



**LOCALIZACION DEL LOCUS DE LA ENFERMEDAD DE
CHARCOT-MARIE-TOOTH TIPO IA: ASOCIACION
DE ESTA ENFERMEDAD CON UNA
DUPLICACION DE DNA**

**POR
ROBERTO MONTES DE OCA LUNA**

**TESIS PRESENTADA A LA
FACULTAD DE MEDICINA
DE LA
UNIVERSIDAD AUTONOMA DE NUEVO LEON
COMO REQUISITO PARCIAL PARA
LA OBTENCION DEL GRADO DE**

**DOCTOR EN CIENCIAS
CON ESPECIALIDAD EN BIOLOGIA MOLECULAR
E INGENIERIA GENETICA**

**MONTERREY, N. L.
ABRIL DE 1993**



U ANL

UNIVERSIDAD AUTÓNOMA DE NUEVO LEÓN

®

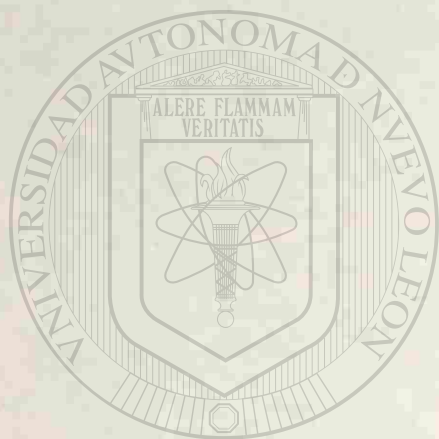
DIRECCIÓN GENERAL DE BIBLIOTECAS

TD
RC935
.C43
M6
c.1

50



1080073228



UANL

UNIVERSIDAD AUTÓNOMA DE NUEVO LEÓN

®

DIRECCIÓN GENERAL DE BIBLIOTECAS



**LOCALIZACION DEL LOCUS DE LA ENFERMEDAD DE
CHARCOT-MARIE-TOOTH TIPO 1A: ASOCIACION
DE ESTA ENFERMEDAD CON UNA
DUPLICACION DE DNA**

POR

ROBERTO MONTES DE OCA LUNA

**TESIS PRESENTADA A LA
FACULTAD DE MEDICINA**

DE LA

**UNIVERSIDAD AUTONOMA DE NUEVO LEON
COMO REQUISITO PARCIAL PARA**

LA OBTENCION DEL GRADO DE

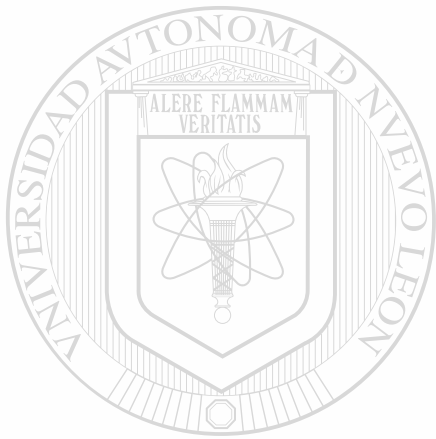
DOCTOR EN CIENCIAS

**CON ESPECIALIDAD EN BIOLOGIA MOLECULAR
E INGENIERIA GENETICA**

Monterrey, N. L.

Abril de 1993

TD
RC935
.C43
M6



UANL

UNIVERSIDAD AUTÓNOMA DE NUEVO LEÓN



DIRECCIÓN GENERAL DE BIBLIOTECAS

**LOCALIZACION DEL LOCUS DE LA ENFERMEDAD DE
CHARCOT-MARIE-TOOTH TIPO 1A: ASOCIACION
DE ESTA ENFERMEDAD CON UNA
DUPLICACION DE DNA**

POR

ROBERTO MONTES DE OCA LUNA

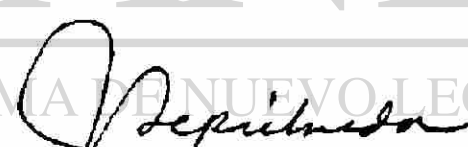
**Tesis presentada a la Facultad de Medicina de la Universidad
Autónoma de Nuevo León como requisito parcial para la obtención
del grado de Doctor en Ciencias con especialidad en Biología
Molecular e Ingeniería Genética**



Comité de Tesis:



Dr. Mario César Salinas Carmona



Dr. Julio Sepúlveda Saavedra



Dra. Herminia G. Martínez Rdz.



Dra. Mirtala Moreno Sepúlveda



Dr. Hugo A. Barrera Saldaña

Esta tesis se realizó en el Instituto de Genética Molecular del Colegio Baylor de Medicina, bajo la asesoría de la Dra. Pragna I. Patel y el Dr. James R. Lupski (Directores externos).

Por parte de la Universidad Autónoma de Nuevo León fungió como asesor de la presente tesis el Dr. Hugo A. Barrera Saldaña (Director Interno), Jefe del Departamento de Bioquímica de la Facultad de Medicina.

DIRECCIÓN GENERAL DE BIBLIOTECAS

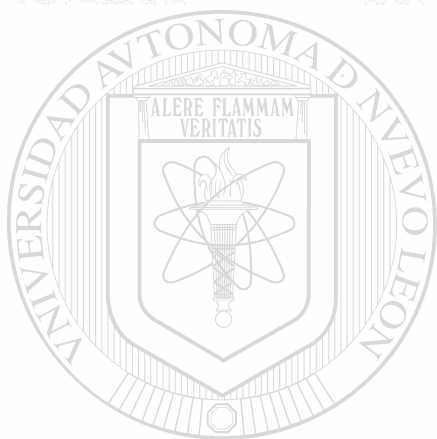
TABLA DE CONTENIDO

INDICE	i
DEDICATORIA	v
AGRADECIMIENTOS	vi
LISTA DE ABREVIATURAS	vii
LISTA DE FIGURAS	viii
LISTA DE TABLAS	ix
RESUMEN	1
INTRODUCCION	2
I. Aspectos Históricos de la Enfermedad de Charcot Marie-Tooth (CMT)	3
II. Características Clínicas del CMT tipo1 (CMT1)	4
III. Electrofisiología del CMT1	4
IV. Genética del CMT	5
A. Herencia Mendeliana	5
B. Penetrancia del fenotipo de CMT	5
C. Variabilidad en la expresión del fenotipo CMT1	6
V. Genética Molecular del CMT	7
A. Estudios de ligamiento genético	7
B. Estudios de ligamiento genético del CMT tipo IA	10
C. Resumen de la búsqueda del locus del CMT1A	10
VI. Planteamiento del proyecto	11
A. Limitante de los estudios de ligamiento utilizando RFLP	11

B. Estrategia alternativa	13
HIPOTESIS	15
OBJETIVO	16
MATERIALES Y METODOS	17
I. Origen de los materiales y reactivos	17
II. Familias y su evaluación clínica	17
III. Marcadores genéticos	20
IV. Aislamiento de cósmidos	20
A. Preparación de las membranas	20
B. Hibridación de las membranas	20
V. Obtención de marcadores genéticos (GT)n	22
A. Identificación de secuencias (GT)n	22
B. Preparación de la sonda	22
C. Hibridación de DNA en gota	22
D. Subclonación y caracterización de las secuencias (GT)n	22
VI. Determinación de genotipos (GT)n	23[®]
A. Aislamiento de DNA genómico de alto peso molecular	24
B. Marcaje de oligonucleótidos	25
C. Reacción de PCR	25
D. Interpretación de los genotipos	25
VII. Obtención de genotipos utilizando RFLP	25
A. Análisis tipo Southern	25
B. Marcaje radiactivo de las sondas	26
C. Preasociación de las sondas	26
D. Cuantificación de las bandas de hibridación	26

VIII. Separación de los alelos polimórficos <i>MspI</i> para el marcador VAW409R1	26
IX. Construcción y análisis de híbridos de células somáticas...	27
RESULTADOS	29
I. Detección de secuencias (GT)_n en los marcadores ligados al CMT1A	29
II. Caracterización de los marcadores (GT)_n	36
III. Evidencia inicial de una duplicación del locus D17S122 en pacientes con CMT1A	41
IV. Confirmación de la duplicación del locus D17S122 mediante el análisis de polimorfismos tipo RFLP	41
V. Demostración de la duplicación del locus D17S122 mediante el uso de marcadores (GT)_n	44
VI. La duplicación de DNA es una mutación común en pacientes con CMT1	54
VII. Demostración de que los alelos RFLP <i>MspI</i> con una señal doble de hibridación en los pacientes con CMT1A contienen dos alelos	54
VIII. El tamaño de la duplicación es mayor de 100 kb	58
IX. Demostración de la herencia de un "cromosoma CMT1A" en pacientes con CMT1A	58
X. Correlación entre fenotipo y genotipo: un individuo severamente afectado con CMT1A es homocigoto para la duplicación	62
XI. Clonación de un nuevo marcador genético para la duplicación de DNA en pacientes con CMT1A	63

IX. Diagnóstico molecular del CMT1A con el marcador pRM11-GT	64
DISCUSION	67
CONCLUSIONES	73
PERSPECTIVAS	74
BIBLIOGRAFIA	76
ANEXOS	87



UANL

UNIVERSIDAD AUTÓNOMA DE NUEVO LEÓN



DIRECCIÓN GENERAL DE BIBLIOTECAS

DEDICATORIA

A la memoria de mi hermano Carlos Montes de Oca Luna.

Con todo mi amor para las siguientes personas: a mis padres Sra. Marcelina Luna Aguilar y Sr. Carlos Montes de Oca Mejía; a mi hijo Carlos Roberto Montes de Oca Saucedo, y a mis hermanos Carmela, Alicia, Saul, Moisés, Claudia, Juan y Fernando.

Con amor e infinita gratitud a mi esposa Odila Saucedo Cárdenas quien incondicionalmente siempre me ha apoyado en mi carrera.

Con eterna gratitud a mi tía Severiana Luna Aguilar y a mis primos Carlos, Rosy, Jesús, Santos, Gloria y Diana por el apoyo que me brindaron durante mis estudios.

A todas las personas de las familias utilizadas en este estudio y a todas aquellas familias afectadas con la enfermedad de CMT1A. Deseo que este trabajo tenga alguna utilidad en sus vidas.

A todas aquellas personas que hicieron posible la realización de este trabajo: Jim Lupski, Pragna Patel, Carlos A. García, James M. Killian, Aravinda Chakravarti, Susan Slaugenhaupt, Odila Saucedo, Vito Guzzeta, Brunella Franco, Robert Wright, Stewart Davis, Pentao Liu y Diego E. Rincón Limas.

Por último para los siguientes doctores que directa o indirectamente han influido en mi formación en el área de investigación: Ramiro González, Carlos H. Leal Garza, Salvador Luis Said Fernández, Hugo A. Barrera Saldaña, Mario César Salinas Carmona, Luis Herrera Estrella y Ariel Alvarez Morales.

AGRADECIMIENTOS

A mis directores de tesis por su apoyo durante el desarrollo de esta tesis para obtener mi grado de doctor en ciencias: Dr. James R. Lupski, Dr. Pragna I. Patel y Dr. Hugo A. Barrera Saldaña.

