ANEXO

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Isolation of a Marker Linked to the Charcot-Marie-Tooth Disease Type 1A Gene by Differential *Alu*-PCR of Human Chromosome 17–retaining Hybrids

Pragna I. Patel,* Carlos Garcia,‡ Roberto Montes de Oca-Luna,* Richard I. Malamut,‡ Brunella Franco,* Susan Slaugenhaupt,§ Aravinda Chakravarti,§ and James R. Lupski*†

*Institute for Molecular Genetics and †Department of Pediatrics, Baylor College of Medicine, Houston; ‡Department of Neurology, Louisiana State University, New Orleans; and §Department of Human Genetics, University of Pittsburgh, Pittsburgh

Summary

We report the isolation of a new marker (S6.1) from band p11.2 of human chromosome 17 by differential *Alu*–polymerase chain reaction (*Alu*-PCR) of both a monochromosomal hybrid retaining a single human chromosome 17 and a hybrid retaining a del(17)(p11.2p11.2) in addition to other human chromosomes. The method is based on the preferential PCR amplification of human DNA in rodent/human hybrids when primers specific to the human *Alu* repeat element are used. *Msp*I and *Sst*I RFLPs associated with S6.1 were identified and used in linkage analysis of both a previously reported and a newly identified French-Acadian kindred segregating autosomal dominant Charcot-Marie-Tooth disease (CMT). A cumulative peak lod score of 3.41 at a peak recombination fraction of .12 indicates that this marker is linked to the CMT 1A locus but is at a distance from the disease gene. Thus, the marker S6.1 will be useful in further delineating the candidate region for the CMT gene when its location with respect to pA10–41 and 1516, two other markers from 17p11.2 which have previously demonstrated close linkage to the CMT locus, has been determined.

Introduction

Charcot-Marie-Tooth disease (CMT) is one type of hereditary motor and sensory neuropathy (HMSN) which is characterized by absent deep tendon reflexes and progressive muscle wasting, particularly in muscles innervated by the peroneal nerves (Charcot and Marie 1886). The disease can be inherited in an autosomal dominant, autosomal recessive, or X-linked manner (McKusick 1988). CMT type 1A (CMT 1A), which is the most prevalent form of this disease, has been recently mapped to the pericentromeric region of chromosome 17 (Raeymaekers et al. 1989; Vance et al. 1989; Middleton-Price et al. 1990; Patel et al. 1990), while CMT type 1B, also inherited in an autosomal dominant manner, has been shown to be linked to the Duffy locus in the q23-q25 region of chromosome 1 (Bird et al. 1982).

We have studied a large French-Acadian pedigree segregating CMT 1A by using markers from the pericentromeric region of chromosome 17 (Patel et al. 1990). Two markers, pA10–41 (D17S71) and 1516 (D17S258), are closely linked to the CMT locus and map to 17p11.2 (vanTuinen et al. 1987; Patel et al. 1990). This chromosomal region is deleted in patients with Smith-Magenis syndrome, a syndrome characterized by multiple congenital anomalies including developmental delay, facial dysmorphology, attention-deficit disorder, and, in some patients, absence of REM sleep (Smith et al. 1986; Stratton et al. 1986). We have used a hybrid cell line, DH110-D1 (Patel et al. 1990), constructed from a Smith-Magenis patient to selectively isolate a marker from 17p11.2 for use in further linkage studies. The probe isolation method is based on the polymerase chain reaction (PCR)-mediated amplification of human DNA in hybrid cell lines by using primers directed to the *Alu*

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Address for correspondence and reprints: Pragna I. Patel, Ph.D., Institute for Molecular Genetics, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030

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repeat sequence (Nelson et al. 1990) and by the comparison of *Alu*-PCR products from hybrid cell lines retaining either an intact or deleted chromosome 17 as described recently for markers in the Xq28 region (Ledbetter et al. 1990). The probe S6.1 was then utilized in linkage analysis with two large CMT 1A pedigrees. Our probe isolation method should have application to the identification of novel probes from specific regions, for use in linkage analysis and disease-gene mapping.

Material and Methods

Cell Lines

The rodent parent of each hybrid cell line is clone 1D, a thymidine kinase–deficient mouse fibroblast cell line. All hybrid cell lines have been described elsewhere (vanTuinen et al. 1987, 1988; Patel et al. 1990). The hybrid MH22–6 retains a single chromosome 17 as its only human material. The hybrids P12.3B, HO-11, and JW-4 retain 17pter→q11.2 or 12, 17p13.100→qter, and 17p13.105→qter, respectively. The hybrid DH110-D1 is derived from a Smith-Magenis patient and retains 17pter→p11.2::p11.2→qter.

PCR Reactions and Primers

The PCR was conducted using the *Alu* primer, TC-65, and the conditions used were essentially those described by Nelson et al. (1989), except that the final concentration of dNTPs was 250 μM.

Gel Electrophoresis and Southern Hybridization

DNAs were visualized with ethidium bromide after electrophoresis in agarose gels. Size markers are a mixture of λ DNA digested with *Hin*dIII and ΦX174 digested with *Hae*III. Southern transfer and hybridization followed procedures described elsewhere (Patel et al. 1990). When PCR products were to be used as probes, they were either ethanol precipitated with ammonium acetate to remove dNTPs or were purified after electrophoretic separation in low-melting-temperature agarose. All probes were preassociated with human placental DNA prior to use in hybridizations. In brief, Southern blots were incubated in a three-fourths volume of prehybridization solution (1 M NaCl, 10% dextran sulfate, 1% SDS) containing 0.1 mg sonicated human placental DNA/ml and used at a final volume of 1 ml/16 cm^2 of membrane. The radiolabeled probe, used at a final concentration of 1×10^6 cpm/ml of prehybridization solution, was mixed with one-fourth volume of the prehybridization solution containing 0.2 mg of human placental DNA/ml, was boiled for 5 min to denature the DNA, and was incubated at 65°C for 2 h to preassociate repeat sequences in the probe prior to use in hybridizations.

Cloning of Alu-PCR Product

The *Alu*-PCR product, S6.1, was digested with *Hin*dIII to purify it from any comigrating contaminants. Two major subfragments, S6.1-HB1 (1.2 kb) and S6.1-HB2 (1.1 kb), were each purified from agarose by using the GeneClean kit (BIO101) and were ligated into *Hin*cII-digested pTZ19R vector (Mead et al. 1986) at a vector:insert molar ratio of 1:2 after repairing the ends. The ligation was performed in One Phor-All Plus buffer with T4 ligase (Pharmacia) for 16 h at 16°C. *Escherichia coli* K-12 strain DH5α competent cells were transformed with the ligation products and plated on LB plates containing 100 μg ampicillin/ml.

CMT Pedigrees

All available members of pedigrees segregating autosomal dominant CMT were subjected to a thorough clinical and electrophysiological examination. Since we had previously demonstrated reduced nerve conduction velocities (NCVs) in asymptomatic individuals (Patel et al. 1990), all individuals used in linkage analysis were examined for NCV. In these kindreds, the diagnosis of CMT 1A was established by the presence of markedly slowed median and ulnar motor NCVs. A single normal motor NCV of the peroneal nerve excluded the diagnosis in patients older than age 5 years.

Pedigree 1 is a 299-person French-Acadian pedigree which has been previously described and of which 33 members have been used in the linkage analysis (Patel et al. 1990). Pedigree 42 is a 149-person French-Acadian pedigree of which 35 members were studied (fig. 1). After informed consent was obtained, blood was collected from each participating family member and was used to establish Epstein-Barr virus–transformed lymphoblasts (Anderson and Gusella 1984) and for isolation of high-molecular-weight DNA (Miller et al. 1988).

Linkage Analysis

The parameters and methods used for linkage analysis for pedigrees 1 and 42 have been described elsewhere (Patel et al. 1990), with the exception that the age-dependent risk of affection was removed, since all critical members of this pedigree were evaluated for NCV. A final penetrance of .99 was assumed for CMT gene carriers.

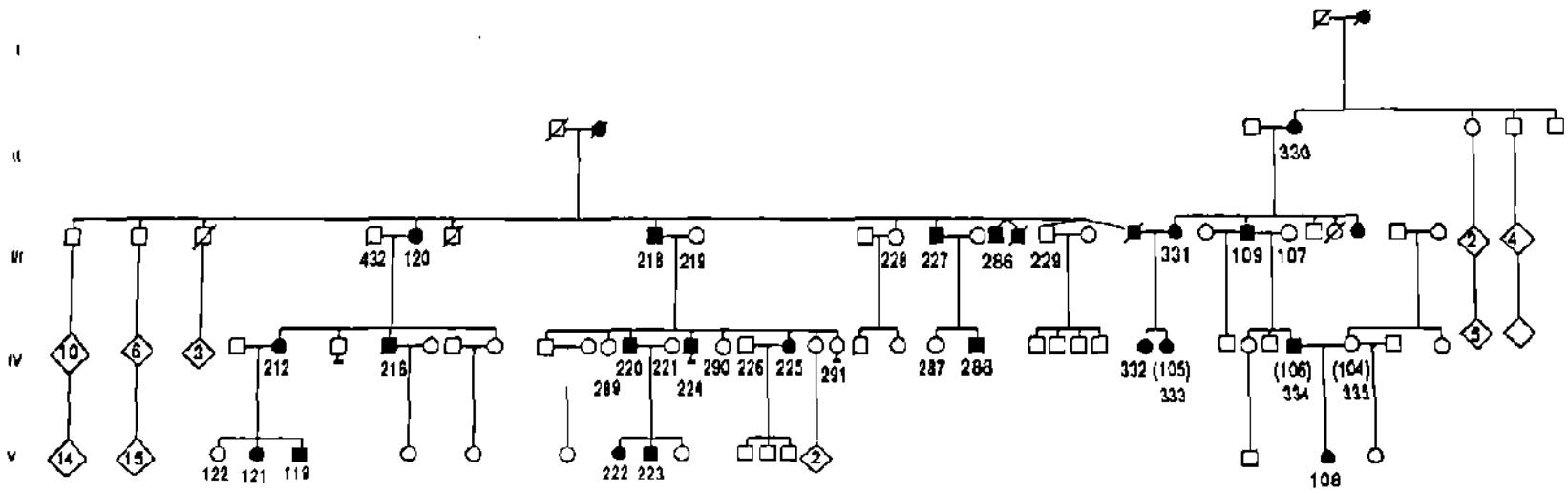

Figure 1 French-Acadian pedigree 42, segregating CMT in an autosomal dominant fashion. □ and ○ = unaffected male and female, respectively; ■ and ● = affected male and female, respectively; ◇ = unaffected progeny, where the enclosed number is the number of individuals. The numbers under the symbols refer to identification numbers of the individuals used in the linkage analysis.

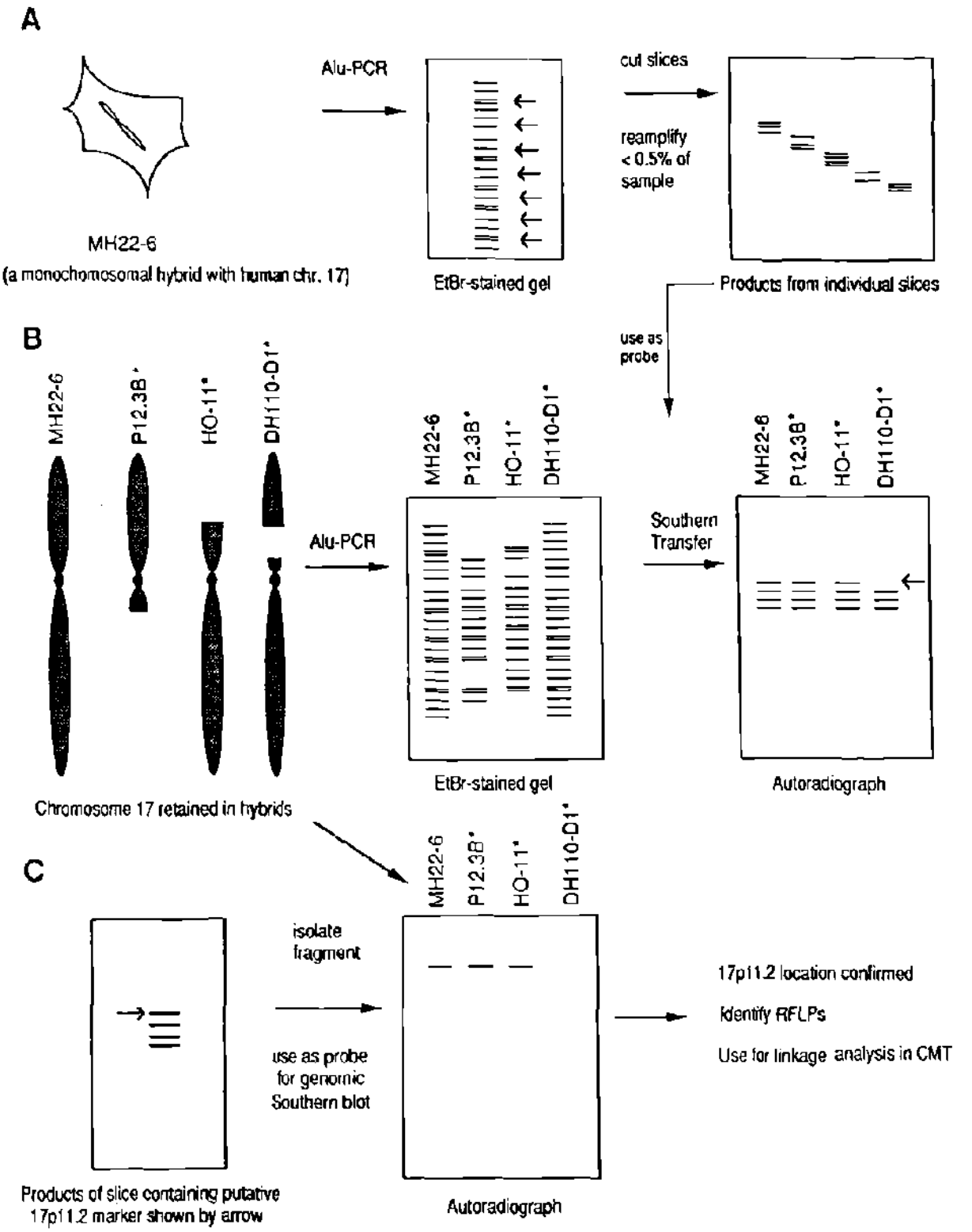

Figure 2 Schematic diagram of method used for isolation of markers from 17p11.2. The hybrids are described in Material and Methods. Hybrids marked with an asterisk retain the indicated derivative chromosome 17 in addition to other human chromosomes.

