of marker alleles demonstrates that individual 85-302 carries the A allele on the CMT1A chromosome if dosage is ignored but the AB allele on the CMT1A chromosome if dosage is considered. Individuals 85-312, 85-320, and 85-326 appear as recombinants with VAW-409R3; however, VAW409R3d shows that this is due to misclassification.

(B) Multipoint linkage mapping of CMT1A on a genetic map of chromosome 17p. The locus positions of the markers are indicated on the horizontal axis. The height of the curves represents the relative likelihood of location (LOD score) at any specified point along the map. When dosage differences are ignored (VAW-409R3), the most likely position of the CMT1A gene is proximal to LEW301; however, the RM11-GT locus data clearly place CMT1A at RM11-GT.

The failure to account for dosage differences at a two-allele RFLP in linkage analysis, when it exists, leads to misinterpretation of the parental origin of alleles, as shown in Figure 8A. These errors appear as multiple, clustered (within sibships) recombination events that reduce the LOD score and increase the recombination value between the disease and the marker. More importantly, when these errors are included, multipoint linkage analysis can seriously distort the positioning of the disease locus. This dramatic effect is shown in Figure 8B, where we present the multipoint LOD score for CMT1A versus a fixed map of the markers LEW301—YNM67-R5—A10-41—VAW409R3/RM11-GT—1517—MYH2. Figure 8B shows the multipoint LOD score curve for two analyses using CRI-MAP that are identical except that VAW409 was first coded as a two-allele RFLP without dosage (VAW409R3) and a second time using the RM11-GT polymorphism. Using the $(GT)_n$ polymorphism, the multipoint LOD score is 31.4 and correctly places the CMT1A locus at VAW409. The 95% confidence limits on the CMT1A location define a 3 cM interval around the $(GT)_n$ locus. On the other hand, ignoring the duplication produces a peak LOD score of 26.81 and incorrectly places CMT1A 1 cM proximal to LEW301. The 95% confidence limits on this location define a 6 cM interval around LEW301. Not only do these two confidence intervals fail to overlap, but the two LOD scores have an odds difference of $10^{4.6}$! Furthermore, the correct location of CMT1 at VAW409 is 50 times less likely than the incorrect location when dosage at VAW409R3 is not scored. The misclassification leads to multiple recombination events with VAW409 and thus places the CMT1A locus toward LEW301, with which no recombinants were observed.

Discussion

We have demonstrated that CMT1A is associated with a DNA duplication using $(GT)_n$ polymorphism and RFLP analysis, FISH analysis, and isolation of parental chromosomes in somatic cell hybrids. Three polymorphic markers at the D17S122 locus have displayed this duplication, namely, VAW409R3, VAW409R1, and RM11-GT, and in each case there is a perfect correlation between the duplication genotype and the CMT1A disease phenotype. PFGE suggests that the duplication includes a large genomic region. We have shown that failure to understand the molecular nature of the polymorphism leads to the mislocalization of CMT1A and reduced evidence for linkage. Preliminary data by RFLP analysis and dosage of polymorphic alleles indicate that two additional markers, VAW-412R3 (D17S125) and EW401 (D17S61) (Wright et al., 1990), which are linked to VAW409, may also be duplicated, while other CMT1A-linked markers do not appear to show evidence for duplication.

The demonstration of an autosomal dominant inherited mutation involving DNA duplication in multiple families is

unprecedented. Several lines of evidence suggest that this duplication is responsible for the CMT1A phenotype. First, the duplication mutation was observed only in CMT1A patients and not observed in 63 control individuals. Second, the duplication was demonstrated in CMT1A patients of French-Acadian descent as well as Ashkenazic Jewish origin. Third, a severely affected offspring of a mating between two affected individuals was shown to be homozygous for the duplication.

An important consequence of our study is the use of RM11-GT for CMT1A diagnosis. With the determination of dosage at the D17S122 locus in CMT1A patients, the positive predictive value of this DNA-based diagnostic test is likely to increase dramatically. Furthermore, the novel SacII fragment observed by PFGE analysis as well as two-color FISH of lymphoblasts or fresh lymphocytes may also be useful diagnostic methods for CMT1A. The availability of highly polymorphic markers similar to those demonstrated in this study from most regions of the human genome may enable the detection of segmental trisomy as the molecular basis for other human diseases.

The mechanism by which a duplication could result in the CMT phenotype is unknown, but possible mechanisms for the disease phenotype include the following: first, overexpression of one or more genes in the region (dosage effect); second, interruption of a candidate gene at the duplication junction leading either to an altered gene product with a dominant deleterious effect or to an absence of the gene product, thus resulting in decreased levels; third, occurrence of a stable dominant mutation in one of the duplicated candidate genes that results in a gene product with a deleterious effect; and fourth, a change in the physical location of the gene(s) within the duplication region, leading to altered regulation of gene expression, secondary to a position effect. The human gene for the β subunit of the muscle nicotinic acetylcholine receptor has recently been mapped to the 17p11.2–p12 region (Beeson et al., 1990). This receptor plays an important role in signal transduction at the neuromuscular junction. Using a highly polymorphic marker within this gene (OS1-βGT), we have demonstrated that it lies outside the duplication region (data not shown). However, the genes for the subunits of the neuronal acetylcholine receptor tend to locate in clustered arrays (Boulter et al., 1990). It is thus possible that altered expression of one or more of such receptor subunits could result in an altered stoichiometry of the subunits and lead to CMT.

The mechanism by which the duplication mutation arose is unknown. De novo mutations that include deletions and duplications have been observed in the proximal region of the short arm of chromosome 17 (Smith et al., 1986; Stratton et al., 1986; Magenis et al., 1986). It is possible that the same recombination mechanisms that result in microdeletion on one chromosome homolog can result in duplication on the reciprocal homolog. Recent studies on chromosomal duplications in Escherichia coli and humans have demonstrated that duplication junctions occur in regions containing repetitive extragenic palindromic (REP) sequences (Shyamala et al., 1990) as well as near Alu sequences (Kornreich et al., 1990; Devlin et al., 1990). Mutations at the *Bar* locus in Drosophila melanogaster are gain of function and semidominant (Lindsley and Zimm, 1985) and are frequently associated with a tandem duplication of chromosome bands 16A1–A7 (Bridges, 1936). Recent molecular analysis has suggested that there is a transposable element (B104) located at the duplication junction. It has been proposed that this element is involved in the generation of the duplication. DNA sequencing analysis of the junctions of the B104 element support a model whereby the duplication is generated by a recombination event between two B104 elements, one in 16A1 and the other in 16A7 (Tsubota et al., 1989). A detailed analysis of the CMT1A duplicated region, particularly the duplication junctions, in CMT1A patients of varied ethnic origin may clarify the molecular mechanism for generating this duplication.

Some probable animal models for CMT are the mouse mutants trembler (*Tr*, dominant) (Falconer, 1951) and trembler-J (Tr^J, semidominant) (Green, 1989). These mice exhibit a demyelinating neuropathy with decreased NCV similar to CMT1, and their mutations map to mouse chromosome 11, which is syntenic with human chromosome 17. It will be interesting to determine if the candidate disease locus is duplicated in these mice as in humans. Determination of the molecular basis of CMT1A will be possible by definition of the limits of the duplicated region, facilitated by identifying overlapping genomic clones that span the region and identifying candidate expressed sequences that map within the duplication. Ultimate confirmation of this finding may require expression of the candidate mutated region in transgenic mice and observation of the phenotype.