A los Drs. Carlos A. García, James M. Killian, R. Malamut, G. Parry and F. Axelrod por la evaluación y diagnóstico clínico de los individuos de este estudio. A los Drs. Aravinda Chakravarti y Susan Slaugenhaupt por los análisis estadísticos de ligamiento genético. Al Dr. David F. Barker por proveer los marcadores genéticos VAW409R1 y VAW409R3. Al Dr. Vito guzzetta por proveer los híbridos celulares y al Biol. Odila Saucedo Cárdenas por el secuenciamiento de los marcadores (GT)_n.

A todos mis sinodales por sus críticas, sugerencias e interés en la revisión de este trabajo.

A la Facultad de Medicina de la U. A. N. L. , por las facilidades otorgadas para la realización de este trabajo, especialmente a todo el personal del Departamento de Bioquímica.

Al Consejo Nacional de Ciencia y Tecnología por la beca de doctorado otorgada.

LISTA DE ABREVIATURAS

0C	Grados centígrados
CMT	Enfermedad de Charcot-Marie-Tooth
CMT1	Enfermedad de Charcot-Marie-Tooth tipo 1
CMT1A	Enfermedad de Charcot-Marie-Tooth tipo 1A
dATP	Desoxiadenosina trifosfatada
dCTP	Desoxicitidina trifosfatada
dNTPs	Desoxinucleósidos trifosfatados
EDTA	Acido etilendiamin tetracético
g	Gramo
h	Hora (s)
Kb	Kilopares de bases
M	Molar
μCi	Microcuries
mg	Miligramos
ml	Mililitros
μg	Microgramos
μl	Microlitros
MEMD	Medio Eagle modificado por Dulbecco
min	Minuto (s)
ml	Mililitro
mM	Milimolar
ng	Nanogramo
nmol	Nanomoles
pb	Pares de bases
PCR	Reacción de polimerización en cadena
pH	Logaritmo negativo de la concentración de H ⁺
RFLP	Polimorfismo (s) en la longitud de fragmentos de restricción
SRDGT	Secuencia repetida del dinucleótido guanina y timina
SRT	Secuencia repetida en tandem
SSC	Solución salina citratos
SDS	Dodecil sulfato de sodio
V	Voltios
VCN	Velocidad de conducción nerviosa
Z	"Lod Score", símbolo que representa el grado de ligamiento genético

LISTA DE FIGURAS

- Figura 1. Ligamiento entre una enfermedad y un marcador genético.
- Figura 2. Falta de informatividad de los RFLPs en los estudios de ligamiento genético.
- Figura 3. Árboles genealógicos segregando la enfermedad de CMT1A.
- Figura 4. Identificación de marcadores genéticos conteniendo secuencias (GT)_n mediante el procedimiento de hibridación en gota.
- Figura 5. Patrón de restricción del marcador genético cH3.
- Figura 6. Detección de secuencias (GT)_n en el marcador genético cH3 mediante hibridación tipo Southern.
- Figura 7. Detección de secuencias (GT)_n en el marcador genético c1517 mediante hibridación tipo Southern.
- Figura 8. Detección de secuencias (GT)_n en el marcador genético c1541 mediante hibridación tipo Southern.
- Figura 9. Detección de secuencias (GT)_n en el marcador genético VAW409R1 mediante hibridación tipo Southern.
- Figura 10. Identificación de las clonas recombinantes del marcador cH3 que contienen la secuencia (GT)_n.
- Figura 11. Secuencia nucleotídica del marcador pRM11-GT.
- Figura 12. Evidencia inicial de una duplicación en pacientes con CMT1A.
- Figura 13. Detección de tres alelos (GT)_n en pacientes con CMT1A utilizando el marcador RM11-GT.
- Figura 14. A, B, C, D y E. Genotipos (GT)_n del marcador pRM11-GT para los árboles genealógicos segregando la enfermedad autosómica dominante CMT1A.

Figura 15. Demostración de dos alelos (GT)_n en alelos polimórficos *MspI* del locus D17S122.

Figura 16. Demostración de un individuo homocigoto para el CMT1A.

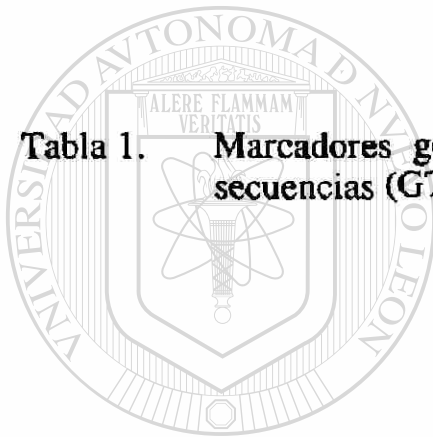
Figura 17. Identificación de un nuevo marcador para la región duplicada.

Figura 18. Diagnóstico molecular del CMT1A utilizando el marcador pRM11-GT.

Figura 19. Observación de falsos recombinantes debido a la duplicación.

LISTA DE TABLAS

Tabla 1. Marcadores genéticos utilizados para la identificación de secuencias (GT)_n.



UANL

UNIVERSIDAD AUTÓNOMA DE NUEVO LEÓN

®

DIRECCIÓN GENERAL DE BIBLIOTECAS

RESUMEN

La enfermedad de Charcot-Marie-Tooth (CMT) es la neuropatía hereditaria más común del sistema nervioso periférico. El CMT tipo 1A (CMT1A) es la forma más prevalente de CMT y se hereda de una manera autosómica dominante, ligada al cromosoma 17. Esta enfermedad se caracteriza clínicamente por una atrofia de los músculos distales y una disminución en la velocidad de conducción nerviosa.

Mediante estudios de ligamiento genético se determinó que el locus para la enfermedad de CMT1A se localiza en la región p11.2-p12 del cromosoma humano número 17. En el presente trabajo se describe el aislamiento de un marcador genético muy polimórfico designado con el nombre de pRM11-GT, el cual se localiza en la región cromosómica 17p11.2-p12. Este marcador detecta un nuevo tipo de polimorfismo que se presenta a nivel de secuencias repetidas en tandem del dinucleótido guanina y timina. Con este marcador pRM11-GT se analizaron 194 individuos que conforman siete árboles genealógicos (seis de origen Acadio-Francés y uno de origen judío) y se encontró que, únicamente en aquellos individuos afectados con CMT1A, había un alelo extra para este marcador. Esto significa que los pacientes con CMT1A tienen una duplicación de DNA que involucra el locus D17S122 al cual pertenece el marcador RM11-GT. Esta duplicación se confirmó con el análisis de los polimorfismos en la longitud de fragmentos de restricción (RFLP) que se obtuvieron con otros dos marcadores de este mismo locus: VAW409R1 y VAW409R3. Nuevamente, solo en los individuos afectados se pudo detectar la presencia de un alelo extra para estos marcadores. Además con el uso de RFLP se determinó que la duplicación también involucra otros dos locus: VAW412 y EW401; esto implicó que el tamaño de la duplicación debe ser al menos de 100 kb. Por otro lado, se demostró que un paciente severamente afectado con CMT1A, hijo de un matrimonio entre dos personas afectadas, era homocigoto para la duplicación de DNA. Esto representa una evidencia indirecta de una correlación entre el genotipo y el fenotipo. Este hallazgo de la asociación de la enfermedad de CMT1A con una duplicación de DNA, ha permitido por primera vez el diagnóstico molecular de la enfermedad de CMT1A, y representa un paso muy importante para la identificación del gen (o de los genes) responsable de esta enfermedad.

INTRODUCCION

Existen descritas más de 4000 enfermedades genéticas en el humano, para la mayoría de las cuales aún se desconocen sus bases moleculares (1). La tecnología del DNA recombinante ha permitido dilucidar las bases moleculares de algunas de estas enfermedades, al identificar los genes responsables y sus correspondientes mutaciones que conducen a la aparición del fenotipo de la enfermedad. Esto se ha logrado principalmente para aquellas enfermedades genéticas en donde previamente se conocía que tipo de proteína estaba alterada en la enfermedad, de tal manera que la identificación de sus respectivos genes se facilitó al usar estrategias de clonación basadas en la secuencia total o parcial de la proteína, en su función y/o en el uso de anticuerpos contra la misma. Tal es el caso por ejemplo de las precisas descripciones moleculares de síndromes de Talasemias (2) e Hipercolesterolemia familiar (3).

Sin embargo, para la mayoría de las enfermedades genéticas humanas, incluyendo la enfermedad de CMT1A, se desconocen sus bases bioquímicas y por ende las proteínas posiblemente involucradas en esta enfermedad. En este tipo de casos se identifica el locus del gen responsable de la enfermedad, a través de estudios de ligamiento genético. Este tipo de estudios se designan con el nombre de "clonación por su posición", e involucra estrategias para el aislamiento del gen, aún y cuando no se conozca ni su producto proteico o/y su función. La lógica de este enfoque es el de establecer la posición del locus de la enfermedad con un marcador genético que previamente se haya determinado que está ligado a la enfermedad; la región cromosómica donde se localiza este marcador corresponde al locus de la enfermedad y en esta área se debe encontrar un gen con alguna mutación que se correlacione con la enfermedad. De esta manera se logró la identificación del gen responsable de la Fibrosis Quística (4,5,6), y establecer al menos la localización cromosómica de diversas enfermedades genéticas como por ejemplo la Corea de Huntington (7) y la enfermedad del Riñón Poliquistico Crónica (8). Algunas veces se pueden encontrar pacientes en donde la enfermedad está asociada con alguna anomalía cromosómica; por ejemplo una translocación o delección. Esto facilita la localización del gen ya que la búsqueda del mismo se restringe en aquella región donde se encuentra la anomalía cromosómica. Así se logró la identificación de los genes responsables de algunas enfermedades genéticas como son la Distrofia muscular tipo Duchenne (9,10), el Retinoblastoma (11), la Granulomatosis Crónica (12) y la Neurofibromatosis (13,14).

El presente trabajo se enfocó a la identificación del gen responsable de una mas de las tantas enfermedades genéticas humanas: la enfermedad de

CMT1A.

I. Aspectos Históricos de la Enfermedad de Charcot-Marie-Tooth.

En 1886, Charcot y Marie en Francia (15) y Tooth en Inglaterra (16), describieron una enfermedad en la cual los individuos presentaban una debilidad muscular como consecuencia de un desorden neurológico. Además, observaron que esta enfermedad se presentaba en varios miembros de la misma familia con lo cual determinaron posteriormente que se trataba de una enfermedad hereditaria. Originalmente esta enfermedad recibió el nombre de atrofia muscular peroneal, sin embargo actualmente ésta se designa con el nombre de sus descubridores: enfermedad de Charcot-Marie-Tooth (CMT). A partir de estas primeras descripciones del CMT han aparecido en la literatura diversos reportes sobre enfermedades con un fenotipo similar al descrito originalmente por Charcot, Marie y Tooth. Actualmente, el CMT comprende un grupo heterogéneo de varias enfermedades que tienen en común un desorden degenerativo y hereditario de los nervios periféricos. Por esta razón es más apropiada la designación de Síndrome de Charcot-Marie-Tooth.