Results

Isolation of Marker S6.1 from 17p11.2

The method used for isolation of markers mapping to 17p11.2 is shown schematically in figure 2. MH22–6 is a hybrid cell line retaining a single copy of chromosome 17 as its only human complement. Human-specific sequences in the cell line were selectively amplified by conducting PCR using the primer TC-65 representing the *Alu* repeat sequence as described by Nelson et al. (1989). The amplification products comprising >50 fragments ranging in size from ~400 bp to ~4 kb were separated by electrophoresis in a 1% low-melting-temperature agarose gel. The gel was sliced at 0.5-cm intervals, each slice was melted, an equal volume of water added, and 5 μl (<0.5%) of each slice was used for a reamplification reaction using the primer TC-65.

The primer TC-65 was also used to amplify the human DNA in the hybrids P12.3B, HO-11, and DH110-D1, which retain a derivative chromosome 17 in addition to several other human chromosomes. The amplification products obtained both with TC-65 and either each of these hybrids separately or hybrid MH22–6, which are displayed in figure 3*A*, were subjected to Southern analysis using amplification products from each of the slices of the low-melting-temperature agarose gel as a probe. The results obtained with the products of slice 6 are shown in figure 3*B*. One of the amplification products was missing in the hybrid DH110-D1, suggesting that it originated from a sequence mapping to 17p11.2. To confirm this, the fragment designated S6.1 was isolated and hybridized to a Southern blot of *Eco*RI-digested genomic DNA from the hybrid DH110-D1 and other human chromosome 17–retaining hybrids. As shown in figure 4, no signal is evident when S6.1 is hybridized to DH110-D1 DNA, while a hybridization signal is seen when 17p11.2-specific DNA sequences are present, thus confirming that this sequence originated from 17p11.2. Figure 4 also shows that S6.1 hybridized to four *Eco*RI fragments, all of which map to 17p11.2.

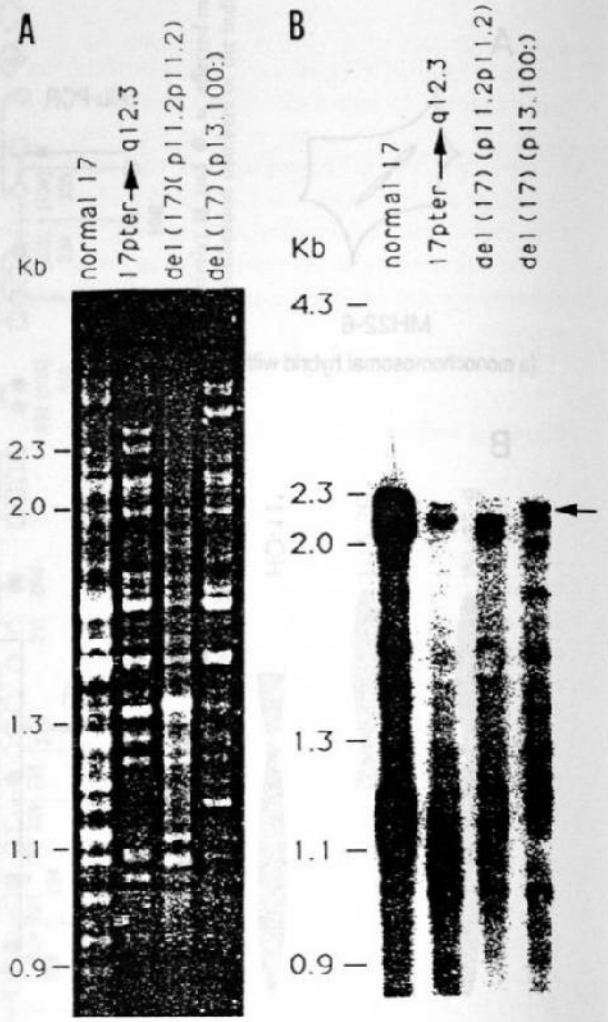

Figure 3 Southern analysis of *Alu*-PCR products from hybrids MH22–6, P12.3B, DH110-D1, and HO-11. One-half microgram from each of these hybrids was amplified with primer TC-65 as described in Material and Methods. Ten percent of the products were electrophoresed in a 1.2% agarose gel, and a Southern blot was prepared. The blot was hybridized with the amplification products from slice 6. *A*, Ethidium bromide–stained gel. *B*, Autoradiograph of Southern blot. The arrow indicates the fragment missing in the Smith-Magenis hybrid, DH110-D1, suggesting that it maps to 17p11.2.

RFLP Analysis of Marker S6.1

To determine whether the marker S6.1 showed linkage to the CMT locus, we attempted to identify RFLPs. DNA from eight unrelated individuals was digested with *Apa*I, *Ava*II, *Bam*HI, *Ban*I, *Ban*II, *Bcl*I, *Bgl*I, *Bgl*II, *Dra*I, *Eco*RI, *Eco*RV, *Hin*cII, *Hin*dIII, *Hin*fI, *Msp*I, *Mbo*I, *Pst*I, *Pvu*II, *Rsa*I, *Sca*I, *Sph*I, *Sst*I, *Stu*I, *Taq*I, and *Xba*I and was used for Southern analysis with S6.1. An *Sst*I polymorphism, with allelic fragments of 12.2 and 7.1 kb at a frequency of 39% and 61%, respectively, was detected. An RFLP was also detected for *Msp*I, with allelic fragments of 1.1 and 1.0 kb at a frequency of 78% and 22%, respectively. Both Mendelian segregation of these RFLPs and the invariant fragments recognized by a cloned subfragment of S6.1 are shown in figure 5. Since a limited number of unrelated individuals were typed for the *Sst*I and *Msp*I RFLPs, it could not be determined whether these RFLPs are in linkage

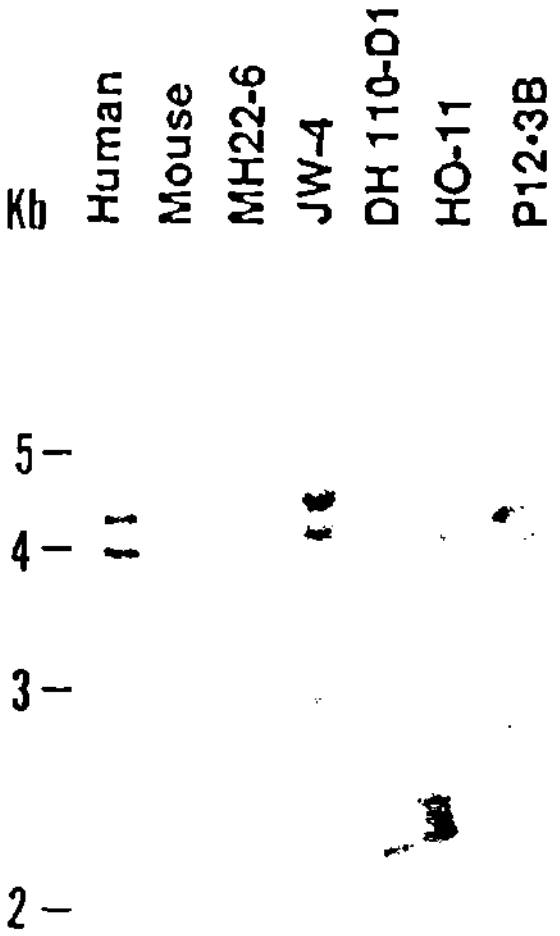

Figure 4 Southern analysis of genomic DNA with *Alu*-PCR product, S6.1. Two and one-half micrograms of human DNA, 5 μg of mouse (clone 1D) DNA, and 10 μg of DNA from the somatic cell hybrids MH22–6, JW-4, DH110-D1, HO-11, and P12.3B were digested with EcoRI and hybridized with S6.1, which is the fragment identified as missing in the hybrid DH110-D1 as shown in fig. 3*B*. The hybrids are described in Material and Methods.

disequilibrium. However, no recombinants were detected between the *Sst*I and *Msp*I RFLPs within two large pedigrees which were analyzed (see below).

A New French-Acadian Kindred Which Segregates CMT 1A

We have previously reported a French-Acadian kindred (pedigree 1) segregating autosomal dominant CMT and demonstrating linkage to markers from the pericentromeric region of chromosome 17 (Patel et al. 1990). A second French-Acadian kindred (pedigree 42; fig. 1) segregating autosomal dominant CMT was sampled, and 35 of the individuals in it were used in the present study. To determine whether pedigree 42 segregated CMT 1A, members were typed for the markers pA10–41, 1516, and EW301, which have been shown to be closely linked to the CMT 1A locus (Raeymaekers et al. 1989; Vance et al. 1989; Middleton-Price et al. 1990; Patel et al. 1990). The individual and cumulative lod scores obtained for pedigrees 1 and 42 by using pA10–41, 1516, and EW301 are presented in table 1. In pedigree 42 the marker pA10–41 was more informative than 1516 or EW301 and yielded $\hat{Z} = 2.33$ at $\hat{\theta} = 0$, suggesting that pedigree 42 segregates CMT 1A.

Linkage Analysis of Pedigrees 1 and 42 with Marker S6.1

To determine the genetic distance of S6.1 with respect to the CMT locus, both pedigree 1 and pedigree 42 were typed for the *Msp*I and *Sst*I RFLPs described above, and haplotypes were constructed. Lod scores were calculated as described in Material and Methods and are presented in table 1 both individually for pedigrees 1 and 42 and as a total for these families, under the assumption of homogeneity of linkage. These results suggest that 1516 and pA10–41 are both closely linked to the CMT 1A gene, but the marker S6.1 is linked at a distance ($\hat{\theta} = .12$) from the disease gene. Determination of the recombination distance between S6.1, 1516, and pA10–41 will entail examination of a larger number of CMT 1A and reference pedigrees with these markers. This in turn will enable a more precise delineation of the candidate region for the CMT locus on chromosome 17.

Discussion

The ability to amplify human-specific sequences in somatic cell hybrids by using primers to repeated sequence elements such as *Alu* (Nelson et al. 1989) and LINE (Ledbetter et al. 1990) has greatly facilitated the identification of markers mapping to specific chromosomal regions. We have described in the present report an application of *Alu*-PCR which has enabled us to rapidly identify a new marker in a specified region of chromosome 17p and to demonstrate the capacity for its direct use in linkage analysis for CMT 1A. This region contains gene(s) responsible for the various phenotypic manifestations in Smith-Magenis syndrome (del(17)(p11.2)), as well as two markers (1516 and pA10–41) closely linked to the gene responsible for CMT 1A (Patel et al. 1990). Only three other markers have been previously physically mapped to this deletion interval—namely, D17S71 (pA10–41) and D17S29 (YNM67)

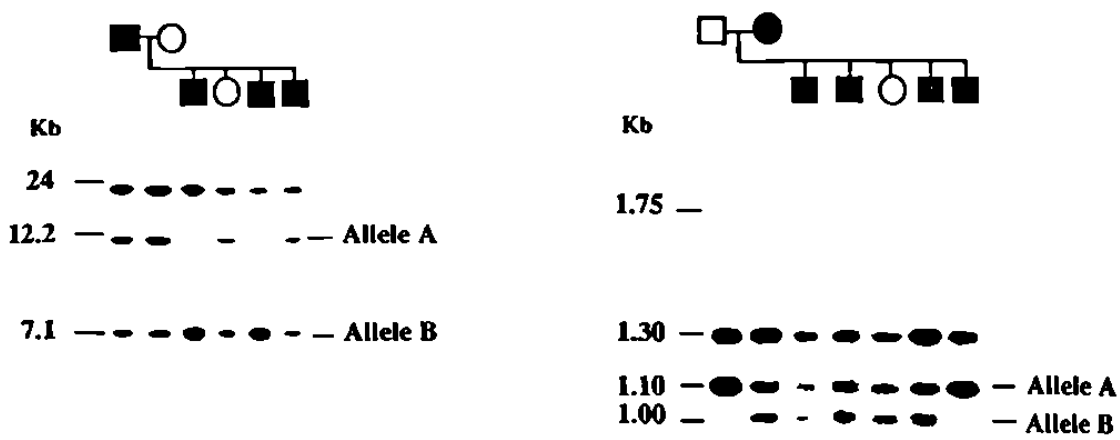

Figure 5 Mendelian segregation of RFLPs revealed by enzymes *SstI* (*A*) and *MspI* (*B*). A cloned subfragment of the marker S6.1 designated S6.1-HB2 (see Material and Methods) was used for Southern analysis. The variant fragments are indicated as allele A and allele B, respectively. The *SstI* pattern obtained with S6.1-HB2 is identical to that seen with S6.1. The *MspI* pattern obtained with S6.1-HB2 is simpler and allows distinction of overlapping *MspI* fragments. Occasionally a strongly hybridizing 1.75-kb *MspI* fragment was seen with this probe, but it could never be conclusively established whether this represented a variant allele of the 1.3-kb *MspI* fragment and/or an incomplete digestion product.

Table 1

Lod Scores for CMT 1A versus Chromosome 17p Markers

Marker (enzyme[s]) and Pedigree[a]	Lod Score at Recombination Fraction of								
	0	.05	.10	.15	.20	.30	.40	$\hat{\theta}$	$\hat{Z}$
A10-41 (*MspI*):									
1	.89	.79	.67	.55	.43	.20	.05	...	...
42	2.33	2.11	1.88	1.65	1.40	.91	.42	...	...
Total	3.22	2.90	2.55	2.20	1.83	1.11	.47	.00	3.22
1516[b]: *Hin*dIII and *MspI*:									
1	3.72	3.39	3.02	2.64	2.23	1.37	.50	...	...
42	−4.13	.64	.73	.70	.61	.38	.15	...	...
Total	−.41	4.03	3.75	3.34	2.84	1.75	.65	.04	4.04
EW301 (*TaqI*):									
1	2.04	2.33	2.39	2.29	2.07	1.44	.45	...	...
42	.92	.78	.65	.55	.46	.31	.17	...	...
Total	2.96	3.11	3.04	2.84	2.53	1.75	.62	.06	3.12
S6.1 (*SstI* and *MspI*):									
1	−3.70	1.76	2.20	2.26	2.12	1.65	.89	...	...
42	−3.39	1.11	1.16	1.07	.92	.54	.16	...	...
Total	−7.09	2.87	3.36	3.33	3.04	2.19	1.05	.12	3.41

[a] Lod scores for pA10-41, 1516 (*Hin*dIII), and EW301 in pedigree 1 were published by Patel et al. (1990); the remaining results are from the present study.