Experimental Procedures

Clinical Evaluation and Sampling of Families

All available at-risk members of pedigrees were subjected to a thorough clinical and electrophysiological examination. In pedigree HOUT, NCVs were initially determined only for clinically affected individuals. Further evaluation indicated that the clinically unaffected individuals 1-13, 1-37, and 1-38 had abnormal NCVs; therefore, the disease status of these individuals is different from that reported in the original pedigree (Patel et al., 1990a). In all other pedigrees at-risk individuals, whether clinically affected or unaffected, had motor NCVs determined. Diagnosis of CMT1 was established by slowed median and ulnar motor NCVs bilaterally (<40 m/s). A single normal motor NCV of the peroneal nerve excluded the diagnosis of CMT1 in patients 5 years or older. Blood was collected from each participating family member, under informed consent, and used to establish EBV-transformed lymphoblasts (Anderson and Gusella, 1984) and for isolating high molecular weight DNA (Miller et al., 1988). The variable numbers of tandem repeat locus YNH24 (D2S44) (Nakamura et al., 1987a, 1987b), in addition to the marker loci used in linkage analysis, were used to check parental origins for each individual in the seven pedigrees in Figure 1. Some parental exclusions were detected; these individuals were not incorporated in the linkage analysis.

Linkage Analysis

The chromosome 17p markers comprise 17 standard RFLPs and were detected using nine DNA probes and Southern analysis as previously described (Patel et al., 1990a, 1990b). The DNA probe FcγRII (chromosome 1q) was studied by Southern analysis to exclude linkage to chromosome 1. LOD score analysis used two point or multipoint methods (Morton, 1956; Ott, 1985; Lathrop and Lalouel, 1988) and the computer programs LINKAGE version 4.7 (Lathrop and Lalouel, 1988) and CRI-MAP version 2.4 (Donis-Keller et al., 1987). CMT1 was considered as

a fully penetrant autosomal dominant trait with a mutant gene frequency of 0.0001. The following markers, where alleles were codominant systems with frequencies as described in the literature, were used: LEW301 (D17S58) (Fain et al., 1987), pA10-41 (D17S71) (Barker et al., 1987); pYNM67-R5 (D17S29) (Ray et al., 1990); c1516 (D17S258) (Patel et al., 1990a); p1516-R4 (D17S258) (Franco et al., 1990); pS6.1-HB2 (D17S445) (Patel et al., 1990b); c1517 (D17S259) (Patel et al., 1990a) MspI allele lengths = 6.2/4.0/2.4 kb; c1541 (D17S260) (Patel et al., 1990a) BglII allele lengths = 3.4/2.0 + 1.4 kb; HindIII 14.0/13.0 kb; BamHI 11.0/7.6 kb (allele lengths for probes c1517 and c1541 reflect changes from the original report); p10-5 (MYH2) (Schwartz et al., 1986; Nakamura et al., 1988); VAW409R1, VAW409R3 (D17S122) (Wright et al., 1990); FcγRII, HFc3.0 (FCG2) (Hibbs et al., 1988; Grundy et al., 1989).

Haplotypes were constructed for multiple DNA polymorphisms detected by the same DNA probe, except for VAW409, and assumed to be equifrequent, as were the alleles at RM11-GT. For markers with five or more alleles, haplotypes were recoded into four-allele systems (Ott, 1978). LOD scores $Z(\theta)$ at assumed recombination values (θ) of 0.0, 0.05, 0.10, 0.20, 0.30, and 0.40 were calculated for individual pedigrees and pooled. The maximum likelihood estimate of θ ($\hat{\theta}$) and the peak LOD score ($\hat{Z} = Z(\hat{\theta})$) were estimated using the ILINK program in LINKAGE. Linkage was accepted if the LOD score was 3 or greater; the exclusion criterion was a LOD score of −2 (odds 100:1 against linkage) at a specified recombination value. Approximate 95% confidence intervals on location were calculated by including all points on a map that have LOD scores at most one unit lower than the peak LOD score (Conneally et al., 1985).

The χ^2 test of homogeneity (Morton, 1956) with 4 degrees of freedom was calculated for each of the eight 17p DNA polymorphisms in Table 1. The χ^2 values ranged from 0.00 to 7.14 and were not statistically significant ($P > 0.10$).

The order between the proximal chromosome 17p markers was established by analyses of these markers in the CEPH (Centre d'Etude du Polymorphisme Humain, Paris, France [Dausset, 1986]) reference families, and from analyses of somatic cell hybrids (Patel et al., 1990a). The distances between adjacent markers in centimorgans were estimated from the CEPH panel (P. Fain, personal communication, 1991) except for 1517, whose distance from YNM67-R5 was estimated from the five CMT1A kindreds described in this paper. The map is as follows: LEW301—2.6 cM—[YNM67-R5, 1516]—1.8 cM—[A10-41, S6.1-HB2]—1.3 cM—VAW409—4.2 cM—1517—7.5 cM—MYH2, where [. . .] indicates markers for which the order is unknown. CRI-MAP is more efficient in likelihood calculations than LINKAGE, since it ignores population allele frequencies and the genotypes of specific individuals in analyses. In comparing the results of identical analyses in these five kindreds using both CRI-MAP and LINKAGE, a 20% information loss was observed for two-point LOD scores but only a 4% loss for multipoint LOD scores. For efficient calculations, only CRI-MAP was used for multipoint analysis.

Detection of $(GT)_n$ Polymorphic Markers and Genotype Determination

$(GT)_n$ repeat sequences were identified by Southern hybridization of dot blots of the plasmid or cosmid DNA to synthetic nick-translated poly(dC-dA)·poly(dG-dT) (Pharmacia) using [α-^{32}P]dCTP (New England Nuclear). Hybridizations were performed in 1 M NaCl, 1% SDS, 10% dextran sulfate at 65°C, and the filters were washed at room temperature in 2× SSC, 0.1% SDS. A $(GT)_n$ repeat sequence was identified in an 11 kb EcoRI fragment cloned in pUC18 (VAW409R1). A 250 bp HaeIII fragment contained the $(GT)_n$ repeat and was further subcloned into pTZ19 (pRM11-GT) and sequenced by the dideoxy chain-termination method (Sanger et al., 1977) using the Sequenase Kit (United States Biochemical Corporation). The repeat sequence present in pRM11-GT was $(TA)_3(GT)_{17}(AT)_6$. Analysis of 83 unrelated individuals identified at least eight different alleles, ranging in size from 153 bp to 167 bp, with an observed heterozygosity of 74%.

For PCR amplification either the GT strand (CAGAACCACAAAATG-TCTTGCATTC) or CA strand (GGCCAGACAGACCAGGCTCTGC) oligonucleotide primer flanking the $(GT)_n$ repeat sequence was end-labeled at 37°C in a 15 μl reaction volume containing 1.2 μM primer, 100 μCi of [γ-^{32}P]ATP at 6000 Ci/mmol, 1× One Phor-All Plus buffer (Pharmacia), and 10 U of polynucleotide kinase (Pharmacia). The kinase was inactivated at 65°C for 10 min and the primer used directly in the PCR reaction (0.4 μl per reaction). PCR was performed using standard conditions in a 25 μl reaction volume in a mixture containing 1 μM each oligodeoxynucleotide primer, 250 μM each dATP, dCTP, dGTP, and dTTP, 2.5 μl of 10× PCR buffer (500 mM KCl, 120 mM Tris–HCl [pH 8.0], 1.5 mM $MgCl_2$, and 0.01% gelatin), 0.63 U of Ampli-Taq (Cetus) DNA polymerase, and 0.4 μl of end-labeled GT primer reaction mix. The amplification conditions were an initial denaturation at 94°C for 5 min followed by 30 cycles of 94°C denaturation for 1 min, 55°C annealing for 1 min, and 72°C extension for 2 min in an automated thermal cycler (Perkin-Elmer/Cetus). Reaction products (1.5 μl) were mixed with 2 μl of formamide stop solution (United States Biochemical Corporation) and electrophoresed in a 6% polyacrylamide DNA sequencing gel at 40 W for 3.5 hr. Gels were dried and autoradiographed for 2–12 hr by exposure to Kodak XAR-5 film at −70°C.