De acuerdo con estudios electrofisiológicos realizados por Henriksen (17), y Gilliat y Thomas (18), el CMT descrito por Charcot, Marie y Tooth, se dividió en dos grupos: CMT1, el cual comprende pacientes con una severa disminución en la velocidad de conducción nerviosa (VCN), y CMT2 el cual incluye pacientes con una VCN normal. Estos grupos se subdividieron, por Dyck and Lambert (19,20), en base a sus características electrofisiológicas, biopsias de nervio y al patrón de herencia. El CMT1 se dividió en dos grupos, ambos con una neuropatía hipertrofica, pero uno con un patrón de herencia dominante y el otro con un patrón de herencia recesivo. El CMT2, con VCN normales y sin hipertrofia de los nervios, también llamado el tipo neuronal, se dividió en cinco grupos con un diferente patrón de herencia. Posteriormente, Brust y cols. (21), y Davis y cols. (22), basándose en los mismos aspectos, describieron un tipo intermedio de CMT, con VCN similares al CMT1, pero sin hipertrofia de los nervios.

La clasificación de esta enfermedad ha sido confusa debido a los diversos criterios utilizados por los diferentes investigadores (23). Sin embargo la clasificación más utilizada para designar el CMT es la propuesta por Dyck y cols. (24), como neuropatías hereditarias motoras y sensitivas (HMSN): HMSN tipo I o CMT1 es una neuropatía hipertrofica que presenta

una desmielinización de los nervios periféricos con una reducción muy severa en la conducción nerviosa, y HMSN tipo II o CMT2 es la forma neuronal en la cual ocurre una degeneración a nivel de los axones, pero la velocidad de conducción nerviosa es prácticamente normal.

II. Características Clínicas del CMT1 (25).

La enfermedad comienza a manifestarse por una atrofia y debilidad de los músculos peroneales, y progresa hasta afectar los músculos distales de las piernas y los brazos. Estos músculos distales pierden progresivamente fuerza y masa, de tal manera que en algunos pacientes la atrofia de las piernas da el aspecto de piernas de cigüeña. Otras características incluyen una pérdida en los reflejos tendinosos, un arco muy pronunciado en los pies (pie cavo) e hipertrofia de los nervios periféricos a manera de bulbo de cebolla. Las deformidades en los pies y las manos puede ser tan severa que se requiere de una intervención quirúrgica para corregirlas. La aparición de los síntomas del CMT ocurre principalmente al final de la primera década o inicios de la segunda década de vida. La penetrancia del fenotipo en la primera década puede ser baja pero incrementa con la edad. La posibilidad de que un paciente muestre los primeros síntomas después de la tercera década se disminuye marcadamente con el incremento de la edad.

IV. Electrofisiología del CMT1 (25).

La expresión del fenotipo de la enfermedad de CMT1 es muy variable de acuerdo a los criterios clínicos convencionales. Sin embargo, todos los pacientes con CMT1 tienen una disminución muy severa en la velocidad de conducción del nervio motor (VCN), de tal manera que la VCN es la característica más confiable y universalmente utilizada para definir el CMT1. Inclusive los pacientes asintomáticos también tienen un grado similar en la disminución de la VCN. Los pacientes con CMT2 pueden manifestar una disminución leve en la VCN, pero no tan severa como en el CMT1.

En un estudio efectuado por Dyck y Lambert (19,20), el promedio en la VCN fué menor de 25 m/seg, y sólo en algunos pacientes alcanzó 42 m/seg. Davis y cols. (22) analizaron la VCN de 116 individuos afectados con la forma autosómica dominante de CMT, y en base a estos estudios la dividieron en dos grupos. En un grupo incluyeron aquellos pacientes con VCN menores de 25 m/seg, los cuales correspondían claramente al tipo CMT1. En el otro grupo

incluyeron pacientes con VCN entre 25 y 45 m/seg el cual incluía pacientes con CMT2.

La VCN es normal al nacimiento en los pacientes con CMT1 (25,26). No existe una correlación entre el grado de disminución de la VCN y el defecto neurológico. Muchos niños y algunos adultos afectados con CMT, aún y cuando tienen una severa disminución en la VCN no presentan signos de la enfermedad, o sea que son asintomáticos. Durante el proceso de la enfermedad no se manifiestan cambios en la VCN. Sin embargo, la severidad en la disminución de la VCN puede reflejar la severidad en el proceso de la enfermedad, de manera que los pacientes con los niveles más bajos en la VCN pueden desarrollar los defectos neurológicos más severos. Dyck et al (28) encontraron que aquellos pacientes que mostraron a edad temprana una severa disminución en la VCN presentaron un fenotipo muy severo.

IV. Genética de la Enfermedad de Charcot-Marie-Tooth (25).

A. Herencia Mendeliana. La mayor parte de las familias con CMT presentan la forma autosómica dominante, sin embargo existen familias en las cuales la enfermedad se hereda ligada al X y algunas veces en forma recesiva (1). En la forma dominante ligada al X, la enfermedad es más severa en los varones que en las mujeres, y en estas últimas el fenotipo clínico es muy heterogéneo aun y cuando forman parte del mismo árbol genealógico (29); esta variabilidad se debe posiblemente a la variación somática en la inactivación del cromosoma X. Por otro lado, existe descrito en la literatura un individuo afectado con la forma autosómica dominante quien presentó un fenotipo muy severo y que se le manifestó en el primer año de vida; puesto que ambos padres también estaban afectados con CMT se postuló que dicho paciente era homocigoto para el alelo dominante de CMT (30). Varios estudios apoyan el hecho de que la forma autosómica dominante es la más frecuente, mientras que la ligada al X es la más rara. En un estudio realizado por Lupski y cols. (25) se determinó que de 72 diferentes familias afectadas con CMT, 68 mostraron el patrón de herencia autosómico dominante.

B. Penetrancia del fenotipo de CMT. El CMT es una enfermedad en donde el fenotipo progresa lentamente con la edad, y por lo tanto la penetrancia del mismo es un fenómeno que depende del tiempo. En un estudio realizado por Bird and Kraft (31), se analizó la penetrancia del fenotipo en 109 individuos que conformaban 15 familias heredando el CMT en una forma autosómica dominante. El resultado fué que la penetrancia del gen(es) del

CMT es de un 28% en la primer década, y casi completa en la mitad de la tercera década. La edad promedio en la cual se manifestó el fenotipo fué a los 12.2 años de edad con una desviación estandar de 7.3 años. Además en este estudio se concluyó que los individuos con riesgo (hermanos de pacientes con CMT) sin manifestaciones clínicas y mayores de 27 años de edad, tienen una probabilidad menor del 3% de que hayan heredado el gen del CMT. En resumen, la penetrancia del genotipo del CMT, en cuanto a su manifestación clínica, independientemente de la edad, es casi completa (no es del 100%); y de acuerdo con los estudios electrofisiológicos, la disminución en la VCN es una característica con una penetrancia completa (25).

C. Variabilidad en la expresión del fenotipo clínico del CMT1. El fenotipo clínico del CMT1 muestra una variabilidad muy notable, ya que se observa una heterogeneidad tanto interfamiliar como intrafamiliar. Esta heterogeneidad intrafamiliar se demostró en un estudio realizado sobre 299 individuos de un árbol genealógico Acadio-Francés afectado con la forma de CMT1 autosómica dominante. En este estudio se le determinó a los individuos la VCN para diagnosticar correctamente la enfermedad de CMT1. De los pacientes diagnosticados con CMT1, mientras que algunos tenían un pie cavo muy severo y con gran dificultad para caminar, otros prácticamente carecían de los rasgos clínicos de la enfermedad (32). Puesto que los individuos afectados deberían portar la misma alteración genética, se concluye que existe una heterogeneidad intrafamiliar en la enfermedad de CMT1.

Otra línea de evidencia sobre la heterogeneidad del CMT1 fué obtenida por García y cols. (33), con el estudio de gemelos idénticos afectados con CMT1. Como se sabe, los gemelos idénticos son muy importantes en los estudios de heterogeneidad, ya que son individuos que poseen el mismo componente genético, y por ende se evita la confusión que se origina por otros genes que pudiesen alterar la expresión del fenotipo de la mutación en cuestión. De acuerdo con esto si los gemelos idénticos afectados con CMT1 tienen un fenotipo diferente apoyan el fenómeno de la heterogeneidad. Como resultado se encontró que en un caso de gemelos idénticos, hubo discordancia en cuanto a los calambres, a la debilidad de los músculos intrínsecos de las manos, debilidad de los pies, y en la hipertrofia de los nervios peroneal, cubital, y auricular mayor. Otro caso de gemelos idénticos afectados con CMT1, fué también discordante en los calambres, debilidad de las manos, e hipertrofia de los nervios. Uno de estos dos gemelos tenía unos pies tan débiles que a la edad de 14 años requirió el uso de muletas. Lo interesante fué que la VCN se mantuvo al mismo nivel de afección para cada uno de los cuatro gemelos analizados (18-20 m/seg). Esto es congruente con una penetrancia

completa del fenotipo de VCN y sugiere que su expresión es menos variable. Estos estudios en gemelos idénticos indican que los factores ambientales juegan un papel importante en la severidad de la expresión clínica del CMT1. Otra evidencia de la influencia del medio ambiente es la diferencia en la susceptibilidad de los pacientes con CMT a los efectos neurotóxicos colaterales de la terapia con vincristina (34-37).

V. Genética Molecular del CMT (25).

A. Estudios de ligamiento genético. Como se mencionó anteriormente los estudios de ligamiento genético han sido muy importantes en la localización de los genes responsables de diversas enfermedades genéticas (13,14). El propósito de un estudio de ligamiento es el de determinar si existe una correlación positiva entre la herencia de una región cromosómica específica (representada por un marcador genético) y la herencia del fenotipo de una enfermedad (Figure 1). Si el locus de la enfermedad y un marcador genético en particular se localizan en diferentes cromosomas, ambos se segregarán independientemente, es decir al azar, y no mostrarán una correlación en su patrón de herencia. Similarmente, si los dos están en lugares opuestos en un mismo cromosoma es muy alta la probabilidad de que el proceso natural de la recombinación los separe, uno en cada cromosoma homólogo. De esta manera al estar en diferentes cromosomas se segregarán independientemente y nuevamente no existirá ninguna correlación entre ellos. Sin embargo si se encuentran muy cerca uno del otro en la misma región cromosómica, es decir si están "ligados", será muy baja la probabilidad de que la recombinación los separe, y por ende tanto la enfermedad como el marcador genético se heredarán en conjunto (cosegregarán) (Figura 1). Por lo tanto el número de recombinaciones entre la enfermedad y el marcador genético a través de un árbol genealógico determinará la distancia que existe entre ellos (en promedio, en el genoma humano, 1% de recombinación corresponde a un millón de pares de bases de DNA). Si la enfermedad y el marcador se encuentran ligados, el gen responsable de la enfermedad se identifica y aísla de aquella región cromosómica donde se localice el marcador genético. Estos estudios de ligamiento son más precisos si se utilizan diferentes marcadores genéticos y se realiza un análisis de ligamiento múltiple. De esta manera se determina la posición del locus de la enfermedad con respecto a los diferentes marcadores. Posteriormente de la región cromosómica comprendida entre los marcadores genéticos que flanquean el locus de la enfermedad se aíslan genes, y se caracterizan para encontrar alguna mutación que se correlacione con la