[b] *Hin*dIII RFLP described by Patel et al. (1990); *Msp*I RFLP described by Franco et al. (submitted).

(van Tuinen et al. 1987) and D17S258 (1516) (Patel et al. 1990).

Ledbetter et al. (1990) have recently described the identification of markers from Xq28 by a comparison of the amplification products obtained from a hybrid retaining a single human X chromosome and from hybrids retaining a del(X)(q28) or only the Xq24-qter region. The patterns of amplification products used for the identification of these markers were simpler than that obtained with the hybrid MH22–6. We therefore used the approach of examining pools of amplification products created by dividing electrophoretically separated amplification products. This approach for identifying *Alu*-PCR amplification products which are absent in a hybrid retaining a chromosome 17 specifically deleted for band p11.2 may be further refined by modifications which reduce the total number of *Alu*-PCR amplification products in the hybrids.

The marker S6.1 appears to hybridize to at least two loci in the genome, as determined by Southern analysis of genomic DNAs digested with a number of restriction enzymes, including *Sst*I (fig. 5), *Kpn*I, *Ava*II, *Bam*HI, and *Pvu*II (data not shown). Both of these loci, which each appear to contain at least one *Eco*RI site, map to 17p11.2, as shown by their absence in a hybrid retaining del (17) (p11.2p11.2) (fig. 4). Identical results were obtained with cloned subfragments of S6.1, thus ruling out the possibility that S6.1 represented a mixture of comigrating *Alu*-PCR products (data not shown). Similar results have been observed with phage clones originating from Xp and have been attributed to the presence of a family of very-low-copy-number repeats whose members are located within close proximity on the X chromosome (Ballabio et al., in press). Use of the marker S6.1 to screen a genomic library to isolate larger homologous genomic sequences—along with physical mapping of these sequences—will determine the relative proximity of these sequences in 17p11.2.

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GENOMICS 9, 31–36 (1991)

Isolation of Region-Specific and Polymorphic Markers from Chromosome 17 by Restricted *Alu* Polymerase Chain Reaction

VITO GUZZETTA,* ROBERTO MONTES DE OCA-LUNA,* JAMES R. LUPSKI,*,† AND PRAGNA I. PATEL*,[1]

*Institute for Molecular Genetics and †Department of Pediatrics, Baylor College of Medicine, Houston, Texas 77030

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We demonstrate that the digestion of template DNAs with restriction endonucleases prior to *Alu* polymerase chain reaction ("restricted *Alu*-PCR") reduces the complexity of the *Alu*-primed amplification patterns of human DNA in somatic cell hybrids and allows a direct informative comparison of these patterns. A comparison of restricted *Alu*-PCR patterns of a monochromosomal hybrid retaining a human chromosome 17 (MH22-6) and a hybrid retaining a human chromosome 17 deleted for band p11.2 (DH110-D1) revealed four *Alu*-PCR products that were present in the former but absent in the latter hybrid. Hybridization of these fragments to the total *Alu*-PCR amplification products of the two hybrids confirmed their absence in DH110-D1 amplification products. Hybridization to a panel of somatic cell hybrids indicated that two of these fragments were deleted in the hybrid DH110-D1 and mapped to 17p11.2, as expected. However, two additional fragments were not deleted in the hybrid DH110-D1 and mapped to other regions of chromosome 17. An insertion–deletion polymorphism was associated with one of the latter fragments, which may be the mechanism for the lack of its amplification in the hybrid DH110-D1. Restricted *Alu*-PCR should enhance the applications of *Alu*-PCR and provides a new method for the identification of chromosome-specific polymorphic markers.

INTRODUCTION

The ability to isolate markers rapidly from a defined human chromosomal region has been greatly enhanced by the *Alu* polymerase chain reaction (*Alu*-PCR). This approach uses primers directed to human *Alu* repetitive sequence elements to amplify human DNA sequences situated between *Alu* repeats (Nelson *et al.*, 1989). Several types of primers based on the consensus *Alu* sequence have been designed (Nelson *et al.*, 1989; Cotter *et al.*, 1990). These primers can be used alone or in combination with other *Alu* primers or primers to other interspersed repeat elements such as L1Hs (Ledbetter *et al.*, 1990) to amplify inter-*Alu* sequences from human DNA present in complex backgrounds, such as rodent–human hybrids.

Direct comparison of the amplified human sequences of a monochromosomal hybrid retaining a human X chromosome to that of hybrids retaining human derivative X chromosomes as the only human material has allowed the isolation of markers mapping to Xq28 (Ledbetter *et al.*, 1990). We sought to isolate markers from 17p11.2 by comparing the *Alu*-PCR amplification patterns of a monochromosomal hybrid retaining human chromosome 17 (vanTuinen *et al.*, 1987) to that of a hybrid retaining a human chromosome 17 deleted for the p11.2 region (Patel *et al.*, 1990). However, the large number of *Alu*-primed amplification products obtained with these hybrids was a limiting factor in the direct comparative analysis of these products from hybrids for the isolation of markers mapping to 17p11.2. In this report, we demonstrate the reduction in complexity of the *Alu*-PCR amplification patterns obtained using *Alu* primers by digesting genomic DNAs from chromosome 17-retaining hybrids with various restriction enzymes prior to *Alu*-PCR. This allowed us to isolate two markers from the 17p11.2 region. A second application resulting from this study is a method for the direct identification of polymorphic markers from specific chromosomal regions.

MATERIALS AND METHODS

Cell Lines

The rodent parent of all somatic cell hybrids is clone 1D, a thymidine kinase-deficient mouse fibroblast cell line. All hybrids used in this study have been described previously. Briefly, the hybrid MH22-6 contains a single chromosome 17 as the only human

[1] To whom correspondence should be addressed at Institute for Molecular Genetics, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030.

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material (vanTuinen *et al.*, 1987), DH110-D1 retains a del(17)(p11.2p11.2) derived from a patient with Smith–Magenis syndrome (Patel *et al.*, 1990), 88-H5 contains 17pter–p11.2 (vanTuinen *et al.*, 1987), LS-1 contains 17cen–qter (Elder *et al.*, 1985) SP-3 contains 7q11.2 (vanTuinen *et al.*, 1987), and HO-11 contains 17p13.1–q25.3 (vanTuinen *et al.*, 1988).

Primers and PCR Conditions

Templates for restricted *Alu*-PCR were prepared by digesting 1 μg of genomic DNA for >4 h with 10 units of restriction enzyme added in two aliquots. The DNAs were precipitated in 0.3 *M* sodium acetate with 2 vol of 95% ethanol, washed in 75% ethanol, and resuspended in 50 μl of water. The PCR was conducted using *Alu* primers TC-65 and 517 as described by Nelson *et al.* (1989) with minor modification. Briefly, the PCR was carried out in a final volume of 50–100 μl with 1 μg of DNA, primer TC-65 at 1 μ*M*, or primer 517 at 0.1 μ*M* in 50 m*M* KCl/10 m*M* Tris–HCl, pH 8.0/1.5 m*M* $MgCl_2$/0.01% gelatin, all four dNTPs (Pharmacia) at either 250 μ*M* (TC-65) or 125 μ*M* (517), and 2.5 units of *Taq* polymerase (Perkin–Elmer/Cetus). The reaction was conducted for 35 cycles of 94°C denaturation (1 min), 55°C annealing (1 min), and 72°C extension (4 min) in an automated thermal cycler (Perkin–Elmer/Cetus). Initial denaturation was at 95°C for 5 min and a final extension was at 72°C for 7 min. Primers were used in reactions after deprotection without further purification.

PCR Products: Gel Electrophoresis, Purification, and Cloning

To analyze the PCR products, they were separated on a 1.3% agarose gel in Tris–borate buffer at 40–50 V for 12–16 h. To isolate individual fragments, the PCR product was electrophoresed on a 1.3% low-melting agarose gel. The fragment was excised from the gel and melted at 65°C, an equal volume of water was added, and approximately 1 μl was reamplified for 35 cycles to allow generation of preparative quantities of the fragment.

To clone the PCR product FG1, it was purified from agarose using the GeneClean Kit (BIO 101) and ligated into *Hinc*II-digested pTZ19R vector (Mead *et al.*, 1986) at a molar ratio of 1:2 vector to insert. The ligation was performed in One Phor-All Plus buffer with T4 ligase (Pharmacia) for 16 h at 16°C. *Escherichia coli* K-12 strain DH5α competent cells were transformed with the ligation products and plated on LB plates containing 100 μg/ml ampicillin. The PCR product FG2 was purified from comigrating contaminants by digestion with *Msp*I and electrophoretic separation of a subfragment of 700 bp.

Southern Analysis

For Southern analysis (Southern, 1975) of genomic DNAs, 10 μg of DNA from each hybrid and 5 μg of human and mouse DNA were digested for >4 h with 3–4 units/μg of the appropriate restriction enzyme. Samples were electrophoresed in Tris–acetate agarose gels with buffer recirculation. For Southern analysis of PCR products, 10% of the amplification products were electrophoresed as described above. Southern transfer, preassociation of all probes, and hybridization were performed as described previously (Patel *et al.*, 1990).

RESULTS

To identify markers from 17p11.2, high-molecular-weight DNA from the hybrid MH22-6 (retaining a single human chromosome 17) and the hybrid DH110-D1 [retaining a del(17)(p11.2p11.2)] was used as a template for amplification with either primer TC-65 or 517. When DNA from MH22-6 was used as a template, approximately 50 fragments, ranging in size from 400 bp to 4 kb, were obtained with primer TC-65 and approximately 30 fragments, ranging in size from 600 bp to 4 kb, were obtained with primer 517. The patterns obtained with DNA from DH110-D1 were more complex, probably because this hybrid retains other human chromosomes.

To reduce the complexity of *Alu*-PCR amplification patterns, DNA from the hybrid DH110-D1 was digested with a number of restriction enzymes, either singly or in combination, prior to performing the *Alu*-PCR. Figure 1 shows the amplification patterns obtained with unrestricted and restricted DNA from DH110-D1. The amplification products of the restricted DNAs are composed of subsets of the products obtained with undigested DNA. It is interesting to note the remarkable reduction in the number of amplification products observed when an enzyme with a four-base recognition sequence, such as *Rsa*I, is used to digest the template. We use the term "restricted *Alu*-PCR" to describe this modification of *Alu*-PCR.

Having demonstrated a reduction in the complexity of the *Alu*-PCR patterns of hybrid DH110-D1, we examined this method for its ability to identify and isolate markers present in one hybrid but absent in another. DNA from hybrids MH22-6 and DH110-D1 was digested with several restriction enzymes and subjected to amplification with either primer TC-65 or 517. Figure 2 shows normal and restricted *Alu*-PCR patterns for hybrids MH22-6 and DH110-D1. The arrows indicate four fragments that are present in the restricted *Alu*-PCR products of MH22-6 but absent in that of DH110-D1.

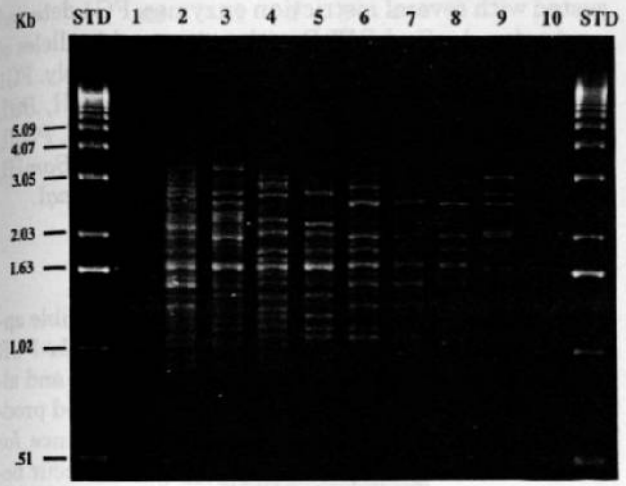

FIG. 1. Simplification of the *Alu*-PCR pattern of the hybrid DH110-D1. One microgram of DNA from the rodent cell line clone 1D and from the hybrid DH110-D1, either uncut or digested with several restriction endonucleases, was subjected to PCR with the *Alu* primer TC-65 and electrophoresed as described under Materials and Methods. The templates are lane 1, clone 1D DNA; lane 2, uncut DH110-D1 DNA; lanes 3–10, DNA from DH110-D1 digested with *Eco*RI + *Bgl*I, *Taq*I, *Ava*II, *Eco*RI + *Pst*I, *Eco*RI + *Pst*I + *Bam*HI, *Bcl*I + *Pvu*II, *Bcl*I + *Bgl*II, and *Rsa*I, respectively. The size standard is the 1-kb ladder (BRL).

These fragments could truly represent inter-*Alu* sequences that fail to amplify when DH110-D1 DNA is used as template. Alternatively, they might represent an artifact associated with restricted *Alu*-PCR. To examine these possibilities, *Alu*-PCR products obtained with undigested DNA from hybrids MH22-6 and DH110-D1 were examined by Southern analysis using each of the isolated fragments FG1, FG2, FG3, and FG4 individually as probes. Each of these probes hybridized to a fragment of similar size in the total amplification products of undigested MH22-6 DNA but was absent in the DH110-D1 products. The efficiency of amplification and transfer was verified by hybridization with a control fragment present in the *Alu*-PCR products of both MH22-6 and DH110-D1. The results obtained for FG3, which are representative of those seen with FG1, FG2, and FG4, are shown in Fig. 3.

To determine the regional localization of these fragments, each was used as a probe for Southern hybridization with a panel of somatic cell hybrids representing different regions of chromosome 17. Both FG1 and FG2 were localized to the 17p11.2 region, as expected, but appeared contaminated with a minor comigrating fragment (data not shown). They were therefore either cloned (FG1) or further purified (FG2) as described under Materials and Methods. Hybridization of purified FG1 and FG2 to the somatic cell hybrid mapping panel confirmed their localization to 17p11.2 (Fig. 4).