Southern Analysis and Dosage Determination

Samples (5.5 μg) of genomic DNA were digested with 3–4 U of the appropriate restriction endonuclease under conditions specified by the manufacturer. A 0.5 μg aliquot was examined by gel electrophoresis to determine completeness of digestion. The digested DNAs were electrophoresed in a 1% agarose gel in 1× TAE buffer (40 mM Tris–HCl [pH 8.5], 40 mM sodium acetate, 2 mM EDTA) for ~16 hr. The DNA was transferred to a nylon membrane (Sureblot, Oncor) and hybridized to the probe after preassociation of repeats as described previously (Patel et al., 1990b). Dosage of alleles was determined by visual inspection of autoradiographs and comparison of the intensity of one polymorphic allele to the other within each lane. Alternatively, such comparisons were made on autoradiographs using a densitometer (LKB Ultrascan) or by direct quantitation of radioactivity in the polymorphic alleles on the nylon membrane using the Betascope analyzer (Betagen) (Sullivan et al., 1987).

Allele Separation for PCR Analysis

Samples (5 μg) of genomic DNA from members of a nuclear family in HOU42 were digested with MspI and electrophoresed in a 1% agarose gel in 1× TAE buffer at 20 V overnight to allow separation of 3 kb and 6 kb alleles. The gel was sliced to isolate these fractions in a minimal volume, and the DNA was purified using Geneclean (BIO101). Approximately 1/30th of the isolated DNA was subjected to PCR analysis with the RM11-GT primers as described before.

Construction and Analysis of Somatic Cell Hybrids

Somatic cell hybrids were used to separate the maternal and paternal chromosomes 17 of individuals 42-331, 42-332, and 42-333. Hybrids were constructed as described by Zoghbi et al. (1989) using a23, a thymidine kinase–deficient Chinese hamster cell line (Westerveld et al., 1971) as the rodent parent. Briefly, two 100 mm plates were seeded with 10^7 a23 cells per plate 16–20 hr before fusion. The cells were washed with Dulbecco's modified Eagle's medium (DMEM). To a 10 ml suspension of 5 × 10^7 lymphoblasts in Hanks' balanced salt solution (GIBCO), 250 μl of a 1 mg/ml phytohemagglutinin (Sigma) solution was added. Five milliliters of this cell suspension was added to each plate of a23 cells, and the plates were incubated for 15 min at 37°C. The solution was aspirated, and 2 ml of 50% polyethylene glycol 1500 (Boehringer Mannheim Biochemicals) was spread over the surface of the plate. After 1 min the polyethylene glycol was aspirated, and the cells were washed three times with DMEM and incubated with 10 ml of DMEM for 30 min at 37°C. The medium was aspirated and the plates were incubated overnight with 10 ml of DMEM with 10% fetal calf serum (FCS). Hybrids were selected by growth in DMEM containing 10% FCS, 0.1 mM hypoxanthine, 0.001 mM aminopterin, and 0.01 mM thymidine. Hybrids were isolated with cloning rings 10–14 days later and transferred to 24-well microtiter plates.

For analysis of the hybrids, cells from each confluent well were collected and lysed by boiling in 30 μl of 1× PCR buffer. Three microliters of the lysate was used for PCR amplification with primers flanking a $(GT)_n$ repeat (OS1-βGT) at the locus for the gene for the β subunit of the nicotinic acetylcholine receptor in 17p11.2. The sequence of the GT strand primer is AACTTTACTACAGGAGTTACACCC, and that of the CA strand primer is CTCGAGCCCCCGCATTCAAGAA. The PCR was conducted as described before using 3 μl of the cell lysate or

<100 ng of genomic DNA from the individual patients. The successful separation of the chromosome 17 homologs in hybrids was noted by comparison of the $(GT)_n$ allele in each hybrid to that of the corresponding human parent.

PFGE

Lymphoblasts were used for preparation of plugs as described (Herrmann et al., 1987). Briefly, exponentially growing lymphoblasts were collected and counted using a hemacytometer. The cells were resuspended at 1×10^7/ml in lysis buffer I (0.1 M EDTA, 0.02 M NaCl, 0.01 M Tris-HCl [pH 7.8]), and an equal volume of 1% Incert agarose (FMC Corporation) was added. The mixture was aliquoted into plug molds kept on ice. The plugs were suspended in lysis buffer II (lysis buffer I with 1.0% N-lauroylsarcosine and 2 mg/ml proteinase K). The digestion was carried out at 50°C for 48 hr. The plugs were dialyzed extensively against 10 mM Tris-HCl (pH 7.5), 1 mM EDTA. Approximately one-fifth of each plug (4 μg of DNA) was digested with ~20 U of restriction endonuclease in a 150 μl volume and electrophoresed in a CHEFII-DR PFGE apparatus (Bio-Rad) for 24 hr in 0.5× TBE buffer using pulse times of 50–90 s ramp at 200 V. The gel was transferred to a nylon membrane, and the Southern blot was hybridized with the probe VAW409R3 as described above with the exception that 0.5 mg/ml human placental DNA was used for preassociation of repeats in the probe.

FISH

Two-color FISH was performed as described previously (Trask et al., 1991). Briefly, VAW409R1 and VAW409R3 were combined and biotinylated using a nick translation kit (BRL). The cosmid c1516 was similarly labeled with digoxigenin (Boehringer Mannheim). The probes were mixed and hybridized to nuclei from post-log phase but unsynchronized lymphoblasts fixed on slides after hypotonic swelling and methanol-acetic acid fixation. After hybridization, hybridization sites of biotinylated and digoxigenin-labeled probes were labeled with Texas red and fluorescein, respectively, by sequential incubation of slides, alternated with wash steps, in avidin-Texas red, biotinylated goat anti-avidin and sheep anti-digoxigenin antibodies; and avidin-Texas red and fluoresceinated rabbit anti-sheep IgG antibodies. Slides were viewed on a Zeiss Axiophot microscope (100× magnification) through a dual band-pass filter (Omega, Brattleboro, VT), which allows fluorescein and Texas red to be viewed simultaneously. Slides were coded before analysis. Nuclei were scored randomly for the number of red and green hybridization sites on each chromosome. Photographs of representative nuclei were taken on 3M Scotch 640T color slide film (15–20 s exposures).

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Somatic Cell Hybrids, Sequence-Tagged Sites, Simple Repeat Polymorphisms, and Yeast Artificial Chromosomes for Physical and Genetic Mapping of Proximal 17p

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Somatic cell hybrids retaining the deleted chromosome 17 from 15 unrelated Smith–Magenis syndrome (SMS) [del(17)(p11.2p11.2)] patients were obtained by fusion of patient lymphoblasts with thymidine kinase-deficient rodent cell lines. Seventeen sequence-tagged sites (STSs) were developed from anonymous markers and cloned genes mapping to the short arm of chromosome 17. The STSs were used to determine the deletion status of these loci in these and four previously described human chromosome 17-retaining hybrids. Ten STSs were used to identify 28 yeast artificial chromosomes (YACs) from the St. Louis human genomic YAC library. Four of the 17 STSs identified simple repeat polymorphisms. The order and location of deletion breakpoints were confirmed and refined, and the regional assignment of several probes and cloned genes were determined. The cytogenetic band locations and relative order of six markers on 17p were established by fluorescence *in situ* hybridization mapping to metaphase chromosomes. The latter data confirmed and supplemented the somatic cell hybrid results. Most of the hybrids derived from [del(17)(p11.2p11.2)] patients demonstrated a similar pattern of deletion for the marker loci and were deleted for D17S446, D17S258, D17S29, D17S71, and D17S445. However, one of them demonstrated a unique pattern of deletion. This patient is deleted for several markers known to recognize a large DNA duplication associated with Charcot–Marie–Tooth (CMT) disease type 1A. These data suggest that the proximal junction of the CMT1A duplication is close to the distal breakpoint in [del(17)(p11.2p11.2)] patients. © 1992 Academic Press, Inc.