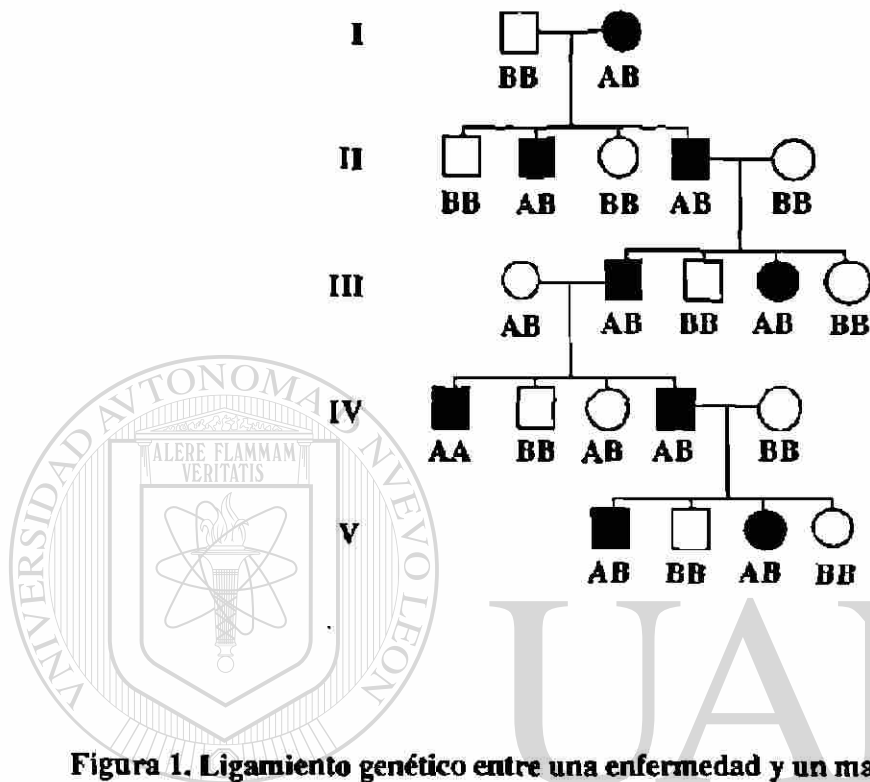


Figura 1. Ligamiento genético entre una enfermedad y un marcador genético.

Ejemplo hipotético de un árbol genealógico segregando una enfermedad autosómica dominante. Los símbolos en negro indican los individuos afectados con la enfermedad y el par de letras debajo de cada uno de los símbolos indican los alelos (el genotipo) presentes en cada uno de ellos, obtenidos por RFLP, utilizando un marcador genético que se ha postulado que está ligado al locus de la enfermedad. Los números romanos indican las generaciones familiares.

Si analizamos el genotipo de los descendientes afectados en la generación II podemos deducir que heredaron la enfermedad en combinación con el alelo A del padre. Uno de estos descendientes nuevamente transmitió a dos de sus hijos la enfermedad ligada a su alelo A; y así sucesivamente.

Este árbol genealógico ejemplifica el resultado esperado en caso de que un marcador genético esté ligado a la enfermedad. En este caso el marcador genético está ligado a la enfermedad puesto que su locus (identificado por el alelo A) siempre se heredó conjuntamente con la enfermedad, es decir que no se separaron por recombinación. Esto indicaría que el locus de la enfermedad y el del marcador están físicamente muy cercanos en el cromosoma (están ligados).

enfermedad.

Para llevar a cabo un estudio de ligamiento genético y concluir que un marcador genético está ligado a una enfermedad se requiere del análisis de árboles genealógicos de varias generaciones familiares en los cuales se herede la enfermedad en cuestión. Además, puesto que cada individuo posee dos cromosomas homólogos, es decir dos copias para un marcador genético determinado (dos alelos), es necesario diferenciar sus alelos para determinar cual de ellos se hereda a los individuos normales y cual se segrega con la enfermedad. Esto implica que se deben utilizar marcadores genéticos polimórficos, es decir marcadores que detecten dos o más diferentes alelos en la población para un mismo locus. Entre más polimórfico sea un marcador, más fácil será distinguir cual de los dos alelos se hereda con el fenotipo de la enfermedad.

El advenimiento de la tecnología del DNA recombinante hizo posible la identificación de cambios en la secuencia nucleotídica (polimorfismos) que se presentan normalmente en el DNA. Estos polimorfismos se detectan mediante el uso de enzimas de restricción, las cuales realizan cortes endonucleotídicos en el DNA a nivel de secuencias muy específicas (sitios de restricción) y producen fragmentos de un tamaño determinado. Los citados cambios en la secuencia de DNA pueden originar la formación o alteración de sitios de restricción, de manera que al cortar el DNA con una enzima de restricción determinada se generarán fragmentos de DNA de diferente tamaño al original. La inserción o pérdida de varios nucleótidos, sin que se modifique el sitio de restricción, también variará el tamaño de los fragmentos resultantes. Estas diferencias en el tamaño de los fragmentos de DNA, que se generan con la digestión de enzimas de restricción, provenientes de un mismo locus de los dos cromosomas homólogos se denominan RFLP (Polimorfismos en la longitud de fragmentos de restricción) (38). Este tipo de polimorfismo, RFLP, es el que principalmente se utiliza en los estudios de ligamiento genético para diferenciar los alelos de un mismo individuo y éstos se identifican utilizando la técnica de Southern (39). En este procedimiento se corta el DNA genómico con una enzima de restricción, se separan los fragmentos resultantes en un gel de agarosa, se transfieren a una membrana de nylon, y el DNA en la membrana se hibrida contra una sonda específica del marcador en cuestión. El patrón de alelos que se obtienen para cada individuo de un árbol genealógico se correlaciona con la presencia de la enfermedad para determinar si existe una cosegregación (ligamiento) entre el marcador y la enfermedad. Este grado de correlación o de ligamiento genético se calcula estadísticamente y se expresa como Z ("lod score"). Por convención, se considera que existe un

ligamiento significativo entre el marcador y la enfermedad, cuando la Z a cualquier fracción de recombinación es mayor de 3 (40,41).

B. Estudios de ligamiento genético en el CMT1. Con los estudios de ligamiento genético en las familias afectadas con el tipo de CMT autosómico dominante (CMT1) se ha determinado que existe una heterogenidad genética. En 1980, Bird y cols. analizaron dos familias con CMT1 y encontraron que existía un ligamiento entre ellas y el locus Duffy que se localiza en la región cromosómica q12-q21 del cromosoma 1 (42,43). Guiloff y cols. confirmaron este mismo ligamiento (44), y posteriormente Stebbins y Conneally (45) también llegaron a la misma conclusión, pero utilizando un árbol genealógico de ascendencia Indú. Además el gen Fc gammaRII, que también se localiza en la misma región que el locus Duffy, se encontró ligado al CMT1 (46). Sin embargo, la heterogenidad genética se estableció al determinar que la mayor parte de las familias con CMT1 no están ligadas al locus Duffy (47,48,49,50). De acuerdo a lo descrito por Bird y cols. (48), el tipo de CMT1 ligado al locus Duffy se denomina CMT1B, mientras que el tipo de CMT1 que no está ligado a este locus se denominó CMT1A. Este último es la forma autosómica dominante más frecuente de CMT1 y está ligada al cromosoma 17.

Vance y cols. (51) fueron los primeros en demostrar que la enfermedad de CMT1A está ligada a dos marcadores genéticos localizados en la región proximal del brazo corto del cromosoma 17 (17p): D17S58 (EW301) y D17S71 (pA10-41). Posteriormente, en forma independiente varios grupos confirmaron que estos marcadores están efectivamente ligados a la enfermedad del CMT1A (32,52,53,54,55,56). Sin embargo, aún y cuando la Z es de 3 para estos marcadores, el grado de ligamiento no es muy grande y ocurren recombinaciones entre estos marcadores y la enfermedad de CMT1A. Debido a esto, se ha seguido con el aislamiento de nuevos marcadores genéticos de esta región del cromosoma 17 y se les ha evaluado su grado de ligamiento. En los trabajos realizados por Patel y cols. (32,55) se aislaron de la región cromosómica 17p11.2 los marcadores genéticos D17S258 (1516), D17S445 (S6.1-HB2) y D17S29 (YNM67-R5), los cuales se demostró que también están ligados al CMT1A con una Z de 3.

C. Resumen de la búsqueda del locus de la enfermedad de CMT1A. De acuerdo con los resultados de ligamiento genético efectuados por los diferentes grupos se concluye lo siguiente: aún no se ha identificado un marcador genético estrechamente ligado al locus del CMT1A. En nuestro laboratorio llevamos a cabo un estudio con el propósito de determinar con mayor precisión la localización de todos los marcadores que habían mostrado

ligamiento al CMT1A. Para esto se utilizaron híbridos de células somáticas que retenían el cromosoma 17 completo o con deleciones y se determinó que los marcadores genéticos que muestran ligamiento con el locus de la enfermedad de CMT1A, se localizan en la región cromosómica 17p11.2-p12 (57,58). Tomando esto en cuenta se concluye lo siguiente: el locus de la enfermedad del CMT1A se localiza dentro de la región cromosómica 17p11.2-p12. Cabe señalar que esta región comprende aproximadamente 10 millones de pb.

VI. Planteamiento del proyecto.

A. Limitación de los estudios de ligamiento utilizando RFLP. Para localizar el gen de una enfermedad mediante un estudio de ligamiento genético, se trata de responder la misma pregunta para diferentes regiones cromosómicas: ¿existe una correlación positiva entre la herencia de una región cromosómica específica y la enfermedad?. Si es así, el gen se localiza en aquella región cromosómica ligada a la enfermedad. Sin embargo, para determinar que el ligamiento resultante no es casual, es necesario analizar varios árboles genealógicos. Además cabe recordar que es necesario distinguir cual de las dos copias de dicha región cromosómica del progenitor son heredadas a sus descendientes. Por lo tanto, el marcador genético de una región cromosómica determinada debe ser polimórfico. Hasta la fecha, el polimorfismo de los marcadores utilizados para localizar el locus del CMT1A se han basado en RFLP. Puesto que con este tipo de polimorfismo se detectan generalmente sólo dos alelos diferentes en la población, la información que proporcionan es limitada. Esta limitación se ejemplifica en la Figura 2 en donde se representan familias que segregan una enfermedad autosómica dominante como lo es el CMT1A, con genotipos que comúnmente se observan en los estudios de ligamiento al utilizar RFLP. Si al progenitor afectado no se le distinguen los dos alelos (Figura 2A) o si ambos padres presentan el mismo genotipo (Figura 2B), no será posible determinar cual de los dos alelos se segrega con la enfermedad y por ende si ha ocurrido algún evento de recombinación. Cabe recordar que los eventos de recombinación son los que determinan la distancia entre el marcador y el locus de la enfermedad. Por el contrario, en una situación hipotética en la cual se utilice un marcador muy polimórfico, de manera que todos los alelos de los padres sean diferentes, se podrá determinar con exactitud cual alelo se segrega con la enfermedad (Figura 2: C y D) y por ende detectar eventos de recombinación (Figura 2D). Por lo tanto la falta de un mayor polimorfismo de los marcadores al utilizar RFLPs, aunado con el hecho de que no siempre se cuenta con árboles

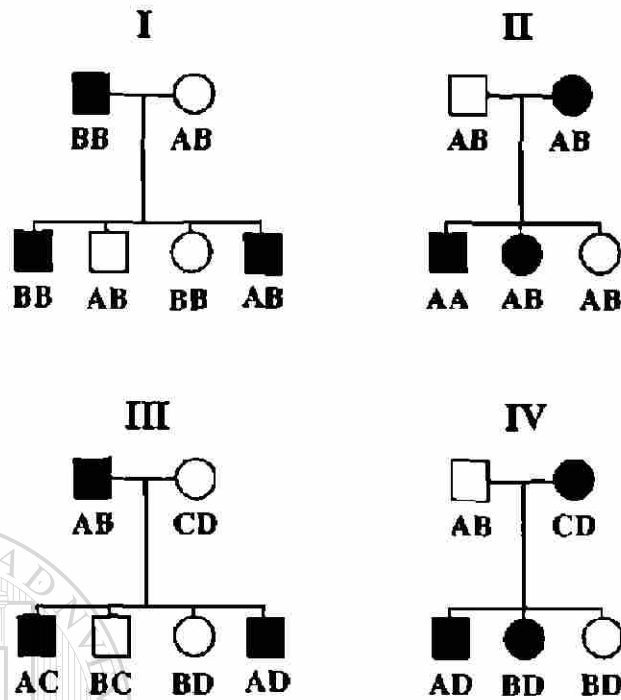


Figura 2. Falta de información de los RFLPs en los estudios de Ligamiento Genético. Esta figura representa el caso de una enfermedad autosómica dominante, como lo es la enfermedad de CMT1A. Las letras corresponden a genotipos hipotéticos para un marcador genético, y los símbolos en negro indican los individuos afectados con la enfermedad.