The fragments FG3 and FG4 did not map to 17p11.2, as was initially expected. The fragment FG3 mapped to 17q11.2–qter and FG4 to 17p12 (Fig. 4). The failure to amplify inter-*Alu* sequences such as FG3 and FG4 in the DH110-D1 DNA could be due to alterations in the sequences flanking or involving the *Alu* element, which might be detectable as RFLPs. To determine this, FG3 was hybridized to DNA from eight unrelated individuals digested with *Bam*HI, *Bcl*I, *Bgl*II, *Dra*I, *Hin*dIII, *Pst*I, *Rsa*I, *Sst*I, or *Taq*I. FG3 detected an insertion–deletion polymorphism discernible with the enzymes *Bcl*I, *Taq*I, *Pst*I, and *Sst*I as shown in Fig. 5. The difference in the size of the polymorphic alleles with each of these three enzymes was approximately 250 bp. In addition, the polymorphisms appeared to be in complete linkage disequilibrium, as indicated by a similar pattern of allele distribution in unrelated individuals (Fig. 5). The hybrids MH22-6 and DH110-D1, when examined for these polymorphic alleles by hybridization of

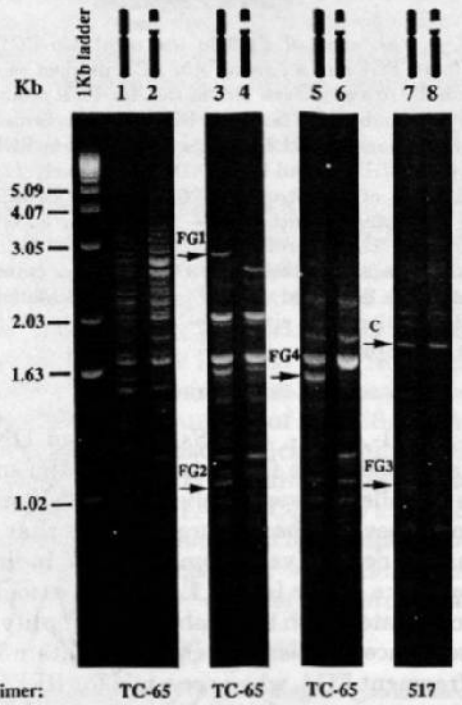

FIG. 2. Comparative restricted *Alu*-PCR of the chromosome 17-retaining hybrids MH22-6 and DH110-D1. Lanes **1, 3, 5, 7** and **2, 4, 6, 8** represent the amplification products from hybrids MH22-6 and DH110-D1, respectively. The status of the template in each lane is: lanes **1** and **2**, uncut; lanes **3** and **4**, digested with the enzymes *Bcl*I, *Pst*I, and *Eco*RI; lanes **5** and **6**, digested with the enzyme *Ava*II; lanes **7** and **8**, digested with the enzymes *Pst*I and *Eco*RI. The *Alu* primer used for each set of amplifications is indicated at the bottom of the figure. FG1, FG2, FG3, and FG4 represent fragments present in MH22-6 amplification products but absent in DH110-D1 amplification products; C represents a control fragment amplified in both hybrids with primer 517, which is relevant to the experiment described in Fig. 3.

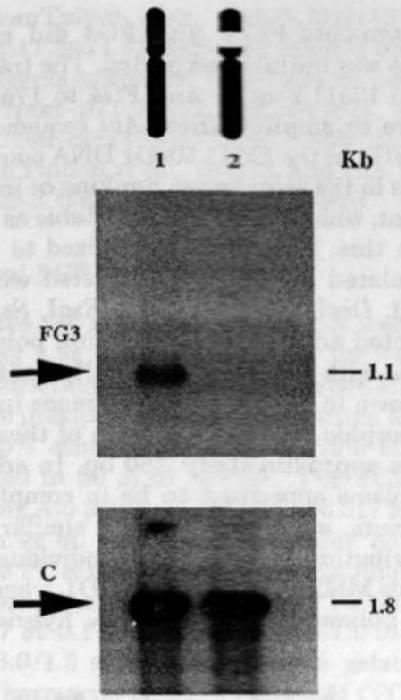

FIG. 3. Absence of FG3 in the total *Alu*-PCR product of DH110-D1. FG3 and a control *Alu*-PCR product were hybridized sequentially to a Southern blot of the *Alu*-PCR products obtained with 1 μg of undigested template DNAs from hybrids MH22-6 and DH110-D1. Lanes 1 and 2 represent the total *Alu*-PCR products of the hybrids MH22-6 and DH110-D1, respectively; C indicates the hybridization of a control *Alu*-PCR product identified in Fig. 2 which was obtained with *Pst*I + *Eco*RI-digested template from both hybrids. The control fragment was isolated from the electrophoretically separated restricted *Alu*-PCR products of DH110-D1, reamplified as described under Materials and Methods and used for hybridization. The size of the fragments in kilobases (kb) is shown on the right.

*Bcl*I-, *Taq*I-, *Pst*I-, and *Sst*I-digested DNAs using FG3 as probe, were found to contain the smaller and the larger allele, respectively (data not shown). Examination of seven other reduced hybrids that retained a normal or derivative chromosome 17 indicated that the presence of the larger *Taq*I allele associated with FG3 correlated with the inability to amplify the inter-*Alu* sequence represented by FG3 (data not shown). The fragment FG4, when screened for RFLPs, did not detect any polymorphism. FG4 was amplified in several other hybrids, including the hybrids 88H5 and HO-11, which all contained the 17p12 region (data not shown), and the mechanism for the lack of amplification in hybrid DH110-D1 is unknown.

FG1 and FG2 mapped to 17p11.2, a region of interest for the study of Charcot–Marie–Tooth disease (Patel *et al.*, 1990) and Smith–Magenis syndrome (Smith *et al.*, 1986; Stratton *et al.*, 1986). They were therefore screened for RFLPs by hybridization with DNAs from eight unrelated individuals that were digested with several restriction enzymes. FG1 detected an *Apa*I and a *Rsa*I RFLP with polymorphic alleles of 12 and 7.5 kb and of 1.5 and 1.1 kb, respectively. FG1 was not polymorphic for *Ava*I, *Ava*II, *Bam*HI, *Bcl*I, *Bgl*I, *Bgl*II, *Eco*RI, *Hin*dIII, *Hin*fI, *Msp*I, *Pst*I, *Pvu*II, *Rsa*I, or *Sst*I. FG2 was not polymorphic for *Bam*HI, *Bcl*I, *Bgl*II, *Eco*RI, *Hin*dIII, *Msp*I, *Rsa*I, or *Taq*I.

DISCUSSION

This study demonstrates a highly reproducible approach for decreasing the complexity of the *Alu*-PCR amplification pattern of somatic cell hybrids and allows a direct comparative analysis of amplified products from hybrid DNA. A recognition sequence for one or more restriction endonucleases can occur between two *Alu* sequence elements juxtaposed in opposing orientations. Digestion of the template with restriction enzyme(s) prior to *Alu*-PCR prevents that particular inter-*Alu* sequence from being amplified. To determine which enzymes would be optimal to use, we considered a previously reported frequency of recognition sequences for restriction enzymes in the human genome (Drmanac *et al.*, 1986). As expected, the reduction in complexity of the amplification pattern

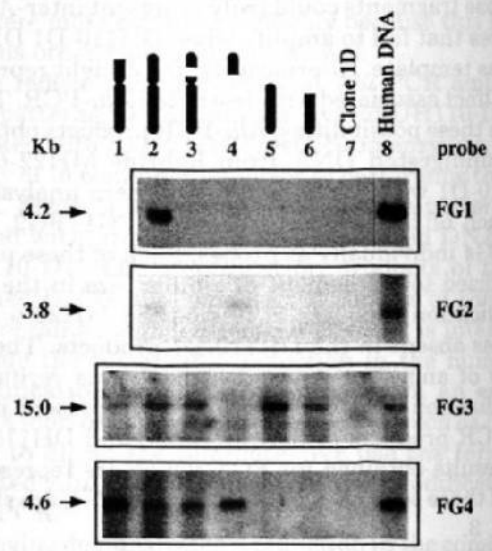

FIG. 4. Hybridization of fragments FG1, FG2, FG3, and FG4 to Southern blots of a somatic cell hybrid mapping panel. For ease of interpretation, hybrid lanes are identified by schematic idiograms representing the portions of chromosome 17 retained in the hybrids. Lanes 1–6, 10 μg of genomic DNA from the hybrids HO-11, MH22-6, DH110-D1, 88H5, LS-1, and SP3, respectively; lane 7, 10 μg of DNA from the parental mouse cell line, Clone 1D; lane 8, 5 μg of human genomic DNA. The individual probes are indicated on the right and the size of the bands in kilobases (kb) is shown on the left. FG1 and FG2 were hybridized to *Pvu*II-digested DNAs, while FG3 and FG4 were hybridized to *Hin*dIII-digested DNAs. FG1 and FG2 map to 17p11.2, FG3 maps to 17q11.2–qter, and FG4 maps to 17p12.

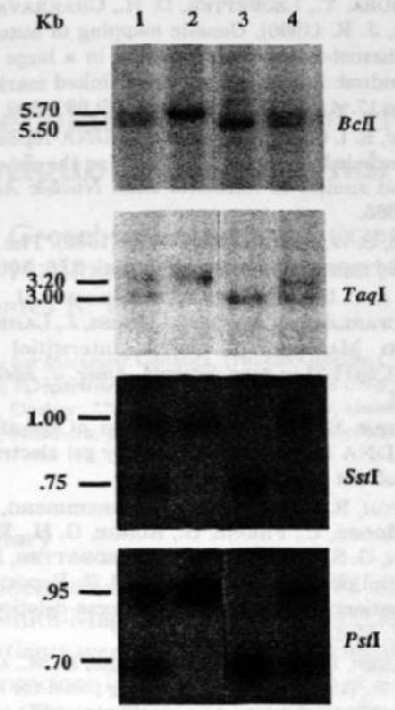

FIG. 5. Insertion–deletion polymorphism detected by FG3. Lanes 1–4 represent DNA from each of the same four unrelated individuals digested with the enzymes indicated on the right. The size of the alleles in kilobases (kb) is shown on the left and indicates that the polymorphic alleles in each case differ in size by about 250 bp and show the same pattern of zygosity in each individual.

was proportional to the frequency of the recognition sequence of the particular enzyme or combination of enzymes used. The presence of new discrete bands in the restricted *Alu*-PCR patterns is probably due to a decrease in competition for the template, which allows significant amplification of regions not obtained with the more complex template. An analogous phenomenon, attributed to template competition, was observed in comparisons of the amplified human sequences in X-chromosome-retaining hybrids (Ledbetter *et al.*, 1990).

The reproducibility of the method allowed us to compare the subsets of *Alu*-PCR products of two chromosome 17-retaining hybrids and to isolate two new markers from the 17p11.2 region. In addition, simplification of the amplification pattern allowed us to detect fragments representing inter-*Alu* sequences that failed to amplify in the hybrid DH110-D1. This failure to amplify an inter-*Alu* sequence may be due to *Alu*-associated polymorphisms. In fact, the fragment FG3 detected an insertion–deletion polymorphism (Anagnou *et al.*, 1984; Frossard *et al.*, 1986; Woods-Samuels *et al.*, 1989) involving a sequence of approximately 250 bp. The difference in the size of the polymorphic alleles, which approximates the length of a single *Alu* repeat, suggests that the polymorphism could have arisen by the insertion or deletion of a single *Alu* element. The FG3 allele of the chromosome 17 retained in MH22-6 is 250 bp smaller than the corresponding allele in DH110-D1. We speculate that the presence of the "inserted" sequence in the chromosome 17 retained in DH110-D1 impairs the amplification of the inter-*Alu* sequence represented by FG3. Thus, restricted *Alu*-PCR may be applicable to the direct isolation of polymorphic markers.

The fragment FG4 was also absent in the amplification products of hybrid DH110-D1, even though it did not map to 17p11.2. A limited survey with the enzymes *Bgl*II, *Hind*III, *Hin*fI, *Msp*I, *Rsa*I, and *Taq*I did not reveal any RFLPs associated with FG4. The lack of amplification of FG4 using DH110-D1 DNA may be due to other types of polymorphisms associated with *Alu* elements (Economou *et al.*, 1990; Zuliani and Hobbs, 1990; Rogaev, 1989), which are not detectable by genomic Southern analysis. These polymorphisms may affect the amplification because of differences in the base composition at the primer annealing site, secondary structure effects, or other mechanisms. The inversion of the orientation of a single *Alu* repeat might also be an additional mechanism that impairs the amplification of a specific *Alu*-PCR product.

The digestion of template DNA with restriction enzymes can enhance the usefulness of *Alu* primers containing degenerate sequences, which have a high frequency of target reiteration and yield a very large number of amplification products. This method can also be used to analyze hybrids that retain a large number of human chromosomes. It may be used to reduce selectively the number of amplification products resulting from a yeast artificial chromosome vector or a radiation hybrid containing a large segment of human DNA. Restricted *Alu*-PCR may also facilitate the detection and isolation of PCR products obtained using specific primers coupled with *Alu* primers by reducing the background of inter-*Alu* products.