INTRODUCTION

Recently, much attention has been focused on the proximal region of the short arm of chromosome 17. Systematic acquisition of useful reagents for genetic and physical mapping in 17p is an important step in understanding the structure of this genomic region and the identification of genes that are candidates for human diseases. At least two clinically important disorders resulting from DNA rearrangements have been mapped to this region. (i) Charcot-Marie-Tooth disease (CMT; Charcot and Marie, 1886; Tooth, 1886) type 1A (CMT1A), which had been previously mapped by linkage analysis to the pericentromeric region of chromosome 17 (Raeymaekers *et al.*, 1989; Vance *et al.*, 1989, 1991; Middleton-Price *et al.*, 1990; Patel *et al.*, 1990a,b), has been recently shown to be associated with a duplication of markers D17S122, D17S125, and D17S61 in the 17p11.2–17p12 region (Lupski *et al.*, 1991; Raeymaekers *et al.*, 1991). Charcot-Marie-Tooth disease, which shows a prevalence rate of 1/2500 (Skre, 1974), is the most common inherited peripheral neuropathy. (ii) Smith–Magenis syndrome (SMS) is a recently described multiple congenital anomaly/mental retardation syndrome that is associated with an interstitial deletion of band p11.2 of the short arm of chromosome 17 (Smith *et al.*, 1986; Stratton *et al.*, 1986) and may represent a contiguous gene syndrome (Schmickel, 1986; Greenberg *et al.*, 1991). The consistent clinical features of SMS patients include characteristic dysmorphic features, microbrachycephaly, brachydactyly, short stature, and developmental delay. Variable clinical features include cleft lip/palate, congenital heart defects, microcornea, sleep disturbances including absent REM sleep, signs of peripheral neuropathy, and aggressive and self-destructive behavior, particularly onychotillomania (pulling out of finger and toe nails) and polyembolokoilamania (insertion of foreign objects into the body). These variable features may be due to varying extents of deletions in different patients.

We initiated the molecular dissection of this chromosomal region by constructing a somatic cell hybrid panel for efficiently sublocalizing large numbers of chromosome-specific probes. In addition, this panel will be use-

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ful for identifying the deletion junctions in SMS patients (Carlock *et al.*, 1985; Oberle *et al.*, 1986). The use of a somatic cell hybrid panel for the mapping of DNA markers is especially suited for chromosome 17, since the construction of somatic cell hybrids is facilitated by the presence of the selectable marker thymidine kinase (TK). To conduct deletion analysis of hybrids by the polymerase chain reaction (PCR), 17 STSs (sequence-tagged sites) were developed from probes known to map to 17p11.2–p12. Fluorescence *in situ* hybridization (FISH) was used to map six loci to cytogenetic bands. Using two-color FISH, these loci were ordered with respect to each other to confirm the results of somatic cell hybrid analyses.

The recent development of artificial chromosome vectors (Burke *et al.*, 1987) has provided a system for cloning several hundred-kilobase fragments of DNA as yeast artificial chromosomes (YACs). As a first step toward identifying the gene(s) involved in SMS and CMT1A, we used the STSs to isolate large genomic clones from a human genomic YAC library. These will be used to build a contig of YACs spanning the entire chromosomal segment. In addition, simple repeat polymorphisms were identified and characterized in markers from 17p11.2–p12, which will be useful for genetic mapping of the region.

MATERIALS AND METHODS

Construction and characterization of somatic cell hybrids. Standard polyethylene glycol fusions were performed between lymphoblastoid cell lines from SMS patients (Greenberg *et al.*, 1991) and a thymidine kinase-deficient (TK⁻) hamster cell line, a23 (Westerveld *et al.*, 1971) as described by Lupski *et al.* (1991). Hybrids from patients 147 and 254 were constructed using a TK⁻ mouse cell line, Cl-1D. For each fusion, 20–30 independent clones were isolated with cloning rings 10–14 days later and transferred to a 24-well microtiter plate. The clones were screened for the retention of the maternal or paternal homolog of human chromosome 17 by a polymerase chain reaction-based screening strategy. Cells representing each clone were obtained by trypsinization of a confluent well from the 24-well plate. One-fifth of the cells were washed with Hanks buffered saline and resuspended in 30 μl of lysis buffer (10 m*M* Tris, pH 8.4, 50 m*M* KCl, 1.2 m*M* MgCl, 0.01% gelatin, 0.45% NP-40, 0.45% Tween 20), heated at 55°C for 1 h and at 95°C for 10 min, and centrifuged for 5 min to eliminate the debris. One microliter of lysate from each clone was used in a 100-μl PCR reaction with STSs from two different loci mapping inside (D17S258) and outside (D17S124) the SMS critical region. It was possible to distinguish between clones containing the normal chromosome 17, which enabled amplification of both loci, and those retaining the deleted 17, which enabled the amplification of only one of the two STSs (D17S124). One clone identified as retaining the deleted chromosome 17 was selected from each fusion for more detailed analysis and for inclusion in the panel. The clone was expanded in a T75 flask, and genomic DNA was isolated as described by Miller *et al.* (1988).

Four previously described hybrids were also used in this study: hybrid DHA4, which is a clonal derivative of hybrid DH110-D1 from a SMS patient (Patel *et al.*, 1990a) and retains only a del(17)(p11.2) chromosome, hybrid HO-11, which retains 17p13.100 → 17qter (van Tuinen *et al.*, 1988), hybrid JW-4, which retains 17p13.105 → 17qter (van Tuinen *et al.*, 1987), and hybrid 88H5, which retains 17pter → p11.2 (van Tuinen *et al.*, 1987).

Simple sequence repeats and STSs. To identify $(GT)_n$ repeat sequences within genomic clones from 17p loci, a dot blot of 100 ng of DNA from each of the cosmids or plasmids corresponding to each

TABLE 1A

Sequence-Tagged Sites for 17pter → 17p12 Loci

Marker	Locus	Primer	Sequence (5′-3′)	Size[a] (bp)	Repeats	Heterozygosity	YACs
c1841	D17S260	IMG 3696	AATGGCTCCAAAGGAGATATTG	≈147	$(TGC)_4TTGC(GT)_5AT(GT)_{10}$	50%	Not screened
		IMG 3697	CTCCCCAACATGCTTTCTCTC				
cDNA of β-subunit of NACHR	CHRNB1	IMG 3829[b]	GCGTTGGCAACCCCCGGGCATC	450			No positives
		IMG 3830	CCATAGTGCAATTCTGCCAGTCG				
c16F4	CHRNB1	IMG 4125[c]	CTCGAGCCCCCGGCATTCAAGAA	≈166	$(GT)_{24}$	88%	No positives
		IMG 4126	AACTTTACTACAGGAGTTACACCC				
pVAW411R2	D17S124	IMG 3125	GGACCTCTTTAGTTTCAGAACCCA	350			B164E1, B86B9, B164D11, B189A10, B236B9
		IMG 3126	CAAAGGCTACAGCTCAGCCTACAG				
pEW405	D17S121	IMG 6311	GGCAACAGCTTTATGATTTCTAGGTGTG	269			Not screened
		IMG 6312	CTTTCTTTGCCTGTATTTTCTTTTTGTCAC				
pEW401	D17S81	IMG 6269	GTTTACATAGGCAGAATAGGAATC	≈194	$(AT)_6N(GT)_{17}(AT)_6$	14%	No positives
		IMG 6270[d]	GGAGAATCGCTTGAAACTGGGAGG				
pVAW412R3	D17S126	IMG 3925	GTTCTTAAAACTCCCCTTGCCTCCG	200			B240E1, A27A11, A56D6, A30D10
		IMG 3926	TTCATCAGATATTTACAGAAGACC				

[a] Size of the product variable when it contains a repeat.