En el ejemplo I, en el descendiente afectado con genotipo BB no es posible determinar si el alelo mutante (el cromosoma con la mutación) se heredó del padre o de la madre y por lo tanto no se puede obtener una información real de ligamiento.

En el ejemplo II, ocurre lo mismo con el descendiente afectado con genotipo AA, sin embargo podemos deducir que la enfermedad se segrega con el alelo A de la madre. En el descendiente afectado AB tampoco es posible determinar cual alelo está recibiendo de cada padre puesto que tienen los mismos genotipos. En cuanto al descendiente no afectado AB, podría ser el resultado de una recombinación, en donde el alelo A lo está heredando del padre, pero debido a que la madre también posee un alelo A, no es posible identificarlo como un evento de recombinación.

Los ejemplos III y IV corresponden respectivamente a los mismos ejemplos I y II con la diferencia de que en estos casos el marcador hipotético es más informativo (polimórfico). Si analizamos los genotipos de los descendientes en el ejemplo III, podemos deducir que la enfermedad se está segregando con el alelo A del padre. En el ejemplo IV y de acuerdo con los genotipos de los dos descendientes afectados, la enfermedad se segrega con el alelo D de la madre, sin embargo, el individuo no afectado BD también heredó el alelo D y puesto que no está enfermo significa que ocurrió un evento de recombinación. Es decir que durante la meiosis para generar los gametos de su padre, el locus del alelo D y el locus de la enfermedad, originalmente presentes en el mismo cromosoma, fueron separados por recombinación uno en cada miembro del par de cromosomas homólogos. Entre mayor número de eventos de recombinación se observen, será mayor la distancia entre el locus de la enfermedad y el locus del marcador que se está analizando.

En resumen, entre mayor sea el polimorfismo del marcador, será posible determinar con mayor precisión la segregación de los alelos del marcador con respecto a la enfermedad y con ello el resultado del estudio de ligamiento será más confiable.

genealógicos de varias generaciones, hacen que el análisis estadístico del ligamiento genético sea menos preciso y origine resultados de ligamiento muy diferentes, aún para el mismo marcador genético. Por ejemplo, la Z obtenida por diferentes grupos de investigación entre el locus del CMT1A y los marcadores genéticos EW301 y A10-41 fluctúa respectivamente entre 2.37 a 10.67 y 0.89 a 5.26 (25). Resultados discrepantes se observan también para los diferentes marcadores genéticos que se han analizado hasta la fecha.

B. Una estrategia alternativa. El propósito del presente trabajo fué el de resolver esta limitación al incrementar el potencial informativo (mayor grado de polimorfismo) de cada marcador ligado al CMT1A, mediante el uso de polimorfismos que se presentan en las secuencias repetidas en tandem tales como aquellas formadas por un dinucleótido de guanina y timina. Estas secuencias se designan en este trabajo como secuencias (GT) n .

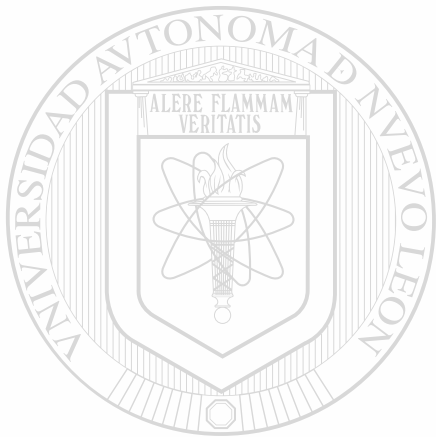
Secuencias repetidas del dinucleótido guanina y timina (SRDGT) Las SRDGT son un tipo de secuencia de DNA que se repite en tandem, cuya fórmula es (dC-dA) n .(dG-dT) n y se designan como secuencias (CA) n o (GT) n . Las SRDGT son muy abundantes y se ha calculado que existen dispersas en el genoma humano de 50,000 a 100,000 secuencias (GT) n , en donde $n \approx 10-60$. Si se considera una distribución uniforme de estas secuencias, la posibilidad de encontrar secuencias (GT) n es de una por cada 30-60 kb (59-62).

Una característica notable de las secuencias repetidas en tandem (SRT) es que varía su tamaño, para un mismo locus entre los diferentes individuos, debido a un cambio en el número de repeticiones dentro de la SRT, (63,64). Es decir que el polimorfismo de las SRT es en cuanto a su longitud, y se pueden reconocer en la población múltiples alelos para un mismo locus. Esto se ha demostrado para diferentes SRT, como por ejemplo en el DNA satélite de primates (65,66), en los genes humanos para la visión del color (67), en la SRT de los genes que codifican para antígenos del parásito de la malaria (68), y en los minisatélites (63,64). Este mismo fenómeno de polimorfismo ocurre en las secuencias (GT) n ya que son también un tipo de SRT (69-72).

El grado de polimorfismo de las secuencias (GT) n es muy superior al de los RFLP, ya que mientras que los RFLP distinguen normalmente dos alelos en la población, con las secuencias (GT) n se pueden distinguir hasta 16 alelos. Los marcadores genéticos que basan su polimorfismo en estas secuencias (GT) n se designan como marcadores (GT) n , y sus correspondientes alelos se denominan alelos (GT) n . Su polimorfismo se determina al amplificar por PCR una región de 150 pb que contenga la secuencia (GT) n y el tamaño de sus

alelos (GT)_n se visualizan en geles de poliacrilamida (69-72).

El hecho de que los marcadores (GT)_n son muy polimórficos, aunado a su amplia distribución en el genoma humano, los hacen ideales para los estudios de ligamiento genético, e incluso representan una herramienta nueva muy valiosa en la resolución del mapa genético del genoma humano.



UANL

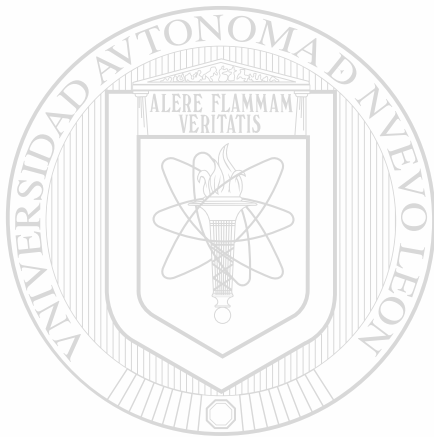
UNIVERSIDAD AUTÓNOMA DE NUEVO LEÓN



DIRECCIÓN GENERAL DE BIBLIOTECAS

HIPOTESIS

Las secuencias (GT)_n son más polimórficas que los RFLP. En un estudio de ligamiento genético, entre mayor sea el grado de polimorfismo del marcador utilizado, el resultado del estudio será más preciso. Por lo tanto, los marcadores (GT)_n determinarán con mayor precisión que los RFLP la localización del locus del CMT1A.



UANL

UNIVERSIDAD AUTÓNOMA DE NUEVO LEÓN



DIRECCIÓN GENERAL DE BIBLIOTECAS

OBJETIVO

Localizar por ligamiento genético el locus para la enfermedad del CMT1A utilizando marcadores (GT)_n.

La estrategia que se siguió para cumplir con nuestro objetivo general estuvo integrada por los siguientes objetivos experimentales:

I. Identificar marcadores genéticos de la región 17p11.2-12 que poseen una repetición (GT)_n.

De acuerdo con los antecedentes, el locus del CMT1A se encuentra dentro de la región cromosómica 17p11.2-12. A todos los marcadores genéticos disponibles de la región 17p11.2-12 se les determinó por hibridación en gota si contenían una secuencia (GT)_n. Puesto que se encuentra una secuencia (GT)_n aproximadamente cada 50 Kb, inicialmente se aislaron cósmidos para algunos marcadores cuyo tamaño era menor de 10 kb. Estos cósmidos portan fragmentos de DNA humano de aproximadamente 40 kb.

II. Obtener marcadores (GT)_n.

De los marcadores positivos para una secuencia (GT)_n se subclonó y caracterizó un fragmento de DNA que contuviera la secuencia (GT)_n, y el cual se designó como un marcador (GT)_n.

III. Determinar el genotipo de los individuos que conforman los árboles genealógicos segregando CMT1A.

A cada uno de los individuos de los árboles genealógicos de la Figura 3, se les determinó el genotipo, es decir el tamaño de sus alelos (GT)_n, para cada uno de los marcadores (GT)_n identificados en el punto II.

IV. Calcular el grado de ligamiento genético de cada marcador (GT)_n.

El cálculo del grado de ligamiento entre el CMT1A y los marcadores (GT)_n se planteó determinarse mediante el programa de computación denominado LINKAGE (61).

MATERIAL Y METODOS.

I. Origen de los materiales y reactivos.

Las enzimas de restricción y modificación de ácidos nucleicos se obtuvieron de diversas casas comerciales: Bethesda Research Laboratories (Bethesda, MD), Boehringer Mannheim Corporation (Indianapolis, IN), New England Biolabs (Beverly, MA), y Pharmacia (Piscataway, NJ), y se utilizaron de acuerdo con las condiciones especificadas por el proveedor.

Los reactivos empleados en la elaboración de amortiguadores, soluciones diversas y medios de cultivo se obtuvieron de distintas casas comerciales: Aldrich (Milwaukee, WI), BIO 101 (La Jolla, CA), Bio-Rad (Richmond, CA), GIBCO BRL (Grand Island, NY), Mallinckrodt Specialty Chemicals Co. (St. Louis, MO), New England Nuclear (Boston, MA), Pharmacia (Piscataway, NJ), Sigma Chemical Company (St. Louis, MO) y United States Biochemical Corporation (Cleveland Ohio).

II. Familias y su evaluación clínica.

Se analizaron 194 individuos pertenecientes a siete árboles genealógicos en los cuales se segrega la enfermedad de CMT1A. En ellos se puede observar la transmisión hombre a hombre, y de generación en generación, característico de una enfermedad autosómica dominante (Figura 3). Seis de estas familias, HOU1 (32), HOU2, HOU42 (55), HOU85, HOU88, y HOU89 son de ascendencia Acadio-Francés, mientras que la familia HOU76 es de ascendencia Judía Ashkenazic. Todos los individuos se sometieron a una meticulosa evaluación clínica y electrofisiológica. Puesto que la disminución en la VCN es un signo con penetrancia completa, inclusive para individuos asintomáticos con CMT1A, se utilizó este parámetro para diagnosticar correctamente la enfermedad. Por tal motivo, a cada uno de los individuos se le determinó la VCN bilateralmente de los nervios mediano y cubital. Una VCN menor de 40 m/seg es diagnóstica de CMT1A.