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Molecular Analysis of the Smith-Magenis Syndrome: A Possible Contiguous-Gene Syndrome Associated with del(17)(p11.2)

Frank Greenberg,*†‡§ Vito Guzzetta,* Roberto Montes de Oca-Luna,* R. Ellen Magenis,‖ A. C. M. Smith,# Sarah F. Richter,** Ikuko Kondo,†† William B. Dobyns,‡‡ Pragna I. Patel,*† and James R. Lupski*†‡§

*Institute for Molecular Genetics, †Human Genome Center, ‡Department of Pediatrics, and §Texas Children's Hospital, Baylor College of Medicine, Houston; ‖Department of Medical Genetics and Child Development Rehabilitation Center, Oregon Health Sciences University, Portland; #Children's Hospital, Denver; **Department of Pediatrics, University of Arizona, Phoenix; ††Department of Human Ecology and Genetics, University of Ryukyus, Nishihara, Japan; and ‡‡Departments of Neurology and Medical Genetics, Indiana University School of Medicine, Indianapolis

Summary

We undertook clinical evaluation (32 cases) and molecular evaluation (31 cases) of unrelated patients affected with Smith-Magenis syndrome (SMS) associated with an interstitial deletion of band p11.2 of chromosome 17. Patients were evaluated both clinically and electrophysiologically for peripheral neuropathy, since markers showing close linkage to one form of Charcot-Marie-Tooth disease (CMT1A) map to this chromosomal region. The common clinical findings were broad flat midface with brachycephaly, broad nasal bridge, brachydactyly, speech delay, and hoarse, deep voice. Fifty-five percent of the patients showed clinical signs (e.g., decreased or absent deep tendon reflexes, pes planus or pes cavus, decreased sensitivity to pain, and decreased leg muscle mass) suggestive of peripheral neuropathy. However, unlike patients with CMT1A, these patients demonstrated normal nerve conduction velocities. Self-destructive behaviors, primarily onychotillomania and polyembolokoilamania, were observed in 67% of the patients, and significant symptoms of sleep disturbance were observed in 62%. The absence of REM sleep was demonstrated by polysomnography in two patients. Southern analysis indicated that most patients were deleted for five 17p11.2 markers—FG1 (D17S446), 1516 (D17S258), pYNM67-R5 (D17S29), pA10-41 (D17S71), and pS6.1-HB2 (D17S445)—thus defining a region which appears to be critical to SMS. The deletion was determined to be of paternal origin in nine patients and of maternal origin in six patients. The apparent random parental origin of deletion documented in 15 patients suggests that genomic imprinting does not play a role in the expression of the SMS clinical phenotype. Our findings suggest that SMS is likely a contiguous-gene deletion syndrome which comprises characteristic clinical features, developmental delay, clinical signs of peripheral neuropathy, abnormal sleep function, and specific behavioral anomalies.

Introduction

Contiguous-gene syndromes are recognizable syndromes and comprise microdeletion and microduplication syndromes (Schmickel 1986; Ledbetter and Cavenee 1989). Specific features of these syndromes may occur individually in families, as phenotypes segregating in a Mendelian fashion. The complex phenotypic abnormalities may result from DNA rearrangements involving several contiguous genes (Schmickel 1986). These syndromes are typically described as clinical entities, prior to establishment of a chromosomal etiology. The cytogenetic abnormality is consistently small and difficult or impossible to detect by routine methods. Some patients with the complete clinical phenotype demonstrate no visible cytogenetic abnormality even after high-resolution analysis.

Contiguous-gene deletion syndromes include retinoblastoma with mental retardation (MR) (del 13q14); Wilms tumor, aniridia, genital abnormalities, and re-

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Address for correspondence and reprints: James R. Lupski, M.D., Ph.D. Institute for Molecular Genetics, Baylor College of Medicine, One Baylor Plaza, T-905, Houston, TX 77030.

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tardation (WAGR) (del 11p13); Langer-Gideon syndrome (del 8q24); Prader-Willi and Angelman syndromes (del 15q11); α-thalassemia and MR (del 16p13.3); Miller-Dieker syndrome (del 17p13); and DiGeorge syndrome (del 22q11) (Schmickel 1986; Ledbetter and Cavenee 1989). In addition, other syndromes associated with terminal deletions of chromosomes, such as Wolf-Hirschhorn syndrome (del 4p16) (Ivens et al. 1990) and cri-du-chat syndrome (del 5p16) (Overhauser et al. 1989), likely represent phenotypes associated with DNA rearrangements involving contiguous genes. Male patients with multiple X-linked disorders that are due to deletion of contiguous genes in the Xp21 region and that lead to various combinations of Duchenne muscular dystrophy, chronic granulomatous disease, McCleod phenotype, retinitis pigmentosa, glycerol kinase deficiency, congenital adrenal hypoplasia, ornithine transcarbamoylase deficiency, and various degrees of MR have been described (Francke et al. 1987). Similarly, deletions of the Xp22 region have been demonstrated to have combinations of steroid sulfatase deficiency, Kallman syndrome, and MR (Ballabio et al. 1989).

Smith-Magenis syndrome (SMS) is a clinically recognizable multiple congenital anomaly/MR syndrome due to an interstitial deletion of chromosome 17p11.2. The disorder was first described by Smith et al. in 1982, and the spectrum of clinical features was delineated in 1986 by Smith et al. and Stratton et al. To date, a total of 27 patients have been reported (Patil and Bartley 1984; Smith et al. 1986; Stratton et al. 1986; Popp et al. 1987; Lockwood et al. 1988; Colley et al. 1990; Hamill et al. 1990). Recently, DNA markers linked to the gene for Charcot-Marie-Tooth disease type 1A (CMT1A) were mapped to 17p11.2 (Raeymaekers et al. 1989; Vance et al. 1989, 1991; Chance et al. 1990; McAlpine et al. 1990; Middleton-Price et al. 1990; Patel et al. 1990*a*, 1990*b*). A somatic-cell hybrid panel (van Tuinen et al. 1987) was used to map these linked markers to 17p11.2 by virtue of absence of Southern hybridization to a hybrid constructed from a del(17)(p11.2) patient (Patel et al. 1990*a*, 1990*b*). Because of the mapping of the CMT1A gene to this region of chromosome 17, we evaluated 32 SMS patients to determine whether they had evidence of peripheral neuropathy consistent with CMT disease. In addition, we evaluated other clinical findings in order to define the common and variable features of the syndrome. A molecular analysis of the deletion in these SMS patients was performed using proximal 17p DNA markers and analysis for DNA polymorphisms. To determine whether genomic imprinting plays a role in the SMS phenotype, as has been discovered to be the case for chromosome 15 in the Prader-Willi and Angelman microdeletion syndromes (Nicholls et al. 1989; Williams et al. 1990), we studied the parental origin of deletion in SMS patients.

Subjects and Methods

Subjects

Of the thirty-two SMS patients evaluated, 22 were ascertained by the cytogenetic laboratories and genetic clinics at Baylor College of Medicine, Denver Children's Hospital, Oregon Health Sciences University, and the University of Arizona at Tucson, and 10 patients were ascertained by other genetic centers. Most patients were ascertained for dysmorphic features and/or developmental delay, and chromosome analysis demonstrated del(17)(p11.2). Six previously reported and 26 newly identified patients were evaluated. Twenty of them were examined by two authors of the present paper (F. Greenberg and J. R. Lupski). The clinical evaluations were done by using standardized forms which included demographic, anthropometric, morphologic, developmental, behavioral, sleep-habit, and neurologic findings. Nerve-conduction studies were performed through the local Muscular Dystrophy Association (MDA) clinics. Data on each of the patients were tabulated and entered into a data base file by using a dBase *III Plus*® program. Data on chromosome analysis, including high-resolution banding patterns for chromosome 17, were available for all patients. Thirty-one of the 32 patients reported here had a deletion of proximal 17p, while one patient (93-360) had a translocation with one breakpoint in 17p11.2. Thirty-one of the 32 patients were analyzed by molecular methods.

DNA Probes

The probes used in the present study, their chromosomal location, the restriction enzyme displaying polymorphisms, expected allele sizes, and source and/or reference are listed in table 1. Probe cH3 is a cosmid identified from a library constructed from flow-sorted human chromosome 17 by using FG-1 (Guzzetta et al. 1991) (D17S446) as a hybridization probe. A 900-bp *Taq*I fragment containing a $(GT)_{19}$ sequence was identified from cH3 and cloned into pTZ19R (pRM7-GT), and the nucleotide sequence was obtained by the dideoxy method using Sequenase® (U.S. Biochemi-

Table 1

DNA Markers

Marker (locus)	Location	RFLP	Allele Size (kb)	Reference
LEW301 (D17S58)	17cen-p11.2	*TaqI*	4.5/3.1	Barker et al. 1987
		BglII	10.0/8.0	Barker et al. 1987
FG1 (D17S446)	17p11.2	*ApaI*	12.0/7.5	Guzzetta et al. 1990
pYNM67-R5 (D17S29)	17p11.2	*TaqI*	3.4/2.0 + 1.3	Ray et al. 1990
		BglII	8.1/6.7	Ray et al. 1990
c1516 (D17S258)	17p11.2	*HindIII*	22.0/12 + 10	Patel et al. 1990*a*
p1516-R4 (D17S258)	17p11.2	*MspI*	3.3/2.4	Franco et al. 1990
pA10-41 (D17S71)	17p11.2	*MspI*	2.4/1.9	Barker et al. 1987
		PvuII	3.2/3.0	Barker et al. 1987
pS6.1-HB2 (D17S445)	17p11.2	*MspI*	1.7/1.3	Present study
			1.1/1.0	Patel et al. 1990*b*
		SstI	12.2/7.1	Patel et al. 1990*b*
VAW409R1 (D17S122)	17p11.2-p12	*MspI*	5.3/2.7 + 2.6	Wright et al. 1990
VAW409R3 (D17S122)	17p11.2-p12	*MspI*	2.8/2.7/1.9	Wright et al. 1990
VAW412R3 (D17S125)	17p11.2-p12	*MspI*	10.5/5.4	Wright et al. 1990
EW401 (D17S61)	17p11.2-p12	*MspI*	5.2/4.4	Wright et al. 1990
c1517 (D17S259)	17p11.2-p12	*MspI*	6.2/4.0/2.4	Patel et al. 1990*a*
VAW410R1 (D17S123)	17p11.2-p12	*BglII*	2.1/2.0	Wright et al. 1990
		TaqI	10.0/9.4	Wright et al. 1990
EW405 (D17S121)	17p11.2-p12	*MspI*	2.0/1.5	Wright et al. 1990
VAW411R2 (D17S124)	17p11.2-p12	*MspI*	10.5/6.1	Wright et al. 1990
		BglII	11.0/10.7	Wright et al. 1990
EW403 (D17S63)	17p11.2-p12	*MspI*	13.5/6.8	Wright et al. 1990
EW503 (D17S67)	17p11.2-p12	*MspI*	6.9/5.7	Wright et al. 1990
EW502 (D17S66)	17p11.2-p12	*BglII*	2.2/1.4	Wright et al. 1990

cals). Flanking PCR primers were synthesized by standard methods and used to analyze $(GT)_n$ polymorphisms as described (Weber and May 1989). The sequence of the priming oligodeoxynucleotide on the GT strand of pRM7-GT is 5′-ATTATTTATTTTG-ATGTCTGAACAC-3′, while that of the priming oligodeoxynucleotide on the CA strand of pRM7-GT is 5′-CTTGGTGAAACGCTGTCTGTAC-3′. The latter primer has homology to the *Alu* repeat sequence.

Southern Analysis and Densitometry

Southern analysis was performed as described elsewhere (Patel et al. 1990*a*; Franco et al. 1991). Equal amounts (5 μg) of digested genomic DNA were included in each lane to ensure reproducibility of densitometric signal. All probes were labeled by the random hexanucleotide priming method (Feinberg and Vogelstein 1983). If a marker was not fully informative for RFLPs, the copy number was determined by dosage analysis of signals obtained by simultaneous hybridization of the experimental marker and the marker DR47, representing a single-copy sequence on chromosome 9. The intensity of the bands in each lane was quantified using an LKB 2400 Gel Ultrascan XL laser densitometer as described elsewhere (Franco et al. 1991). The analysis of segregation patterns for alleles at the VNTR locus YNH24 (D2S44) (Nakamura et al. 1987) demonstrated no instances of false paternity.

PCR and GT Repeat Polymorphism Screening

The unique sequence primer from the GT strand of marker RM7-GT was end labeled at 37°C in a 15-μl reaction volume containing 1.2 μM primer, 100 μCi [γ^{32}P]ATP at 6,000 Ci/mmol, 1 × One Phor-All Plus buffer (Pharmacia), and 10 units polynucleotide kinase (Pharmacia). The T4 polynucleotide kinase was heat inactivated by incubating the reaction mixture at 65°C for 10 min. The end-labeled primer resulting from the kinase reaction was used directly in the PCR reaction, without separating the unincorporated nucleotides (0.40 μl/reaction). PCR was performed using standard conditions in a 25-μl reaction volume. The reaction mixture contained 1 μM of each oligodeoxynucleotide primer, 250 μM each of dATP,

dCTP, dGTP, and dTTP, 2.5 μl 10 × PCR buffer (500 mM KCl, 120 mM Tris HCl [pH 8.0], 1.5 mM $MgCl_2$, and 0.01% gelatin), 0.63 units of AmpliTaq (Cetus) DNA polymerase, and 0.4 μl end-labeled primer, as stated above. The amplification conditions were an initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C denaturation (1 min), 55°C annealing (1 min) and 72°C extension (2 min) in an automated thermal cycler (Perkin Elmer—Cetus). Reaction products (1.5 μl) were mixed with 2 μl formamide stop solution (U.S. Biochemicals) and electrophoresed in a 6% polyacrylamide DNA sequencing gel at 40 W for 3.5 h. Gels were dried and autoradiographed for 2–12 h by exposing them to Kodak XAR-5 film with either one or two intensifying screens at −70°C.

Results

Clinical Spectrum of SMS

In the present series of 32 patients, there were 14 males and 18 females. The age range was 1 mo–72 years, with a mean of 15 years and a median of 10 years. The mean maternal age was 26 years, and the mean paternal age was 30 years. The percentages of findings for the most common physical features of the 26 newly ascertained SMS patients we examined are shown in table 2 and are compared with those of the 27 previously reported patients (Patil and Bartley 1984; Smith et al. 1986; Stratton et al. 1986; Popp et al. 1987; Lockwood et al. 1988; Colley et al. 1990; Hamill et al. 1990).

The most common physical findings were brachycephaly with flat midface, broad nasal bridge, brachydactyly, and short stature (usually 2–3 SDs below the mean for age). Clinical symptoms included failure to thrive in infancy and limitation of movement at the elbow, which in some cases was documented to be associated with radioulnar synostosis. Common but less consistent physical abnormalities included prominent forehead, synophrys, prognathism, posteriorly rotated ears, low-set ears, and/or other ear anomalies. Abnormalities seen in a smaller percentage of patients were congenital heart defects (primarily ventricular or atrial septal defects) in 31% and cleft lip and palate in 7%. Less common findings included cutaneous syndactyly of the fingers or toes, microcornea, iris coloboma, and craniosynostosis. Another unusual and striking feature noted in the older children, adolescents, and adults was a hoarse, deep voice, which was noted in 82% of the patients in the present study.