TABLE 1B

Sequence-Tagged Sites for 17p12 → 17cen Loci

Marker	Locus	Primer	Sequence (5′–3′)	Size (bp)	Repeats	Heterozygosity	YACs
pFVG11	D17S122	IMG 4646	GCGGTATTTGAGCCAAGACTGTTAGG	250			C8H1, C24G1, A22D7, A217H6
		IMG 4646	GCATCTCTTTGGAAGTCTTCTCTGC				
pRM11-GT	D17S122	IMG 3342[a]	CAGAACCACAAATGTCTTGCATTC	≈169	$(TA)_8N(CA)_{11}(TA)_8$	74%	A217H6
		IMG 3343	GGCCAGACAGACCAGGCTCTGC				
pS6.1-HB2	D17S445	IMG 3124	TCTTCCCTCCTCTTCTTCTTAAAG	231			B127H4, A60D11, B227H2, A275H2
		IMG 3194	TATGGAGACAGAAGTCCTGGTGG				
OS2-GA3	D17S71	IMG 4472	GGGTGACAGAGTGAGGCTCTGTC	166	$(GAAA)_{10}$	63%	Not screened
		IMG 4473	GATTACGGAATTCAGCCATGCTC				
pA10-41	D17S71	IMG 1907	ATTGGGCTGGTTACTGTTTCAC	193			A165E7, A166B9
		IMG 3517	CAAAGCCAAATCCTGCCCATG				
pFG2	D17S447	IMG 6313	GCTCTGGTTCATATTACCCTGACAC	231			Not screened
		IMG 6314	GCTCTCCAGCCTGGGCAACAAGGGTG				
pYNM67-R5	D17S29	IMG 2812	TCTTCATCCCTACGTATCACTAGGGCC	400			A25H9
		IMG 2813	CACCCCATTCTCCGTCTGTCCCCTTGC				
pYNM67-R5	D17S29	IMG 2917	CCAGTCTCAGAGATCTTAATAATC	222	$(GTTT)_{16}$		Not screened
		IMG 2918[b]	GTAGTCTCAGCTATTCGGGAGGCTG				
c1516	D17S258	IMG 2915	TCTAACCCCAAGCAAGCCACTCC	209			B167H4, B157H5, A260B4
		IMG 2916	GAGACCCTGGGATGATAATGGATTG				
pFG1	D17S446	IMG 4004	TTTCAACTCAACCTGGGCTGTGG	550			A294E7
		IMG 4066	TTGTTGGTGAATGAATGGGACAAC				
pEW301	D17S68	IMG 3283	GTCTTCTCCCACCACTAATAC	252			A164B2, B172C7, A276B1, B39B11, B11C4
		IMG 3284	GCGTTTGAACCATACTCATCACC				

[a] Primers for pRM11-GT from Lupski *et al.* (1991).
[b] Primer has some homology to the *Alu* repeat element.

locus was hybridized with nick-translated synthetic poly(dC-dA)·poly(dG-dT) (Pharmacia), using [α-^{32}P]dCTP (New England Nuclear). The hybridizations were performed in 1 *M* NaCl, 1% SDS, at 65°C, and the filters were washed at room temperature in 2× SSC, 0.1% SDS. Restriction fragments of 300–500 bp containing the repeat sequence were identified by Southern analysis of DNA from plasmids or cosmids that appeared to be positive in the dot blot assay and were subcloned for sequencing. Restriction fragments to be subcloned were purified from an agarose gel using Geneclean (BIO 101, La Jolla, CA) and ligated to vector DNA that was linearized with the appropriate enzyme and treated with calf intestinal phosphatase as described previously (Patel *et al.*, 1990b). The nucleotide sequence was obtained by the dideoxy method using the Sequenase kit (U.S. Biochemicals). STS information from the 17p markers was obtained by isolation and/or subcloning the following for sequence determination: (1) c1541—A 500-bp repeat-positive *Sph*I fragment from c1541 (Patel *et al.*, 1990a) was subcloned into the *Sph*I site of pTZ19R. (2) c15F4—The cosmid 15F4 was obtained by screening the Los Alamos flow-sorted chromosome 17 library with the cDNA for the β subunit of the mouse muscle nicotinic acetylcholine receptor. A 1.2-kb *Xho*I fragment from c15F4 that hybridized with the cDNA was subcloned into pTZ19R. (3) pVAW411R2—A 500-bp *Hinc*II/*Hin*dIII fragment from pVAW411R2 was subcloned into pTZ18R and pTZ19R, which were digested with *Hinc*II and *Hin*dIII. (4) pVAW401—An 890-bp *Rsa*I fragment from pEW401 was subcloned into the *Hinc*II site of pTZ19R. (5) FVG11—The St. Louis human genomic YAC library was screened by PCR with primers IMG 3342 and IMG 3343 representing an STS in VAW409R1 (Lupski *et al.*, 1991a) to identify YAC A217H6. DNA from this YAC was used for amplification with a primer for the right end of the YAC vector (Nelson *et al.*, 1991) and the *Alu* primer 278 (Nelson *et al.*, 1989). The 550-bp PCR product FVG11 was directly sequenced using the latter primers (Casanova *et al.*, 1990). (6) pS6.1-HB2—A 555-bp *Eco*RI fragment from the cloned *Alu* PCR product S6.1-HB2 (Patel *et al.*, 1990b) was subcloned into the *Eco*RI site of M13mp18. (7) pA10-41—The 345-bp fragment from the clone pUC10-41 (Barker *et al.*, 1987) was subcloned into pTZ19R. (8) OS2-GA3—The STS developed from pA10-41 sequence was used to identify YAC A167E7 from the St. Louis human YAC library. A fragment specific for the right end of this YAC was amplified with a vector-specific primer (Nelson *et al.*, 1991) and the *Alu* primer 517 (Nelson *et al.*, 1989). This fragment OS2-GA3 was subcloned into the *Hinc*II site of pTZ19R. (9) pFG2—A 700-bp *Msp*I fragment from the cloned *Alu* PCR fragment FG2 (Guzzetta *et al.*, 1990) was treated with Klenow fragment to enable cloning into the *Hinc*II site of pTZ19R. (10) pYNM67-R5—A 430-bp *Eco*RI/*Hinc*II fragment and a 360-bp repeat-positive *Hae*III fragment from pYNM67-R5 (Ray *et al.*, 1990) was subcloned into the *Eco*RI/*Hin*dIII and *Hinc*II site of pTZ19R, respectively. (11) c1516—A 284-bp *Eco*RI fragment from c1516 was subcloned into the *Eco*RI site of M13mp18. In addition, the subclones pEW301 (Barker *et al.*, 1987), pEW405, and pVAW412R3 (Wright *et al.*, 1990) were directly sequenced using double-stranded plasmid DNAs after alkaline denaturation of the template DNA.