De cada individuo se obtuvo sangre periférica y se utilizó para aislar DNA genómico de alto peso molecular (73). Además, a cada uno de los individuos se le comprobó el origen paterno y materno utilizando el marcador molecular muy polimórfico denominado YNH24 (locus D2S44) (64,74).

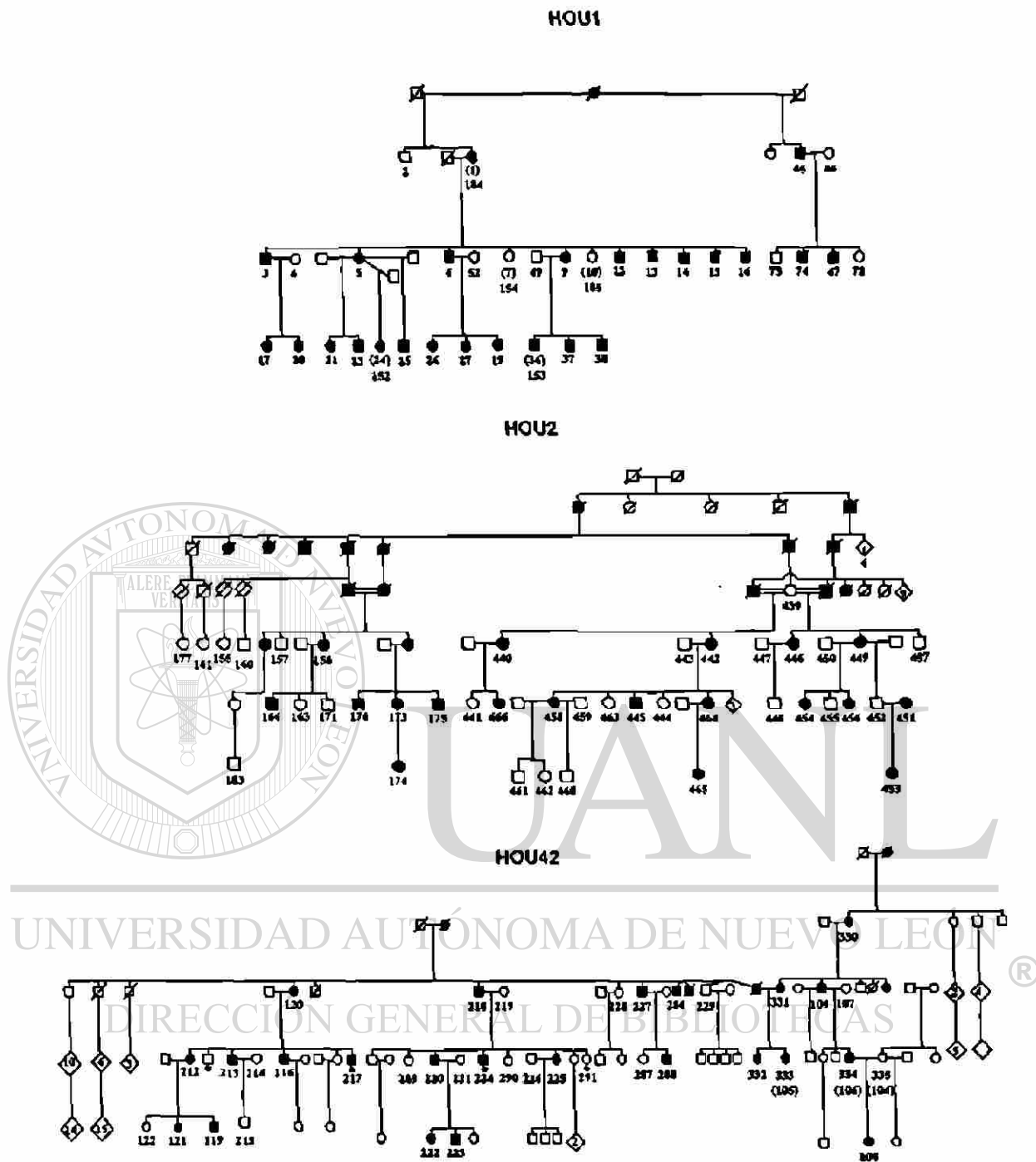


Figura 3A. Árboles genealógicos segregando la enfermedad de CMT1A.

Todos son de origen Acadio-Francés. Los números debajo de cada símbolo corresponden a la identificación del laboratorio para cada individuo. Note la transmisión de la enfermedad hombre a hombre y sin saltar generaciones, característico de una herencia autosómica dominante.

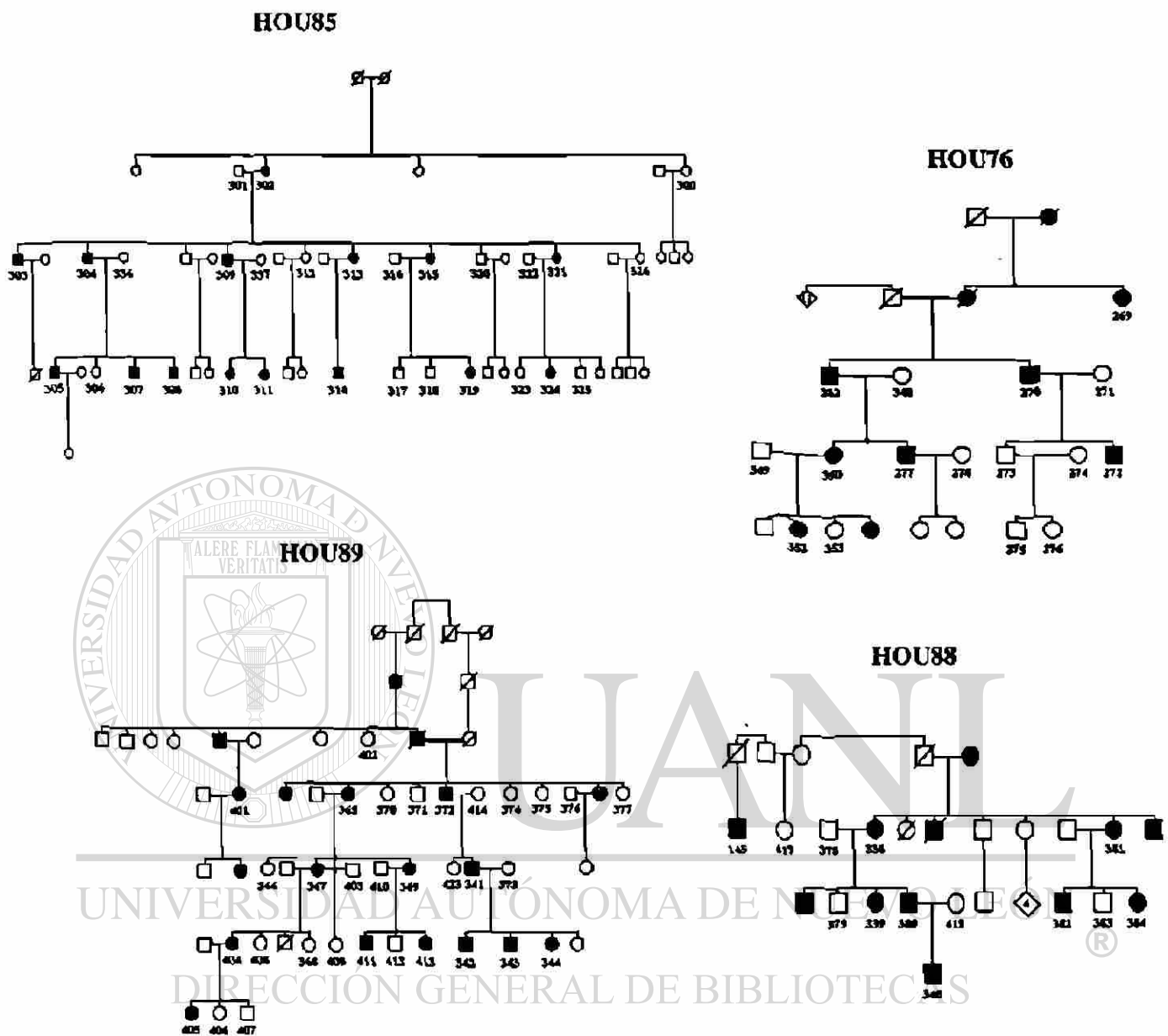


Figura 3B.- Árboles genealógicos segregando la enfermedad de CMT1A.

Todos son de origen Acadio-Francés, excepto HOU72 el cual es de descendencia Judía. Los números debajo de cada símbolo corresponden a la identificación del laboratorio para cada individuo. Note la transmisión de la enfermedad hombre a hombre y sin saltar generaciones, característico de una herencia autosómica dominante.

III. Marcadores genéticos.

Los marcadores genéticos que se utilizaron en este trabajo se describen en la Tabla 1. Para cada uno de ellos se purificó DNA a gran escala por el método de lisis alcalina, seguido por una purificación en gradientes de cloruro de cesio, de acuerdo al procedimiento descrito por Maniatis y cols. (75).

IV. Aislamiento de cósmidos.

Dos de las secuencias (GT)_n descritas en este trabajo (pRM7-GT y OS1-βGT) se identificaron en los cósmidos cH3 y cO15F4, los cuales corresponden respectivamente a los marcadores FG1 y al gen de la subunidad β del receptor muscular de la acetilcolina. Para la obtención de estos cósmidos se utilizó una librería de cósmidos, específica del cromosoma humano 17, construída en el laboratorio National Lawrence Livermore (Livermore, California). El tamizaje de la librería se realizó según se describe brevemente a continuación.

A. Preparación de las membranas. Aproximadamente 10,000 bacterias portando cósmidos se crecieron durante aproximadamente 12 h en membranas de nylon (Genescreen Plus) colocadas sobre cajas de cultivo conteniendo medio Luria-Bertani suplementado con ampicilina a 50 µg/ml. Posteriormente, para amplificar los cósmidos, las membranas se transfirieron a cajas de cultivo conteniendo el mismo medio, pero suplementado con cloranfenicol a 200 µg/ml. Las colonias bacterianas se lisaron directamente sobre la membrana con una solución de SDS al 5% y SSC 2X durante 5 min. Enseguida las membranas se pusieron en un horno de microondas de 700 W durante 5 min en el nivel máximo de poder, se secaron al aire y se hornearon al vacío durante 2 h a 80°C. Finalmente las membranas se lavaron dos veces en una solución conteniendo Tris 50 mM pH 8, NaCl 1M, EDTA 1 mM y SDS al 0.1%, a 50°C durante 15 min cada lavado.

B. Hibridación de las membranas. El procedimiento de hibridación y lavado de las membranas que a continuación se describe, es el mismo que se utilizó en todas las hibridaciones referidas en este trabajo. Las membranas se prehibridaron e hibridaron a una temperatura de 65°C en una solución compuesta de NaCl 1M, SDS 1%, sulfato de dextran al 10% y DNA sonicado de placenta humana a una concentración de 0.25 mg/ml. El tiempo de la prehibridación fué de 2 h al término del cual se adicionó la sonda radiactiva previamente desnaturalizada por calor y se dejó hibridar durante 16 h. El lavado de las membranas consistió en lo siguiente: un lavado inicial de 5 min

Tabla 1. Marcadores utilizados en este estudio para la identificación de repeticiones (GT)ⁿ.