Of the 32 patients evaluated, pes planus or pes cavus was noted in 48%, and 24% had scoliosis. A total of 17 (55%) of 31 patients had clinical signs, including significantly decreased or absent deep-tendon reflexes and insensitivity to pain, which suggested peripheral neuropathy. A summary of the presumed neuropathic changes and nerve-conduction studies in these patients is shown in table 3. On the basis of previous psychometric testing (in most cases), the SMS patients showed varying degrees of MR, with the majority fall-

Table 2

SMS (del 17p11.2) Physical Features

Physical Feature	% of Reported Patients[a] (*N* = 21)	Proportion of Reevaluated Patients (*N* = 6)	Proportion (%) New Patients (*N* = 26)	% of Total Patients (*N* = 53)
Flat midface	95	6/6	24/26 (92)	94
Brachycephaly	86	5/6	21/26 (81)	83
Prominent forehead	81	1/6	16/26 (62)	64
Broad nasal bridge	76	4/6	23/26 (88)	81
Prominent jaw	38	4/6	15/26 (58)	51
Ear abnormalities	67	5/6	17/26 (65)	68
Brachydactyly	81	5/6	21/26 (81)	81
Limitation at elbow	NE	3/6	5/22 (23)	29
Pes planus/cavus	NE	3/6	11/23 (48)	61
Scoliosis	NE	3/6	4/23 (17)	24
Congenital heart defect	38	1/6	6/21 (29)	31

[a] NE = not specifically examined for in other studies.

Table 3

Clinical Signs Suggestive of Peripheral Neuropathy and Peroneal NCV in 19 SMS Patients

Patient (Age in years)	Status of[a]				Peroneal Nerve-Conduction[b] Velocity (m/s)
	Pes Cavus or Pes Planus	Scoliosis	Decreased or Absent DTR	Insensitivity to Pain	
55-200 (8)	+	+	+	+	R39.4 and L45.9
56-203[c] (4)	+	−	−	−	62.5
57-206 (19)	+	+	ND	ND	ND
64-239 (10)	+	−	+	−	ND
65-241 (16)	+	−	+	+	51
66-244 (20)	−	−	+	−	ND
67-246 (21)	+	−	+	−	55
68-248 (6)	−	−	+	−	58
69-251 (12)	+		+	+	59
71-255 (14)	+	−	−	+	ND
75-266 (4)	−	−	−	+	50
78-280 (2)	−	−	+	+	68.7
79-283[c] (18)	+	+	−	−	65
94-362 (72)	−	+	+	−	44
95-363 (40)	+	+	−	+	51
96-364 (26)	−	−	+	−	49
100-389 (2)	+	+	ND	ND	ND
112-474 (35)	−	+	ND	+	ND
112-475 (20)	+	+	−	ND	ND

[a] + = Present; − = absent; ND = not determined.

[b] Normal value, expressed as mean ± SD in 120 nerves from 60 patients who were 16–86 (mean 41) years and who had no apparent disease of the peripheral nerves with site of stimulation below the knee, was 48.3 ± 3.9 (Kimura 1989).

[c] Had no significant signs of peripheral neuropathy, but peroneal nerve-conduction velocity was measured.

ing within the moderate range. Other neurobehavioral abnormalities included infantile hypotonia, seizures, developmental delay with speech delay greater than motor delay, conductive hearing loss, and hyperactivity (table 4). Sixty-two percent of patients had symptoms of a sleep disorder which manifested as difficulty falling asleep, difficulty staying asleep, and frequent awakening during the night. Both one previously reported patient (patient 2 in Stratton et al. 1986) and one of the present study's patients (64-239) had absence of REM sleep, documented by polysomnography. Self-destructive behavior was noted in 67% of patients. This behavior consisted of head banging, wrist biting, onychotillomania (pulling out fingernails and toenails) and polyembolokoilamania (insertion of foreign bodies into body orifices).

Several of the physical and behavioral findings either appeared to be more noticeable with increasing age or demonstrated an age-dependent penetrance; these included frontal prominence, prognathism, brachydactyly, and the hoarse voice. In addition, facial features appeared to coarsen somewhat with age. Although onychotillomania was uncommon under 5–6 years of age, in some patients self-destructive behaviors such as head banging and wrist biting were noted as early as the second year of life.

Five patients were of particular note. Patient 55-200, a 9-year-old severely mentally retarded boy with cleft lip and palate and congenital heart defect (patient 2 in Smith et al. 1986), had clinical evidence of a peripheral neuropathy including decreased deep-tendon reflexes in the arms, absent reflexes in the legs, a stork-leg deformity of the legs, and prominent pes cavus. Nerve-conduction studies showed a velocity of 39.4 m/s in the right common peroneal nerve and 45.9 m/s in the left (table 3). The right and left peroneal nerve had increased distal latencies (R = 4.02 milliseconds; L = 5.12 milliseconds) and decreased base-to-peak amplitude (R = 1.5 K; L = 2.5 K). Sural sensory-nerve responses were absent bilaterally. Patient 92-357 had del(17)(p11.2p12)—a deletion involving 17p11.2—but did not have a phenotype similar to those of the other patients, and the only consistent findings were broad nasal bridge, short-stature,

Table 4

SMS (del 17p11.2) Behavioral/Functional Features

Behavioral/Functional Features	% of Reported Patients[a] (*N* = 21)	Proportion of Reevaluated Patient (*N* = 6)	Proportion (%) of New Patients (*N* = 26)	% of Total Patients (*N* = 53)
Infantile hypotonia	71	2/5	8/15 (53)	66
Seizures	32	1/5	5/24 (21)	30
Short stature/FTT	61	5/6	21/23 (91)	78
Speech delay	92	4/4	21/22 (95)	98
Conductive hearing loss	58	4/5	12/18 (67)	67
Hoarse, deep voice	62	4/5	15/18 (65)	74
Hyperactivity	92	5/5	15/23 (65)	82
Sleep disorder	31	1/3	15/23 (65)	51
Possible peripheral neuropathy	NE	4/5	13/26 (50)	55
Self-destructive behavior	73	5/6	15/24 (63)	70

[a] NE = not specifically examined for in earlier studies (i.e., Smith et al. 1982, 1986; Patil and Barsky 1984; Stratton et al. 1986; Popp et al. 1987; Lockwood et al. 1988; Colley et al. 1990; Hamill et al. 1990).

speech delay, hoarse voice, and hyperactivity. Patient 93-360 had an apparently balanced translocation—46,XY,t(2;17)(p25.3;11.1), with a breakpoint in the Smith-Magenis region—involving chromosome 2 and chromosome 17. He had relatively mild physical findings similar to those seen in SMS patients and had some behavioral problems, including hyperactivity without self-destructive behavior. Patient 94-362 (patient 8 in Smith et al. 1986), 72 years of age at the time of the present study, was noteworthy for being the oldest patient in our study. Patient 112-474 was clinically diagnosed as having SMS and subsequently was confirmed, by high-resolution cytogenetic analysis, to have del(17)(p11.2), demonstrating that SMS is a clinically recognizable syndrome.

SMS Is Associated with Deletion of Proximal 17p DNA Markers

All the patients reported here had cytogenetic evidence of a DNA rearrangement involving 17p11. Previous cytogenetic analysis revealed del(17)(p11.2) in all but three patients. Patient 93-360 had translocation t(2;17)(p25.3p11.1) with one breakpoint in 17p11, while two patients, 92-357 and 100-389, had deletions that appeared to extend distally [del(17)(p11.2p12)] by cytogenetic analysis. In order both to define the deletion interval common to most SMS patients and to identify patients who had novel deletion intervals, genomic DNA isolated either from peripheral lymphocytes or from Epstein-Barr virus–transformed lymphoblastoid cell lines was examined by Southern analysis using several proximal 17p DNA markers as probes. Each DNA marker displays one or more RFLPs (table 1) after digestion of genomic DNA with the appropriate enzyme(s). RFLP analysis was then used to determine deletion status, with parental DNA being used as a control when it was available. The data are tabulated in figure 1. A plus sign indicates the presence of the DNA marker, while a minus sign indicates deletion of that DNA marker in that patient. A boldface plus or minus sign indicates a fully informative RFLP analysis, while the plain plus and minus symbols indicate deletion status determined by measuring the dosage of an allele by using densitometry.

Five DNA markers—FG-1, pYNM67-R5, c1516, pA10-41, and pS6.1-HB2—were deleted in almost all SMS patients. These markers appear to define a region critical to the SMS phenotype. Analysis of the deletion status of these and additional 17p markers in individual SMS patients revealed the following: (1) Patient 92-357 was deleted for the four markers pA10-41, pS6.1-HB2, EW401, and EW405 but not for FG-1, pYNM67-R5, or c1516, indicating a more distal deletion when compared with the other patients. Thus, these data suggest that FG-1, pYNM67-R5, and c1516 are proximal markers in the critical SMS region, that pA10-41 and pS6.1-HB2 are distal in the critical SMS region, and that EW401 and EW405 are distal to the SMS critical region. (2) Except for patient 92-357, the majority of the patients were not deleted

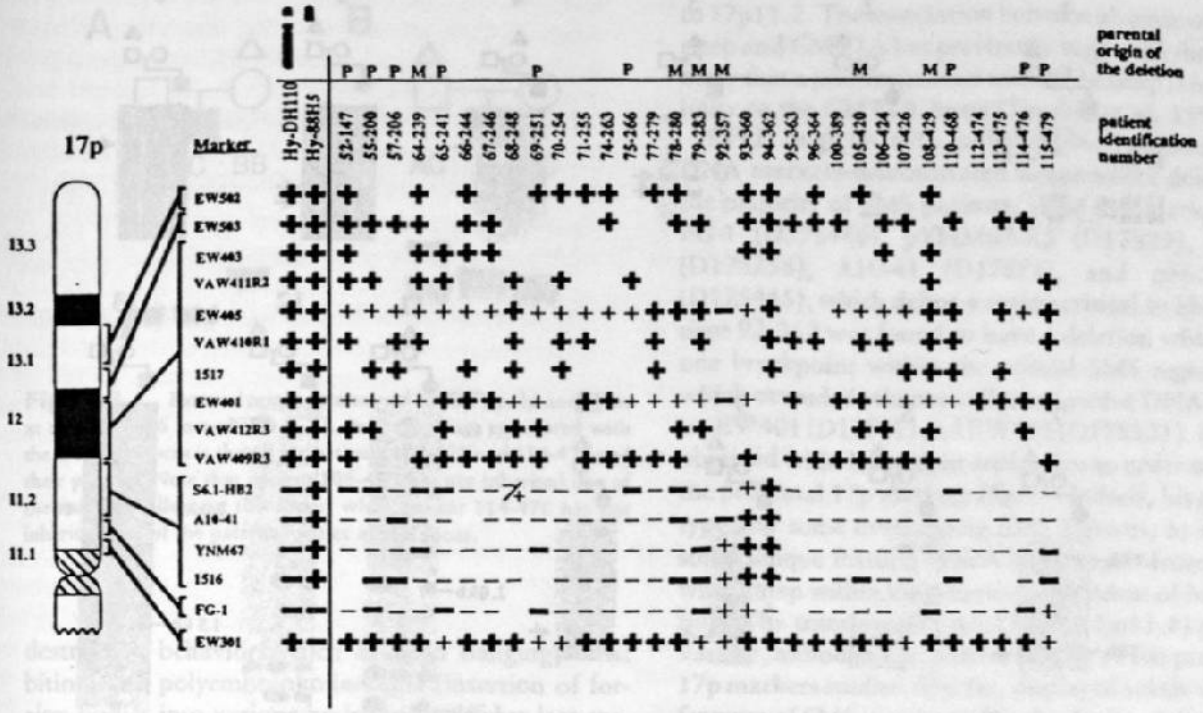

Figure 1 Deletion status of 17p DNA markers in SMS patients. An idiogram illustrating both the short arm of chromosome 17 and the relative position of individual markers is shown on the left. Marker order, from VAW409R3 distal, is taken from the published genetic map of Wright et al. (1990), except for 1517, whose precise position with respect to flanking markers is not known. The order of markers proximal to VAW409R3 is from the present study and is based on the deletion analysis of SMS patients, especially 92-357, and of hybrids DH110-D1 and MH22-6. Within the deletion interval encompassed by 1516, YNM67, A10-41, and S6.1HB2, the relative order of 1516 with respect to YNM67 and of A10-41 with respect to S6.1-HB2 cannot be determined from this analysis. Informative analysis for marker FG-1 (D17S446) was determined by $(GT)_n$ polymorphism analysis as in fig. 3. A blank space within the region encompassed by DNA markers EW301–S6.1-HB2 represents data which were not informative. For most markers distal to VAW409R3, except for EW401 and EW405 where patient 92-357 was deleted by a fully informative analysis, only fully informative heterozygous individuals are listed. If more than one marker was used at a specific locus (e.g., c1516 and p1516-R4 at D17S258; VAW409R1 and VAW409R3 at D17S122) or if more than one polymorphism was recognized by a single probe (e.g., EW301, pYNM67-R5, pA10-41, and pS6.1-HB2), a cumulative deletion status was scored. The first two columns illustrate the results obtained with the 17p DNA markers by using human chromosome 17–retaining somatic-cell hybrids. MH22-6 retains an intact human chromosome 17 as its only human complement, while 88H5 retains the distal portion of 17p with a breakpoint in the SMS region. The other 31 columns are from individual SMS patients. The − / + designation at the S6.1-HB2 locus for patient 68-248 reflects an apparent deletion, by densitometry, with one polymorphism but not with the other. Further studies, using somatic-cell hybrids, are in progress.

for EW301, EW401, EW402, EW403, EW404, EW405, VAW409R1, VAW409R3, VAW410R1, VAW411R2, VAW412R3, EW502, and EW503. (3) Patient 93-360 (with a 2;17 translocation) was found not to be deleted for any of the markers studied. (4) Patient 94-362, the oldest patient, appeared to be deleted for only one DNA probe, FG-1.