Polymerase chain reaction. The PCR was performed using standard conditions in a 50-μl reaction volume in a mixture containing 1 μM of each oligodeoxynucleotide primer with the exceptions noted below: 250 μM of each dNTP, 5 μl of 10× PCR buffer (500 m*M* KCl, 120 m*M* Tris-HCl, pH 8.0, 1.5 m*M* MgCl2, and 0.01% gelatin), and 1.26 units of Amplitaq (Cetus) DNA polymerase. The amplification conditions were an initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 55°C or as specified below, and extension for 2 min at 72°C in a first-generation automated thermal cycler (Perkin-Elmer/Cetus). For STSs developed from c1516 and RM11-GT, an annealing temperature of 60 and 65°C, respectively, was used. When a primer had homology with the *Alu* repeat, it was used at 0.1 μM. The PCR products were visualized by electrophoresing 10 μl of the reaction in a 1.3% agarose gel in 1× TBE buffer.

For PCR amplification of polymorphic repeats, either primer was end-labeled at 37°C as described by Lupski *et al.* (1991). PCR was performed as described above with 0.4 μl of the labeled primer, the same unlabeled primer at 0.1 μM, and the second unlabeled primer at

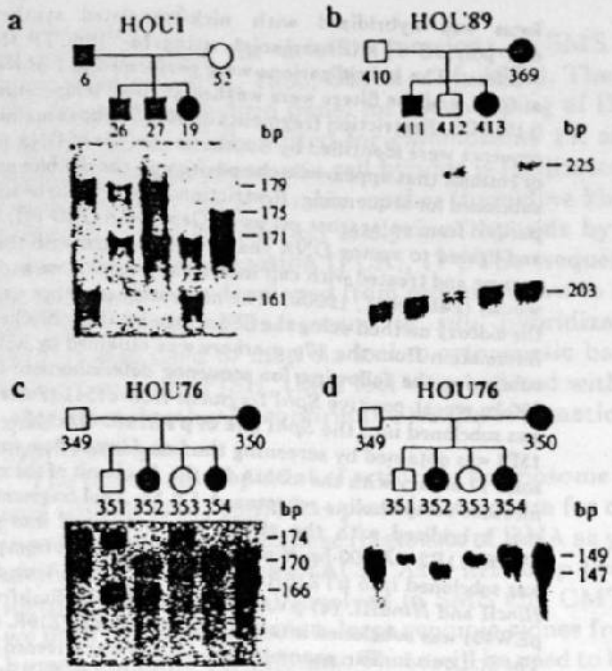

FIG. 1. Mendelian inheritance of simple repeat polymorphisms in 17p. Approximately 100 ng of genomic DNA from members of nuclear families segregating CMT1A was subjected to amplification with primers for the indicated loci and analyzed by electrophoresis in 6% polyacrylamide sequencing gels, as described under Materials and Methods. The individual loci/markers are: (a) CHRNB1 (c15F4), (b) D17S61 (EW401), (c) D17S71 (pOS2-GA3), (d) D17S260 (c1541).

1 μM. A total of 2 μl of the reaction products was mixed with 4 μl of the formamide dye and electrophoresed in a 6% polyacrylamide DNA sequencing gel at 40 W for 3.5 h. Gels were autoradiographed at −70°C for 2–12 h by exposure to Kodak XAR-5 film.

Fluorescence in situ hybridization. One- and two-color fluorescence *in situ* hybridization was performed as detailed elsewhere (Trask, 1992). Probes used for FISH were as follows: c1516 and c1541 were obtained as previously described (Patel *et al.*, 1990a); cYNM67 was kindly provided by Dr. Y. Nakamura; c15F4 was obtained as described above in the section on STSs and c17-68 was obtained by screening the Los Alamos flow-sorted chromosome 17 cosmid library with the cDNA for the ϵ subunit of the mouse muscle nicotinic acetylcholine receptor; probe VAW409 represents a mixture of the subclones pVAW409R1 and pVAW409R3 (Wright *et al.*, 1990). Prometaphase chromosome spreads were prepared from methotrexate-synchronized peripheral blood lymphocyte cultures of healthy donors (Yunis, 1976). DNA was labeled with biotin- or digoxigenin–dUTP by nick translation. To map probes relative to cytogenetic bands, biotinylated probes were hybridized individually to metaphase spreads and labeled with fluorescein-conjugated detection reagents [avidin–fluorescein (FITC), biotinylated goat anti-avidin antibodies, and avidin–FITC]. The chromosomes were incubated in DAPI and actinomycin (Schweizer, 1976) to produce a Q-banding pattern similar to enhanced QFH-type bands (Verma and Babu, 1989). The slides were mounted in an antifade solution containing propidium iodide. Chromosomes were viewed alternately through a filter set transmitting FITC-labeled probe sites and propidium iodide counterstaining and a filter set transmitting DAPI fluorescence. At least 10 metaphase spreads were analyzed to establish the band location of each probe.

To establish relative probe order along the chromosome, two probes, one biotinylated and one digoxigenin-labeled, were mixed and hybridized simultaneously to metaphase chromosomes. The biotinylated probe was subsequently labeled orange-red by incubation in avidin–Texas Red, biotinylated goat-anti-avidin antibody, and avidin–Texas Red. The digoxigenin-labeled probe was labeled green with sheep anti-digoxigenin antibody (Fab fragments, Boehringer-Mannheim) and FITC-conjugated donkey anti-sheep IgG (Molecular Probes). Red and green signals were viewed simultaneously through a dual band pass filter (Omega, Brattleboro, VT). The chromosomes were counterstained with DAPI. The order of red and green hybridization sites was scored relative to the long axes of at least 50 randomly selected chromatids. Ordering information could be obtained from those chromatids that showed red and green signals either proximal or distal relative to each other, whereas side-by-side signals were noninformative (Trask *et al.*, 1991). The significance of the metaphase ordering results was determined by calculating the test statistic, $z = 2(f - 0.5) \times n$, where f is the observed fraction of observations in one of two classes (proximal or distal) and n is the number of observations in these two classes. z was compared with tables of the normal distribution to obtain two-tailed P-values.

RESULTS

Sequence-tagged sites and simple repeat polymorphisms for 17p loci. DNA sequence information was obtained for 15 loci in 17p and was used to design 16 primer pairs for PCR amplification. In addition, published information on sequence for the β subunit for the muscle nicotinic acetylcholine receptor gene (CHRNB1) was used to design primers IMG 3829 and IMG 3830 for PCR. Published primer sequences were used for amplification of the ubiquitin locus mapping to 17p (Fain *et al.*, 1991), and VAW409 (D17S122) (Lupski *et al.*, 1991) loci. These primer sequences and the sizes or size ranges of the corresponding amplification products are displayed in Tables 1A and 1B.

Four new simple repeat PCR-based polymorphisms were identified. One of these, a $(GT)_n$ polymorphism associated with the gene for the β subunit of the muscle acetylcholine receptor, was highly informative with an observed heterozygosity of 88% in 17 unrelated Caucasians and at least 12 different alleles ranging in size from 155 to 181 bp. Mendelian inheritance of the polymorphism was noted in five multigenerational families with 108 individuals. A three-allele polymorphism associated with the D17S71 locus was identified in a subclone pOS2-GA3 from the right end of YAC A165E7. This polymorphism had an observed heterozygosity of 63% in 24 unrelated Caucasians. Mendelian inheritance of the polymorphism was observed in five multigenerational families with 140 individuals. Two two-allele polymorphisms associated with the D17S260 locus (c1541) and the D17S61 locus (EW401) were also identified. Mendelian inheritance of each of these polymorphisms is shown in Fig. 1. $(GT)_n$ repeats were also identified at the D17S258 (c1516) and D17S259 (pYNM67-R5) loci, but were not polymorphic, likely due to the fact that there were fewer than 12 monomer motifs of the repeated element.