MARCADOR	LOCUS	UBICACION	RFLP	ALELOS	REFERENCIAS
EW301	D17S58	17cen-p11.2	<i>TaqI</i> <i>BglII</i>	4.5/3.1 kb 10.0/8.0 kb	Barker et al., 1987
c1516	D17S258	17p11.2	<i>HindIII</i>	22kb/12+10kb	Patel et al., 1989
p1516-R4	D17S258	17p11.2	<i>MspI</i>	3.3/2.4 kb	Franco et al., 1990
pA1041	D17S71	17p11.2	<i>MspI</i> <i>PvuII</i>	2.4/1.9 kb 3.2/3.0 kb	Nakamura et al., 1988
S6.1-HB2	D17S445	17p11.2	<i>MspI</i> <i>SstI</i> <i>MspI</i>	1.0/1.1 kb 12.2/7.1 kb 1.2/1.6kb	Patel et al., 1990
FG-1	D17S446	17p11.2	<i>ApaI</i>	12.0/7.5 kb	Guzzetta et al., 1991
YNM67-R5	D17S29	17p11.2	<i>TaqI</i> <i>BglII</i>	3.4/2.2+1.3 kb 8.1/6.7 kb	Ray et al., 1990
EW401	D17S61	17p11.2-p12	<i>MspI</i>	5.2/4.4 kb	Wright et al., 1990
EW402	D17S62	17p11.2-p12	<i>MspI</i>	8.0/7.0 kb	Wright et al., 1990
EW403	D17S63	17p11.2-p12	<i>MspI</i>	13.5/6.8 kb	Wright et al., 1990
EW404	D17S64	17p11.2-p12	<i>MspI</i>	5.6/5.0 kb	Wright et al., 1990
EW405	D17S121	17p11.2-p12	<i>MspI</i>	2.1/1.4 kb	Wright et al., 1990
VAW409R1	D17S122	17p11.2-p12	<i>MspI</i>	5.3/2.7+2.6 kb	Wright et al., 1990
VAW409R3	D17S122	17p11.2-p12	<i>MspI</i>	2.8/2.7/1.9 kb	Wright et al., 1990
VAW410R1	D17S123	17p11.2-p12	<i>BglII</i> <i>TaqI</i>	2.0/2.1 kb 10/9.4 kb	Wright et al., 1990
VAW411R2	D17S124	17p11.2-p12	<i>MspI</i> <i>BglII</i>	10.5/6.1 kb 11.0/10.7 kb	Wright et al., 1990
VAW412R3	D17S125	17p11.2-p12	<i>MspI</i>	10.5/5.4 kb	Wright et al., 1990
EW502	D17S66	17p11.2-p12	<i>TaqI</i>	2.2/1.4 kb	Wright et al., 1990
EW503	D17S67	17p11.2-p12	<i>BglII</i>	6.9/5.7 kb	Wright et al., 1990
c1517	D17S259	17p11.2-p12			Patel et al., 1990
c1541	D17S260	17p13			Patel et al., 1990

a temperatura ambiente en una solución de SSC 2X y 0.1% de SDS; enseguida un lavado de 20 min a 65°C en una solución de SSC2X y SDS al 0.1%, y finalmente un lavado de 10-20 min a 65°C en una solución de SSC 0.1X y SDS al 0.1%. Las membranas se colocaron con un film sensible a la radiactividad (Kodak XAR-5) durante al menos 12 horas a una temperatura de -70°C.

V. Obtención de marcadores genéticos (GT)n.

A. Identificación de secuencias (GT)n. Para identificar este tipo de secuencias se analizaron por hibridación de DNA en gota (76) todos los marcadores genéticos disponibles en el laboratorio que habían mostrado cierto grado de ligamiento con la enfermedad de CMT1A (Tabla 1).

B. Preparación de la sonda. Se utilizó como sonda un copolímero sintético de doble cadena del dinucleótido guanina y timina denominado poly(dC-dA).poly(dG-dT) (Pharmacia). 100 ng de este copolímero se marcaron radiactivamente con [alfa-³²P]dCTP (New England Nuclear) utilizando el estuche de marcaje por translación de la muesca o "Nick Translation" (Amersham).

C. Hibridación de DNA en gota (76). Para esta hibridación se utilizó el dispositivo Vacusystem (BioRad) y se manejó de acuerdo a las instrucciones especificadas por el proveedor. Básicamente consistió en lo siguiente. Se colocó una membrana de nylon (Genescreen Plus) del tamaño apropiado en el Vacusystem y en cada uno de sus pozos se depositó 1 µg de DNA disuelto en 100 µl de agua. El DNA fué previamente desnaturalizado por ebullición durante 5 min y enfriado inmediatamente en hielo durante 5 min. Se aplicó vacío para succionar la solución y adherir el DNA a la membrana de nylon, enseguida se depositó 400 µl de NaOH 0.4 N en cada uno de los pozos y se aplicó nuevamente vacío. La membrana se separó del Vacusystem, se lavó con SSC 2X y se dejó secar al aire entre dos hojas de papel Whatman 3 MM. La membrana se prehibridó e hibridó de la manera anteriormente descrita, con las diferencias de que el tiempo de la hibridación fué de 5 h y la membrana se lavó únicamente a temperatura ambiente en una solución conteniendo SSC 2X y SDS al 0.1%. Finalmente la membrana se colocó con una película de Rayos X sensible a la radiactividad, a -70°C durante 2 a 12 h. En este tipo de experimentos se utilizó como control positivo un fragmento de DNA de 150 pb que contenía una secuencia (GT)n.

D. Subclonación y caracterización de las secuencias (GT)n. Como se

mencionó en los antecedentes, el polimorfismo de las regiones (GT)_n se determina por la técnica de PCR, y por lo tanto es importante conocer la secuencia nucleotídica de la región que flanquea la secuencia (GT)_n para sintetizar los oligonucleótidos necesarios en la reacción de PCR. Por esto, una vez identificados los marcadores genéticos que contienen una secuencia (GT)_n, es necesario subclonar un fragmento de DNA que contenga la secuencia repetida y que sea del tamaño adecuado para su secuenciación. La estrategia que se siguió para este propósito fue la de cortar los marcadores positivos para secuencias (GT)_n con enzimas de restricción cuyo sitio de reconocimiento era de cuatro y cinco nucleótidos. Los fragmentos de DNA que resultaron de la digestión se separaron en un gel de agarosa al 1.5% y mediante una hibridación tipo Southern (55) se determinó cual de las enzimas producía un fragmento de DNA, portando la secuencia (GT)_n, del tamaño adecuado para secueceo. A diferencia del procedimiento de hibridación en gota seca, en el Southern, el DNA se separó inicialmente en un gel de agarosa y posteriormente se transfirió por capilaridad a la membrana de nylon. Una vez identificada la enzima de restricción que produjo el fragmento de interés, se cortó nuevamente el marcador para purificar dicho fragmento de un gel preparativo de agarosa utilizando el estuche de GeneClean (BIO 101). Finalmente este fragmento de DNA se subclonó en el plásmido pTZ19R mediante una reacción de ligación. La reacción de ligación se efectuó en un volumen final de 15 µl conteniendo: 300 ng del vector pTZ19R, 200 ng del fragmento portando la secuencia (GT)_n, 1.5 µl de amortiguador One Phor-All Plus (Pharmacia), 1 mM de dATP y 1 U de DNA ligasa del bacteriófago T4. Esta mezcla se incubó a 16°C durante al menos 1 h, y el producto de la ligación se utilizó para transformar *Escherichia coli* DH5 alfa Ca⁺⁺ competentes. Las clonas recombinantes se analizaron con enzimas de restricción para corroborar que contuvieran el inserto del tamaño correspondiente y se les determinó la secuencia nucleotídica mediante el procedimiento de terminación de cadena (77,78) utilizando el estuche de "Sequenase Kit" (United States Biochemicals) en combinación con ³⁵S-α-dATP (Amhersam).

VI. Determinación de genotipos (GT)_n.

Para determinar el polimorfismo en el locus de las secuencias (GT)_n, de los diferentes individuos y obtener sus correspondientes genotipos (GT)_n, se analizó el DNA genómico mediante un procedimiento previamente descrito en la literatura (70).

A. Aislamiento de DNA genómico de alto peso molecular. De cada individuo que formó parte de los diferentes árboles genealógicos se obtuvo DNA genómico de alto peso molecular a partir de sangre periférica de acuerdo al procedimiento descrito por Miller y cols. (73). De 15 a 25 ml de sangre se centrifugaron a 2500 rpm por 15 min. en un tubo cónico de 50 ml, se descartó el plasma y se adicionó al tubo una solución conteniendo Sacarosa 0.32 M, Tris pH 7.5 10 mM, MgCl₂ 5 mM y 1 % de Tritón X-100 hasta completar 50 ml, se mezcló por inversión y se incubó en hielo por 30 min. Se centrifugó nuevamente, se descartaron ≈ 42 ml, la pastilla celular se resuspendió después de aforar nuevamente a 50 ml con la misma solución y se colocó nuevamente en hielo por 20 min. Se volvió a centrifugar y el precipitado se resuspendió en 3 ml de una solución conteniendo Tris pH 7.5 10 mM, NaCl 400 mM y EDTA 2 mM, y se le adicionó 0.1 ml de SDS al 20% y 0.5 ml de una solución de proteinasa K a 2 mg/ml. Se incubó por 12 h a 37°C y la proteinasa se eliminó con un tratamiento de fenol. Finalmente, el DNA se precipitó con 2 volúmenes de etanol y un décimo de acetato de sodio 3 M, se recuperó con una pipeta pasteur y se resuspendió en TE (Tris 10 mM pH 7.5 y EDTA 1 mM).

B. Marcaje de oligonucleótidos. Uno de los dos oligonucleótidos utilizados en la reacción de PCR, se marcó radiactivamente en su extremo 5' utilizando la enzima polinucleótido cinasa. El marcaje se realizó en un volumen final de 15 µl conteniendo: 1.2 µM de oligonucleótido, 100 µCi de [γ -³²P]ATP con una actividad de 6000 Ci/mmol, amortiguador One Phor-All Plus (Pharmacia) diluido a una concentración final de 1X, y 10U de la enzima. La mezcla se incubó a una temperatura de 37°C por 30 minutos, y la enzima se inactivó a 65°C por 10 minutos.