Parental Origin of Deletion

Since SMS is purportedly caused by a de novo deletion of chromosome 17 in a parental gamete, we sought to determine whether the deletion occurred preferentially in the paternal or maternal gamete. Parental origin of the deletion was determined by following the inheritance of polymorphic alleles, as shown in figures 2 and 3. Examples of fully informative Southern analyses of patients and parents are shown in figure 2, while similar fully informative $(GT)_n$ polymorphism analyses are shown in figure 3. The parental origin of the deletion could be determined in 15 patients. The deletion was paternally derived in nine individuals and was maternally derived in six individuals. Within this group of 15 patients, there appeared to be no significant clinical differences between indi-

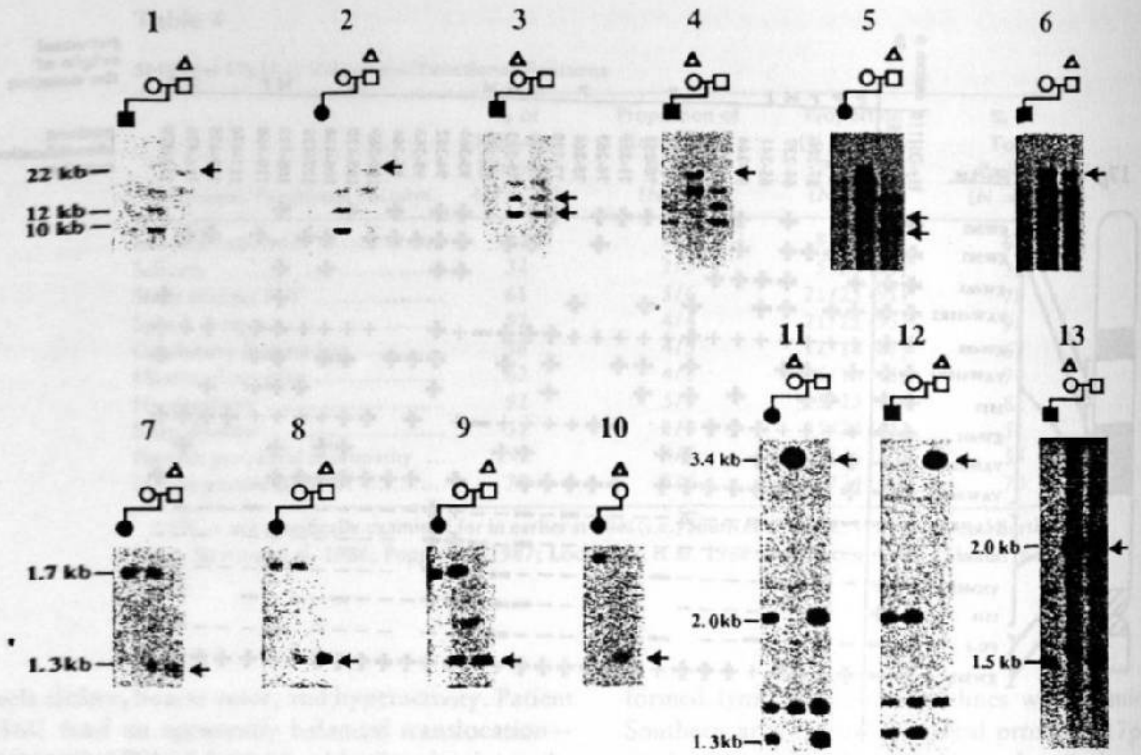

Figure 2 Parental origin of deletion in SMS patients. Genomic DNA from 13 SMS patients and their parent(s) was digested with the indicated restriction endonuclease and subjected to Southern analysis with the indicated probe. Panels 1–6, *Hin*dIII-digested DNA from patients 55-200, 57-206, 79-283, 105-420, 110-468, and 115-479 respectively, that was hybridized to 1516. Panels 7–10, *Msp*I-digested DNA from patients 75-266, 65-241, 52-147, and 78-280, respectively, that was hybridized to pS6.1-HB2. Panels 11 and 12, *Taq*I-digested DNA from patients 64-239 and 69-251, respectively, that was hybridized to pYNM67-R5. Panel 13, *Msp*I-digested DNA from patient 92-357 that was hybridized to EW405. The pedigree structure is shown above each autoradiograph. A triangle above a symbol identifies the parent who was the origin of the deletion. The sizes of the alleles are shown on the left of each blot. The arrow depicts the deleted allele in the SMS patient. The blots shown are the results obtained using a DNA marker that gave a fully informative analysis.

viduals with paternally derived and individuals with maternally derived deletions.

Discussion

SMS, associated with an interstitial deletion of the short arm of chromosome 17, was first described in 1982. Although the number of patients reported is relatively small, there are likely many unreported patients who will be ascertained with improvement in techniques for high-resolution cytogenetic banding. In Harris County, Texas, over a 2-year time period, we have detected four infants with this deletion, suggesting a minimum birth prevalence of approximately 1/25,000. Thus, SMS may be more common than cri-du-chat syndrome (del 5p16) (Niehbuhr 1978), which has an estimated frequency of 1/50,000, and about as common as Prader-Willi syndrome (del 15q12) (Burd et al. 1990). On the basis of the frequency of SMS, our preliminary investigations indicate that this syndrome may be a relatively common cause of MR, because of deletion of a specific chromosomal region.

As determined in the present study, clinical findings in SMS patients were dysmorphic features including brachycephaly, broad nasal bridge, mild synophrys, posteriorly rotated or low-set ears, prognathism, and brachydactyly. Clinical symptoms of the patients included failure to thrive in infancy, short stature, infantile hypotonia, developmental delay, and subsequent MR with speech and language delay greater than motor delay. Variable features included cleft lip and/or palate, congenital heart defect, microcornea, and craniosynostosis. Self-destructive behavior, particularly onychotillomania (pulling out fingernails or toenails) was common in older individuals. Other self-

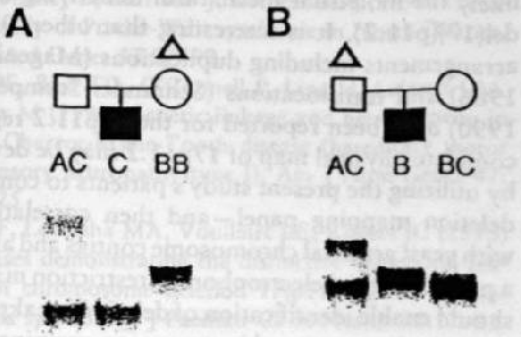

Figure 3 Parental origin determined by $(GT)_n$ polymorphism at the D17S446 locus. The $(GT)_n$ polymorphism associated with the D17S446 locus is shown for patients 108-429 and 114-476 and their parents. Note that patient 108-429 has not inherited one of the maternal alleles at this locus, while patient 114-476 has not inherited one of the paternal alleles at this locus.

destructive behaviors, such as head banging, wrist biting, and polyembolokoilamania (insertion of foreign bodies into various body orifices) were less specific for this disorder. About two-thirds of patients had sleep disturbance, and two patients studied by polysomnography had absence of REM sleep.

Onychotillomania due to picking or manipulation of the nails is a condition which has been reported (*a*) in association with either delusion of infestation or depressive neurosis or (*b*) as an isolated finding (Sait et al. 1985; Colver 1987). SMS patients have more severe manifestations of onychotillomania in that they have been observed to extract the entire nail from the nail bed. The severe expression is probably related to what has been observed in many SMS patients: relative insensitivity to pain. This insensitivity to pain may be a consequence of peripheral neuropathy, altered emotional response to pain, or both. This type of onychotillomania may be relatively specific to this disorder.

Absence of REM sleep is a rare disorder, and its effects are uncertain (Hobson 1990). Although 62% of patients in the present study had clinical histories of sleep disorders, thus far only two patients have had formal sleep evaluations; and both of these patients were found to have absence of REM sleep, without any exposures to medication. The behavioral abnormalities in SMS patients may be related to decreased REM sleep. The observation of the absence of REM sleep in SMS patients suggests that this may be due to a loss of a gene, involved in sleep function, that maps to 17p11.2. The association between absence of REM sleep and CMT1A has previously suggested the possibility that a gene associated with REM sleep is in proximity to the CMT1A locus (Tandan et al. 1990).

RFLP analysis with polymorphic proximal 17p DNA markers demonstrated five markers deleted in the majority of SMS patients; these five markers are FG-1 (D17S446), pYNM67-R5 (D17S29), c1516 (D17S258), A10-41 (D17S71), and pS6.1-HB2 (D17S445), which define a region critical to SMS. Patient 92-357 was found to have a deletion which had one breakpoint within the critical SMS region and which extended telomerically, to involve DNA markers EW401 (D17S61) and EW405 (D17S121). Results obtained with this patient enabled us to order some of the proximal 17p markers (fig. 1). Indeed, his phenotype had some overlapping SMS features, as well as some unique features which likely result from genes which map within the telomeric extension of his deletion. The translocation t(2;17)(p25.3;p11.1) patient 93-360, although not deleted for any of the proximal 17p markers studied thus far, displayed subtle clinical features of SMS, most notably the distinct behavioral disturbances. This suggests that he may have a submicroscopic deletion within the region—but that it is not encompassed by any of the markers used in the present study. Alternatively, the phenotype may result from a position effect secondary to juxtaposition of 17p11.2 genes to a different environment, or the translocation may interrupt a single critical gene in this region. By densitometric analysis, patient 94-362 appeared to be deleted for only one proximal 17p marker; studies using somatic-cell hybrids to confirm this finding are in progress. It is interesting that she is the longest-lived patient and had less severe clinical problems, lending support to our hypothesis that the extent of hemizygosity in this patient may be lower than that in other SMS patients, although we cannot rule out a cryptic translocation of some proximal 17p material in this patient.

The DNA markers deleted in SMS patients are linked to CMT1A (Raeymaekers et al. 1989; Vance et al. 1989; Chance et al. 1990; McAlpine et al. 1990; Middleton-Price et al. 1990; Patel et al. 1990*a*, 1990*b*). CMT1A is the most commonly inherited peripheral neuropathy characterized clinically by (*a*) absence of deep-tendon reflexes, (*b*) distal muscle wasting resulting in either pes cavus or pes planus and in a claw-hand deformity, and (*c*) distal sensory neuropathy (Lupski et al. 1991*a*). CMT1A is characterized electrophysiologically by decreased nerve-conduction

velocity (Kimura 1989; Lupski et al. 1991*a*). SMS patients demonstrate clinical signs suggestive of a peripheral neuropathy, but their peroneal motor-nerve-conduction velocities were normal, except for patient 55-200. Recently, we have demonstrated that CMT1A is completely linked and associated with a large DNA duplication in proximal 17*p*, a duplication which appears to encompass VAW409R3, VAW-412R3, and EW401 (Lupski et al. 1991*b*). These markers border the SMS deletion region. It is interesting that patient 92-357 is deleted for EW401, one of the markers apparently duplicated in CMT1A, and yet displays no clinical signs of peripheral neuropathy.

In a number of human genetic disorders, the phenotypic expression of the disease may depend on paternal or maternal inheritance of the mutation (Hall 1990). It has been hypothesized that genomic imprinting is an epigenetic process that marks the paternal or maternal chromosomes involved in such parental effects. Genomic imprinting has been implicated in Prader-Willi and Angelman syndromes, both caused by cytogenetically indistinguishable deletions of bands q11-q13 of chromosome 15. Molecular studies appear to indicate that, while Angelman syndrome is due to a deletion of the maternal allele, Prader-Willi syndrome is caused by a deletion of the paternal allele (Nicholls et al. 1989; Williams et al. 1990). The extent to which imprinting effects on the human genome may be discerned through the study of the parental origin of the deleted segment in microdeletion syndromes remains to be determined. We analyzed 15 SMS pedigrees by analysis of DNA polymorphisms associated with 17p11 markers which were fully informative for parental origin of the deletion. Nine of the deletions were of paternal origin, while six were of maternal origin. The clinical phenotype was similar, regardless of parental origin of the deletion. Although further clinical studies are needed to investigate this hypothesis, the variability of the SMS phenotype does not appear to be associated with parental origin of deletion. These results are similar to those in a series of Miller-Dieker syndrome patients who have deletion of distal 17p (Dobyns et al. 1991).

In conclusion, in addition to the characteristic previously described features, SMS appears to be a contiguous-gene microdeletion syndrome which is associated with del(17)(p11.2) and which can include clinical signs of peripheral neuropathy, self-destructive behavior, and sleep disorders or absence of REM sleep. A DNA rearrangement leading to the deletion of several contiguous genes in 17p11.2 is likely the molecular mechanism underlying de novo del(17)(p11.2). It is interesting that other DNA rearrangements including duplications (Magenis et al. 1986) and translocations (Schrander-Stumpel et al. 1990) have been reported for the 17p11.2 region. A complete physical map of 17p11.2 may be developed by utilizing the present study's patients to construct a deletion mapping panel—and then correlating this with yeast artificial chromosome contigs and also with a pulsed-field gel electrophoretic restriction map. This should enable identification of deletion breakpoints in individual patients and is a means to examine mechanisms of DNA rearrangements in man. It will also enable both delineation of specific genes which map to this region and correlation of the genotype with the phenotype in individual SMS patients.

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DNA Duplication Associated with Charcot–Marie–Tooth Disease Type 1A

James R. Lupski,*†° Roberto Montes de Oca-Luna,* Susan Slaugenhaupt,‡ Liu Pentao,* Vito Guzzetta,* Barbara J. Trask,§ Odila Saucedo-Cardenas,* David F. Barker,‖ James M. Killian,# Carlos A. Garcia,** Aravinda Chakravarti,‡ and Pragna I. Patel*°
*Institute for Molecular Genetics
°Human Genome Center
†Department of Pediatrics
#Department of Neurology
Baylor College of Medicine
Houston, Texas 77030
‡Departments of Human Genetics and Psychiatry
University of Pittsburgh
Pittsburgh, Pennsylvania 15261
§Biomedical Sciences Division
Lawrence Livermore National Laboratories
Livermore, California 94550
‖Department of Medical Informatics
University of Utah School of Medicine
Salt Lake City, Utah 84130
**Departments of Neurology and Pathology
Louisiana State University
New Orleans, Louisiana 70112

Summary

Charcot–Marie–Tooth disease type 1A (CMT1A) was localized by genetic mapping to a 3 cM interval on human chromosome 17p. DNA markers within this interval revealed a duplication that is completely linked and associated with CMT1A. The duplication was demonstrated in affected individuals by the presence of three alleles at a highly polymorphic locus, by dosage differences at RFLP alleles, and by two-color fluorescence in situ hybridization. Pulsed-field gel electrophoresis of genomic DNA from patients of different ethnic origins showed a novel SacII fragment of 500 kb associated with CMT1A. A severely affected CMT1A offspring from a mating between two affected individuals was demonstrated to have this duplication present on each chromosome 17. We have demonstrated that failure to recognize the molecular duplication can lead to misinterpretation of marker genotypes for affected individuals, identification of false recombinants, and incorrect localization of the disease locus.