Identification of yeast artificial chromosomes corresponding to 17p STSs. Thirteen primer pairs representing 11 loci on the short arm of chromosome 17 were used to screen the human genomic YAC library constructed at Washington University, St. Louis (Burke *et al.*, 1987; Brownstein *et al.*, 1989). The library was screened for

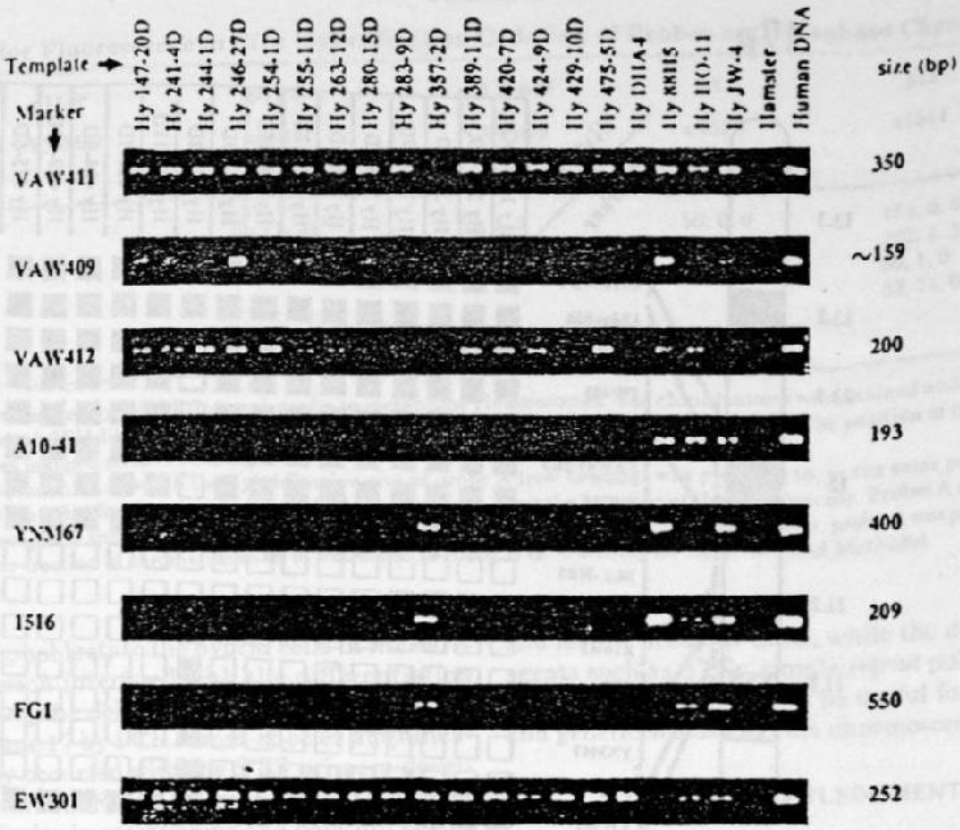

FIG. 2. PCR amplification products from representative loci in 17p obtained from somatic cell hybrids. 150 ng of genomic DNA from each hybrid was subjected to PCR amplification with primers representing each of the loci, as described under Materials and Methods. Ten microliters of the PCR reaction was electrophoresed in a 1.3% agarose gel. The size of the amplification product is indicated on the right. Hy 147-20D through Hy DHA4 represent hybrids derived from individual SMS patients. Hy 88H5, HO-11, and JW-4 retain 17pter → 17p11.2, 17p13.100 → 17qter, and 17p13.105 → 17qter, respectively. Note that Hy 357-2D is the only Smith-Magenis hybrid to have a unique pattern of deletion. The size of the PCR products for the VAW409 locus is variable due to the presence of a $(GT)_n$ repeat.

positive clones by a PCR-based strategy as described by Kwiatkowski *et al.* (1990). YACs were identified with 10 of these primer pairs and are listed in Tables 1A and 1B. The designations for the YAC clones correspond to the original clone designations at Washington University, St. Louis.

Somatic cell hybrids from SMS patients. Fifteen somatic cell hybrids retaining a del17(p11.2) chromosome from previously described (Greenberg *et al.*, 1991) individual patients with Smith–Magenis syndrome were constructed. These hybrids and four previously characterized hybrids, namely DHA4, 88H5, HO-11, and JW-4, were subjected to analysis with the STSs described in Table 1. Representative results with primers from eight of these loci are shown in Fig. 2 and cumulative results are depicted schematically in Fig. 3. The hybrids analyzed comprised a total of six different chromosomal breakpoints, which produced a maximum of seven different recognizable regions that are cytogenetically delineated into only two regions, namely 17p11.2 and 17p12. The majority of the SMS hybrids, with the exception of Hy 357-2D, appeared to have a similar pattern of deletion for the markers tested.

Five markers were deleted in most of the patients (D17S446, D17S258, D17S29, D17S71, and D17S445) defining the Smith–Magenis critical region. One hybrid from a SMS patient demonstrated a unique pattern of deletion. Hybrid 357-2D had a deletion that extended further distal from the centromere, as evidenced by deletion for several 17p12 markers (D17S125, D17S122, D17S124) and retention of several 17p11.2 markers (D17S446, D17S258, D17S29).

Fluorescence in situ hybridization. Seven loci were mapped relative to cytogenetic bands and relative to each other by fluorescence *in situ* hybridization to metaphase chromosomes (Table 2). cYNM67 and c1516 mapped to 17p11.2, VAW409 mapped to 17p11.2–p12, and c15F4, c1541, and c17-68 mapped to 17p13. The hybridization sites of these probes were labeled in different colors so that their relative order could be determined on metaphase chromosomes. For example, the site of cYNM67 was proximal to the site of c1516 on a significantly greater fraction of informative chromatids (73 of 109) than it was distal to c1516 (36 of 109) ($P < 0.0005$). No ordering information was provided by 71 additional chromatids on which the hybridization sites of cYNM67 and c1516 were equidistant from the telomere. FISH ordering on metaphase chromosomes established the order: cen–cYNM67–c1516–VAW409–c15F4–c1541–c17-68.

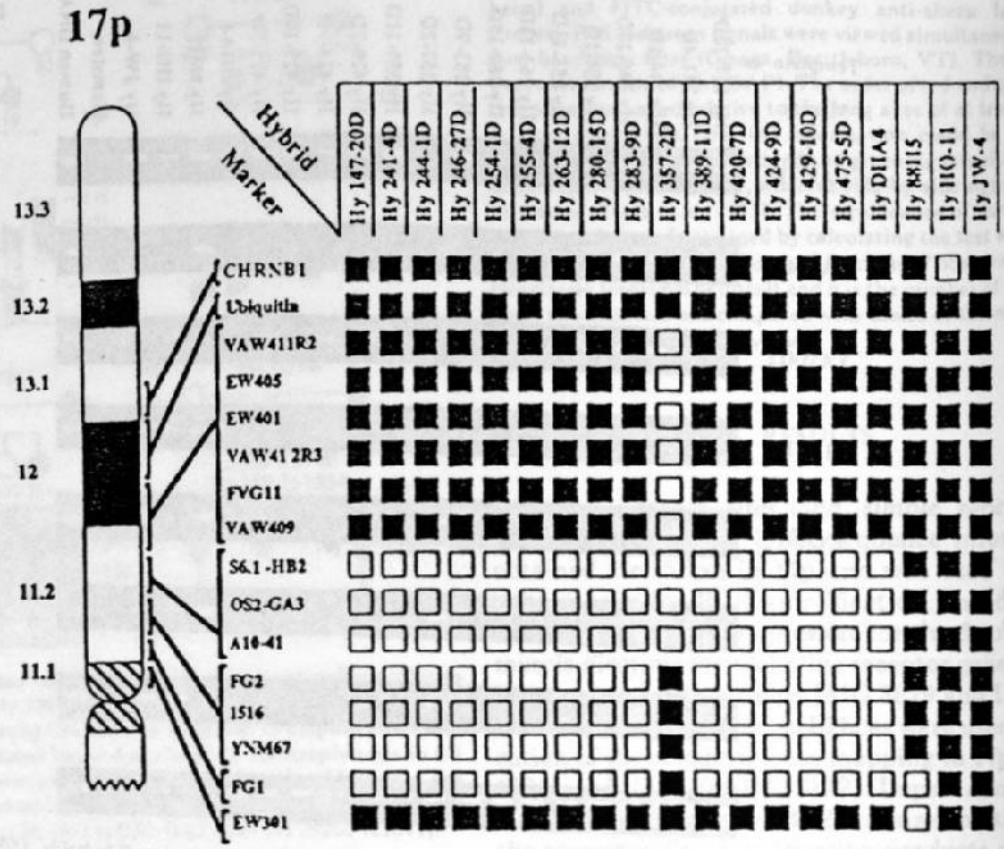

FIG. 3. Deletion status of 17p markers in the hybrids determined by PCR. An idiogram illustrating the short arm of chromosome 17, the deletion intervals, and the relative position of individual markers are shown on the left. An open box indicates that the marker is deleted, while a solid box indicates that the STS yields an amplification product in the hybrid. The order of cYNM67 and 1516 with respect to the centromere was established by FISH mapping.

DISCUSSION

Much attention has been focused on efforts to characterize the human genome at two levels: the gross physical and genetic map of chromosomes and their DNA sequence (McKusick and Ruddle, 1987). The molecular dissection of specific chromosomal segments and the acquisition of region-specific polymorphic sequences are significant steps toward these goals. We describe the development of a somatic cell hybrid panel from SMS patients that should be very useful for the ordering of probes within the 17p11.2–17p12 intervals and provide valuable information on the molecular basis of the syndrome. The density of well-characterized markers available in this region did not allow us to differentiate the deletions in these patients except in patient 92-357. The extent of the region encompassed by the deletion in patient 92-357 has an interesting implication. This patient carries a deletion spanning the markers LEW401 (D17S61), VAW409 (D17S122), and VAW412 (D17S-125), which span a genetic distance of ≃10 cM (Wright *et al.*, 1990) and have been demonstrated to map within a 1.5-Mb region duplicated in patients affected with CMT1A (Lupski *et al.*, 1991; our unpublished observations). This suggests that the proximal junction of the CMT1A-associated duplication may closely flank the distal breakpoint in del(17)(p11.2) patients. Patient 92-357, who is deleted for all three markers, does not sh any clinical signs of CMT1A, although further elect physiological evaluation is required. This suggests th monosomy for the gene(s) in the CMT1A-associated d plication interval does not lead to clinical signs CMT1A. The deletion in this patient extends more te meric to include the VAW411R2 (D17S124) locus, whi is not duplicated in CMT1A patients.

The gene for the β subunit of the muscle acetylcholi receptor had been previously mapped to the 17p11 17p12 region (Beeson *et al.*, 1989). Our studies clea show that this gene maps to 17p13. This is based on P analysis of the hybrid panel using two different prin sets specific for this gene and FISH. Thus, the chrom somal location of this gene, which is much more di than the markers mapping within the CMT1A-as ciated duplication, makes it an unlikely candidate this disease.

FISH mapping confirmed the hybrid panel resul Probes from three of the seven intervals defined by t hybrid panel were assigned to cytogenetic band lo tions. In addition, FISH mapping placed cYNM67 pr imal to c1516, c15F4 (NACHRB) in 17p13, distal VAW409, and placed two additional markers, c1541 c17-68 (CHRNE) distal to c15F4 (CHRNB1) in 17p

The use of PCR for analysis of hybrid DNA provec be a reliable method, although persistent attachment

TABLE 2

Two-Color Fluorescence *in Situ* Hybridization Ordering of Probes on Metaphase Chromosomes

	p11.2[a]	p11.2	p11.2–p12	p13	p13	p13
	B: cYNM67	c1516	VAW409	c15F4	c1541	c17-68
A						
cYNM67		73, 71, 36[b]	52, 8, 3	50, 0, 0	155, 0, 0	n.d.
c1516			44, 28, 13	53, 0, 0	103, 1, 1	102, 0, 1
VAW409				73, 2, 0	50, 1, 0	52, 5, 0
c15F4					33, 14, 0	71, 13, 1
c1541						58, 20, 2
c17-68						

[a] Band location determined by hybridizing each probe to metaphase chromosomes. The chromosomes were stained with DAPI and actinomycin to produce enhanced QFH-like bands (Schweizer, 1976) and counterstained with propidium iodide. The position of the fluorescein-labeled hybridization site was mapped relative to cytogenetic bands on at least 10 metaphase spreads.

[b] The number of chromatids in which the hybridization site of probe A (row heading) was proximal to, at the same position as, or distal to (listed in that order) the hybridization site of probe B (column heading) along the long axis of the chromosome. Probes A and B were hybridized simultaneously to metaphase spreads, and their sites were labeled with different fluorochromes. In all cases, probe A was proximal to probe B on a significantly greater fraction of chromatids than it was distal to probe B ($P < 0.0005$; see Materials and Methods).

the parental lymphoblasts to the hybrid cells in the early phases of the fusion interfered with the differentiation of clones retaining the deleted from those retaining the intact chromosome 17 by PCR. Cross-species amplification occasionally occurred with the STS primers developed from the markers RM11-GT and c1516, thus leading to some difficulty in establishing the deletion status of the hybrids. These two problems were circumvented by reducing the amount of DNA used as template and increasing the annealing temperature to allow selective amplification of human sequences, respectively.

The construction of region-specific STSs allowed us to isolate several YAC clones specific for chromosome 17p11.2–p12. The availability of methods for rapid screening (Kwiatkowski *et al.*, 1990) and isolation of insert-terminal sequences from YACs (Riley *et al.*, 1990; Nelson *et al.*, 1991) provides the possibility of rapid walking and cloning of DNA spanning the entire region. The YACs will also be useful for the direct identification of expressed sequences that may be candidates for CMT1A, Smith–Magenis syndrome, and other diseases that may map to this region. YACs have recently been used to directly screen cDNA libraries (Wallace *et al.*, 1990; Elvin *et al.*, 1990), and in addition, potential HTF islands likely to be associated with expressed sequences (Bird, 1987), may be easily identified on the restriction map.

We also identified and characterized four simple repeat polymorphisms (Weber, 1990), which will be useful for establishing the parental origin of the deletion in SMS patients and for genetic mapping of the region. In particular, the identification of a highly informative polymorphism within the gene for the β subunit of the muscle acetylcholine receptor will be useful for evaluating its importance in various human diseases by linkage analysis.

In conclusion, the use of somatic cell hybrids and PCR as well as fluorescence *in situ* hybridization has proved to be a rapid and reliable method for mapping probes and for the study of SMS, while the development of reagents such as STSs, simple repeat polymorphisms, and region-specific YACs will be useful for further physical and genetic studies of this chromosomal segment.

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