Introduction

Charcot–Marie–Tooth disease (CMT) is an inherited peripheral neuropathy in humans with involvement of both the motor and sensory nerves (Charcot and Marie, 1886; Lupski et al., 1991) and a prevalence rate of 1 in 2500 (Skre, 1974). Most families demonstrate autosomal dominant Mendelian segregation, although autosomal recessive and X-linked forms of the disease have been reported (McKusick, 1990). The most common form of the disease, CMT type 1 (CMT1), is characterized by distal muscle atrophy, decreased nerve conduction velocities (NCV), and a hypertrophic neuropathy on nerve biopsy. CMT1 is inherited as an autosomal dominant disease, the clinical expression of which is age dependent and the penetrance of which is nearly complete (Bird and Kraft, 1978). The average age at onset of clinical symptoms is 12.2 ± 7.3 years. Recent studies provide convincing evidence that abnormal NCV (<40 m/s) is highly diagnostic of CMT1 and is a 100% penetrant phenotype that is essentially independent of age (Lupski et al., 1991).

CMT1 displays marked clinical variability both within and between families, suggesting genetic heterogeneity. Since the molecular basis of this disorder is unknown, linkage studies are indispensable for mapping the gene(s) responsible for CMT1 and to ascertain whether multiple genes, multiple alleles, or both lead to the clinical variation in symptoms. Genetic linkage studies in large pedigrees (see Lupski et al., 1991, for review) suggest the existence of at least three distinct loci causing CMT1: the CMT1A locus maps to human chromosome 17 (region p11–p12) (Vance et al., 1989; Raeymakers et al., 1989; Middleton-Price et al., 1990; Timmerman et al., 1990; McAlpine et al., 1990; Chance et al., 1990; Patel et al., 1990a, 1990b; Vance et al., 1991); the CMT1B locus maps to human chromosome 1 (region q23–q25) (Bird et al., 1982); and a third type is unlinked to both the CMT1A and CMT1B loci (Chance et al., 1990).

These studies provide the basis for isolating the disease gene(s) by virtue of map position. Positional cloning experiments can be aided by the existence of patients with specific chromosomal DNA rearrangements. However, no chromosomal anomaly, indicative of genomic DNA rearrangement, has been described in CMT1A patients. We have now identified a DNA duplication in CMT1A. By a series of molecular and genetic methods, we demonstrate complete linkage and association of this duplication in seven multigenerational CMT1A pedigrees and in several isolated, unrelated patients. The DNA duplication is transmitted to affected offspring without recombination, but failure to recognize this duplication leads to incorrect interpretation of the marker genotypes of affected individuals and an incorrect localization of the disease gene. The discovery of this DNA rearrangement is an important step toward the identification of the gene(s) involved by positional cloning and has implications for disease diagnosis in individuals without a firm family history. Our findings implicate a local DNA duplication, a segmental trisomy, as a novel mechanism for an autosomal dominant human disease.

Results

RFLP and Family Studies

Seven large families segregating autosomal dominant CMT1, as evidenced by vertical male-to-male transmission, were identified. Six of these families, HOU1 (Patel et al., 1990a), HOU2, HOU42 (Patel et al., 1990b), HOU85,

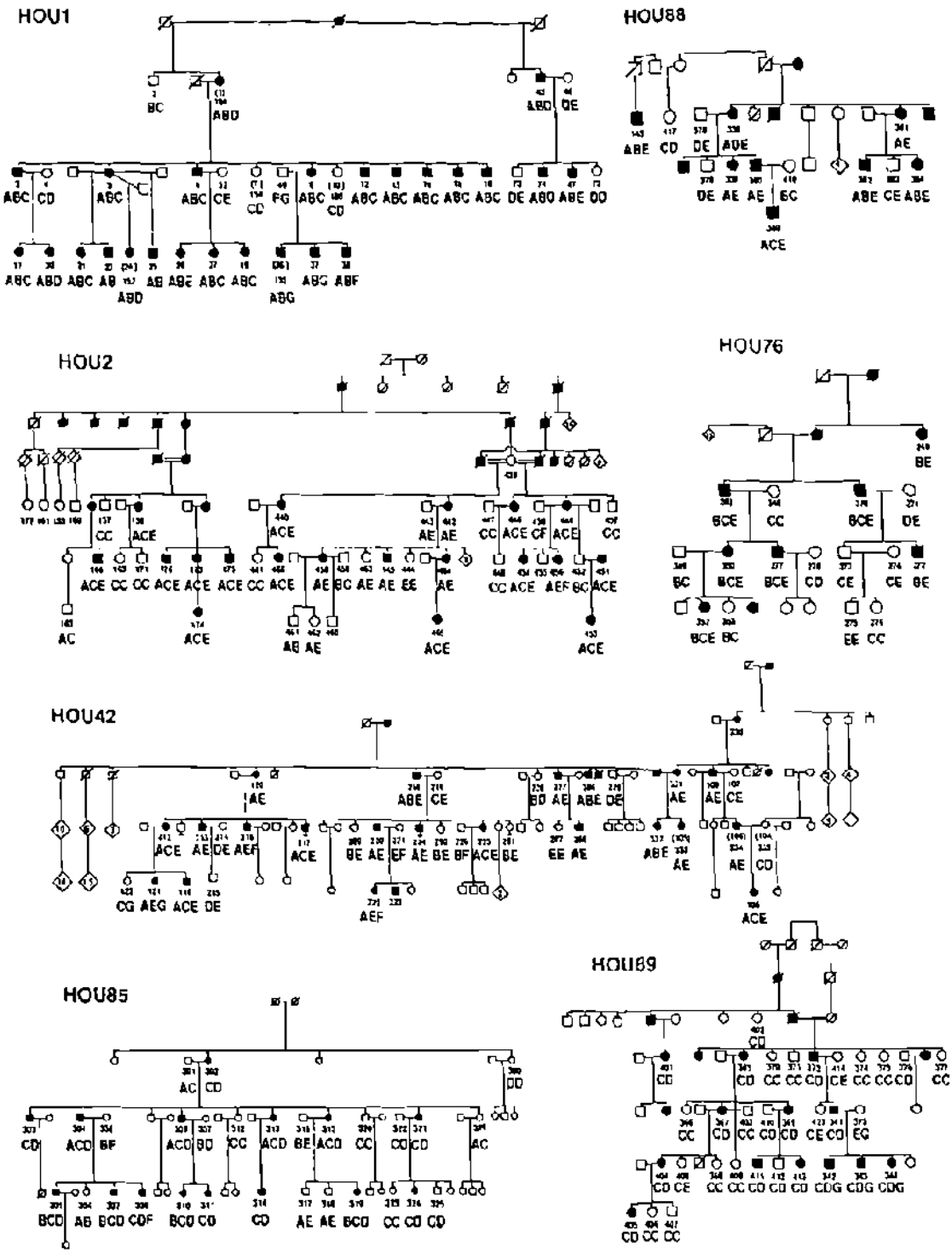

Figure 1. $(GT)_n$ Genotypes at the D17S122 Locus for Kindreds Segregating Autosomal Dominant CMT1A

HOU1, HOU2 (Killian and Kloepfer, 1979), HOU42, HOU85, HOU88, and HOU89 are of French-Acadian descent while HOU76 is of Ashkenazic Jewish descent. Standard pedigree symbols are used, disease is indicated by the darkened symbols. The laboratory identification number and the $(GT)_n$ genotype of each individual are indicated below the pedigree symbols. $(GT)_n$ genotypes were obtained by PCR analysis and were scored for the number of visible alleles using a standardized coding system: A = 165 bp, B = 163 bp, C = 161 bp, D = 159 bp, E = 157 bp, F = 155 bp, G = 153 bp. When a single allele was evident in an individual, it was scored as being present in two copies. Data were scored blind to disease status, and scoring was confirmed by two other investigators. Careful inspection of the relative intensity of the Mendelian inheritance of each allele was conducted to avoid scoring of shadow bands as alleles. The number of alleles evident in an affected individual depends on the number of distinguishable alleles segregating in the parents. In cases where all four parental chromosomes can be distinguished (e.g., unaffected father 1-49 genotype FG and affected mother 1-9 genotype ABC), the three alleles in the affected sons (1-153 and 1-37 ABG; 1-38 ABF) can be easily visualized. On the other hand, in HOU76 the affected father 76-270, with genotype BCE, and his unaffected spouse, 76-271, with genotype DE have an affected

Table 1. LOD Scores between Chromosome 1q and 17p Markers and CMT1A

Marker	Recombination Value							
	0.00	0.05	0.10	0.20	0.30	0.40	$\hat{\theta}$	$\hat{Z}$
FcγRII	$-\infty$	−16.17	−9.42	−3.66	−1.20	−0.16	0.500	0.00
LEW301	14.74	13.38	11.95	8.90	5.65	2.36	0.000	14.74
YNM67-R5	$-\infty$	9.47	8.72	6.67	4.25	1.78	0.023	9.66
1516	15.89	14.63	13.24	10.16	6.75	3.17	0.000	15.89
A10-41	$-\infty$	10.28	9.59	7.46	4.92	2.27	0.035	10.35
S6.1-HB2	$-\infty$	12.69	12.11	9.73	6.59	3.10	0.046	12.70
1517	$-\infty$	15.17	13.71	10.26	6.47	2.65	0.013	15.84
MYH2	$-\infty$	1.10	2.91	3.56	2.81	1.45	0.180	3.56
1541	$-\infty$	−1.57	1.17	2.69	2.38	1.27	0.222	2.72

HOU88, and HOU89, are of French-Acadian origin, while HOU76 is an Ashkenazic Jewish family (Figure 1). To accurately map the CMT1A gene in these pedigrees, 17 DNA polymorphisms localized to the proximal region of chromosome 17p and a highly polymorphic marker on chromosome 1q were studied. In view of the demonstrated genetic heterogeneity, we required that each family provide independent evidence of linkage to a specific chromosomal region. Initial linkage analysis was restricted to the large families HOU1, HOU2, HOU42, HOU85, and HOU89 (Figure 1). Families HOU76 and HOU88 were too small to include or exclude linkage to a specific location but were useful in the association study described below.

The pooled evidence for linkage (LOD scores) from all five pedigrees, the maximum likelihood estimates of the recombination value ($\hat{\theta}$) between CMT1 and various genetic markers, and the peak LOD scores ($\hat{Z}$) for nine loci are shown in Table 1. The immunoglobulin receptor FcγRII on chromosome 1q shows complete linkage to CMT1 in a large Indiana kindred (R. Lebo, personal communication) and is diagnostic of CMT1B. None of our families show linkage to FcγRII ($\hat{\theta} = 0.5$, $\hat{Z} = 0.0$, Table 1). Individually, each pedigree showed negative LOD scores (data not shown), and together these families exclude linkage to a region 20 recombination units ($\theta = 0.20$) on either side of FcγRII.

Linkage analysis was performed using the 17p probes LEW301, YNM67-R5, 1516, A10-41, S6.1-HB2, 1517, MYH2, and 1541. All markers except 1541 showed LOD scores exceeding 3.0 (Table 1), and all loci except MYH2 and 1541 showed recombination values of 4.6% or less, demonstrating tight linkage of the disease to the 17p region. Each individual family, except HOU42, showed a LOD score of 3.0 or greater with one or more DNA markers in this region (data not shown); HOU42 showed a peak LOD score of 2.9 at $\hat{\theta} = 0$ with the DNA probe YNM67-R5. Statistical tests on all the marker data suggested that the disease locus in these families mapped to the same location on chromosome 17p and segregated CMT1A. For further confirmation, we calculated the peak multipoint LOD score for each family, including HOU42, with respect to the map LEW301—YNM67-R5—A10-41—MYH2 using the computer program CRI-MAP; these LOD scores were 6.27, 3.79, 3.98, 4.26, and 4.84 for HOU1, HOU2, HOU42, HOU85, and HOU89, respectively, and confirmed their classification as CMT1A families.

The DNA probes MYH2 and 1541, located on distal chromosome 17p, demonstrated loose linkage to CMT; consequently, multiple recombinants between the disease and these markers are observed in each family. On the other hand, only five recombinants were detected for the markers closely linked to CMT1A. Of these, LEW301 and 1516 show no recombinants. However, individual 89-401 in HOU89 is recombinant for YNM67-R5, individual 85-326 in HOU85 is recombinant for A10-41 and S6.1-HB2 (same event detected), individual 1-13 in HOU1 is recombinant for S6.1-HB2, and individual 2-448 and one of the spouses of 2-439 in HOU2 are recombinant for 1517. The order of the closely linked 17p DNA probes is LEW301—(YNM67-R5, 1516)—(A10-41, S6.1-HB2)—1517 and covers a distance of 9.9 cM. The five families contain approximately 108 meioses, which for the LEW301–1517 interval should contain 9.7 ± 3.1 recombinants. The observed number of recombinants (5) is well within expectations ($\chi^2 = 2.30$, 1 degree of freedom, $P > 0.10$). These recombinants suggest that CMT1A is localized between LEW301 and 1517, which corresponds to an interval of approximately 10 million bp, assuming that recombination is uniform in the human genome. In the following section we report isolation of a highly informative $(GT)_n$ polymorphism that detects multiple alleles in CMT1A patients. Genotypes at this locus are also provided in Figure 1.

son, 76-272, of apparent genotype BE, but shows a double dose for allele E. Since dosage differences were not always reproducible from PCR, we scored absolute number of alleles visualized on the autoradiograph. The disease status of all at-risk individuals was determined by NCV measurements with the exception of individuals 1-45, 1-46, 1-47, 1-72, 1-73, and 1-74, who were diagnosed by clinical examination only. Note the nuclear family of individuals 42-331, 42-332, 42-333, where a mating occurs between two affected individuals. CMT1A segregates with the alleles A and E in HOU2, HOU42, and HOU88, with alleles A and B in HOU1, with alleles C and D in HOU85 and HOU89, and with alleles B and E in HOU76.