C. Reacción de PCR. La reacción de PCR se realizó utilizando condiciones estandar (79) en un volumen final de 25 µl que contenía: 300 ng de DNA genómico, cada uno de los dos oligonucleótidos que flanquean la secuencia (GT)_n a una concentración de 1 µM, 0.2 µl de uno de estos dos oligonucleótidos marcado radiactivamente, 250 µM de cada uno de los 4 desoxinucleótidos (dATP, dCTP, dGTP y dTTP), 2.5 µl de buffer de PCR (KCl a 500 mM, Tris-HCl 120 mM pH 8.0, MgCl₂ a 1.5 mM, y gelatina al 0.01%), y 0.63 U de la enzima DNA polimerasa "Ampli-Taq" (Cetus). Las condiciones de la reacción de PCR fueron una desnaturalización inicial de 5 min a 94°C; seguida por 30 ciclos de amplificación, consistiendo cada ciclo de 1 min de desnaturalización a 94°C, 1 min a 55°C y 2 min de polimerización a 72°C; y una extensión final de 7 min a 72°C. 1.5 µl del producto de la

reacción de PCR se mezcló con 2 µl de formamida (Stop solution, United States Biochemical Corporation) y se analizó en un gel de poliacrilamida al 6% del tipo que se usa para secuenciación de DNA. La electroforesis se desarrolló durante 3.5 hrs a 40 W. Los geles se secaron y se colocaron con un film Kodak XAR-5 durante 2-12 horas a una temperatura de -70°C. Para determinar el tamaño de los alelos (GT)_n de los diferentes individuos, estos alelos se compararon en el gel de secuenciación con el producto del PCR del respectivo plásmido recombinante que contenía la secuencia (GT)_n. Puesto que se obtuvo la secuencia nucleotídica de estos plásmidos, se conocía el tamaño exacto del producto de PCR de cada marcador (GT)_n.

D. Interpretación de los resultados. Los genotipos (GT)_n, es decir los alelos (GT)_n para cada individuo se designaron de acuerdo a su tamaño en pares de bases: A= 165, B=163, C=161, D=159, E=157, F=155 y G=153. A los individuos que se les observó únicamente un alelo se registraron como poseedores de dos copias de ese alelo. Los genotipos para cada individuo se leyeron sin conocer el estado de su enfermedad, y se confirmaron por otros dos investigadores. En este tipo de polimorfismos de secuencias repetidas se observan invariablemente bandas de menor tamaño y menos intensas que la banda principal de los alelos; sin embargo, es posible leer los genotipos sin ambigüedad (72).

VII. Obtención de genotipos utilizando RFLP.

A. Análisis tipo Southern. En este análisis se utilizó el DNA genómico de alto peso molecular purificado anteriormente. Muestras de DNA de los diferentes individuos (5.5 µg), se digirieron con la enzima de restricción *MspI*® de acuerdo a las condiciones especificadas por la compañía, utilizando 3-4 U/µg de DNA. Los DNAs digeridos se separaron por electroforesis en geles de agarosa al 1% en buffer TAE (Tris-HCl 40mM, pH 8.5, acetato de sodio 40 mM, y EDTA 2 mM) durante aproximadamente 16 h con un voltaje constante de 35 volts y se transfirieron a una membrana de Nylon (Genescreen Plus). La transferencia e hibridación del DNA se realizaron de acuerdo al procedimiento ya descrito anteriormente(55). En este caso, después de la hibridación las membranas se lavaron con una solución de SSC 2X y SDS al 0.1% a temperatura ambiente durante 5 min, enseguida con SSC2X y SDS al 0.1% a 65°C durante 30 min y finalmente con SSC 0.1X y SDS al 0.1% a 65°C durante 10-20 min. Las membranas se colocaron con un film sensible a la radiactividad.

B. Marcaje radiactivo de las sondas. El marcaje se realizó con 100 ng de DNA de las sondas ya sea en solución o en agarosa de bajo punto de fusión previamente fundida, mediante el procedimiento de Feinberg and Vogelstein (80) utilizando ^{32}P - α -dCTP (New England Nuclear) . Cada una de las sondas radiactivas se precipitó agregando espermina a 10 mM, para eliminar los nucleótidos libres (81).

C. Preasociación de las sondas. Debido a la relativa abundancia de secuencias repetidas en el genoma humano (por ejemplo la secuencia Alu), es muy alta la probabilidad de que el DNA utilizado como sonda contenga una de ellas. Si este es el caso, la sonda hibridará con un gran número de fragmentos de DNA y como resultado de la hibridación se observará una señal radiactiva a lo largo del carril. Para evitar ésto, antes de que se utilizaran en la hibridación, las sondas radiactivas se mezclaron en una solución de hibridación que contenía DNA sonicado de placenta humana a una concentración final de 0.25 mg/ml. Esta mezcla se calentó a 95°C durante 5 min y la preasociación se realizó a 65°C durante dos horas. Durante este tiempo las secuencias repetidas de la sonda hibridarán con las secuencias repetidas del DNA sonicado de placenta humana, y de esta manera no hibridarán con el DNA adherido a la membrana de nylon.

D. Cuantificación de las bandas de hibridación. La cuantificación de las bandas de hibridación, correspondientes a los alelos *MspI* en las autorradiografías de los Southern se determinó al comparar por inspección visual y por densitometría (LKB Ultrascan) la señal de hibridación entre las dos bandas de los alelos polimórficos *MspI* de individuos heterocigotos.

VIII. Separación de los alelos polimórficos *MspI* del marcador VAW409R1.

La separación y purificación de los alelos se realizó según el método descrito por Bedford y van Helden (82). El DNA genómico (5 μg) correspondiente a los individuos de una familia del árbol genealógico HOU42 se digirió con la enzima de restricción *MspI* y los fragmentos de DNA resultantes de la digestión se separaron por electroforesis en un gel de agarosa al 1% en buffer TAE. Para permitir una buena separación de los fragmentos de DNA, la electroforesis se realizó a 20V, durante aproximadamente 20 horas. Del gel se cortaron por separado los fragmentos de 3 y 6 Kb, correspondientes al tamaño de los alelos polimórficos *MspI* del marcador pVAW409R1, y se purificaron de la agarosa utilizando el estuche de

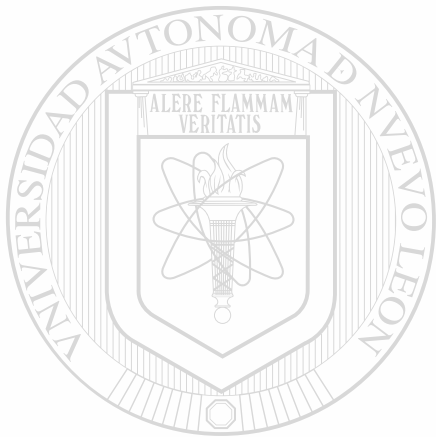
Geneclean (BIO101). A cada uno de estos DNA se le determinó por PCR el número de alelos (GT)_n utilizando los oligonucleótidos del marcador pRM11-GT.

IX. Construcción y análisis de híbridos de células somáticas.

Se utilizaron híbridos de células somáticas para separar los cromosomas homólogos, paterno y materno, número 17, de los individuos 42-331, 42-332 y 42-333. Los híbridos se construyeron de acuerdo a lo descrito por Zoghbi y cols. (83), usando como célula paterna la línea celular de Hamster Chino a23, deficiente en timidina kinasa (84). Brevemente, se crecieron en dos cajas de cultivo de 100mm 10⁷ células a23, durante 16 a 24 horas y posteriormente se lavaron con medio Eagle modificado por Dulbecco (MEMD). 10⁷ linfoblastos de cada individuo se resuspendieron en 10 ml de solución salina de Hanks (GIBCO), adicionada con 250 µl de una solución de fitohemaglutinina (Sigma) a 1 mg/ml. A cada una de ambas cajas de cultivo que contenían las células a23, se les adicionó 5 mls de la suspensión de linfoblastos, y se incubaron a 37°C durante 15 min. Posteriormente se aspiró la solución y se vertieron sobre cada caja 2 ml de una solución de polietilenglicol 1500 al 50% (Boehringer Mannheim Biochemicals). Transcurrido 1 min se aspiró la solución del polietilenglicol, se lavaron las células tres veces con MEMD y se incubaron en el mismo medio a 37°C durante 30 min. Nuevamente se aspiró el medio, y se depositó en cada caja 10 ml de medio MEMD suplementado con 10% de suero fetal de ternera (SFT), y se incubaron nuevamente durante 12 horas. Los híbridos formados se seleccionaron en medio MEMD conteniendo 10% de SFT, 0.1 mM de hipoxantina, 0.001 mM de aminopterina, y 0.01 mM de timidina. Transcurridos de 12 a 14 días se aislaron los híbridos resultantes [®] y se transfirieron a cajas de microtitulación de 24 pozos.

Para determinar cual de los dos cromosomas homólogos 17 se retuvo en los híbridos, se utilizó el marcador genético (GT)_n OS1-βGT (este marcador se localiza fuera de la región duplicada y forma parte de un intrón del gen para la subunidad beta del receptor muscular de la acetilcolina). Las células que crecieron en cada uno de los pozos, se colectaron y se lisaron por ebullición en 30 µl de buffer de PCR. Cada uno de los lisados se sometió a una reacción de PCR utilizando los oligonucleótidos que flanquean la secuencia (GT)_n del marcador OS1-βGT. La reacción de PCR se realizó de la manera anteriormente descrita utilizando ya sea 3 µl de cada lisado celular o 100 ng de DNA genómico de los pacientes seleccionados. Finalmente, para determinar que cromosoma 17 se retuvo, se compararon los alelos (GT)_n de los

linfoblastos del paciente contra el alelo presente en cada híbrido.



UANL

UNIVERSIDAD AUTÓNOMA DE NUEVO LEÓN

®

DIRECCIÓN GENERAL DE BIBLIOTECAS

RESULTADOS.

Con el objetivo de definir con mayor precisión el locus para la enfermedad de CMT1A, se inició la identificación de secuencias (GT)_n en diferentes marcadores genéticos ligados al CMT1A (Tabla 1).

I. Detección de secuencias (GT)_n en los marcadores ligados al CMT1A.

Inicialmente llevamos a cabo un experimento piloto para determinar la factibilidad de identificar secuencias (GT)_n con el procedimiento de hibridación en gota. El resultado se muestra en la Figura 4, donde se observa una señal de hibridación fuerte en los testigos positivos y prácticamente nula con los negativos. Estos resultados nos indicaron que el procedimiento funcionó adecuadamente, por lo que procedimos a determinar cuales de los marcadores citados en la Tabla 1 contenían una secuencia (GT)_n. De esta tabla, los marcadores genéticos S6.1-HB2 y FG1, corresponden a dos nuevos marcadores de la región cromosómica 17p11.2 que se identificaron y clonaron en nuestro laboratorio (57,58).

De todos los marcadores citados en la Tabla 1, únicamente 5 resultaron positivos para la presencia de una secuencia (GT)_n: cH3, c1517, c1541, c1516 y VAW409R1. En la misma Figura 4, se muestra únicamente el resultado de la hibridación en gota para los marcadores cH3 y c1517.

El siguiente paso fué el identificar y subclonar de estos marcadores un fragmento del tamaño apropiado para secuenciación y que contuviera la secuencia (GT)_n. La estrategia que seguimos fué el de cortar estos cinco marcadores con diferentes enzimas de restricción y mediante el procedimiento de Southern identificar los fragmentos de interés que contuvieran una secuencia (GT)_n. Cabe señalar que los marcadores cH3, c1517, c1541 y c1516 corresponden a cósmidos con un inserto de DNA humano de aproximadamente 40 kb, mientras que el marcador VAW409R1 corresponde al plásmido pUC18 con un inserto de DNA humano de 11 kb.

En la Figura 5 se muestra el patrón electroforético de los fragmentos de restricción del cósmido cH3 analizados en un gel de agarosa al 1.5%. Este mismo gel se utilizó en el análisis tipo Southern para identificar cual fragmento de restricción contenía la secuencia (GT)_n (Figura 6). Como se

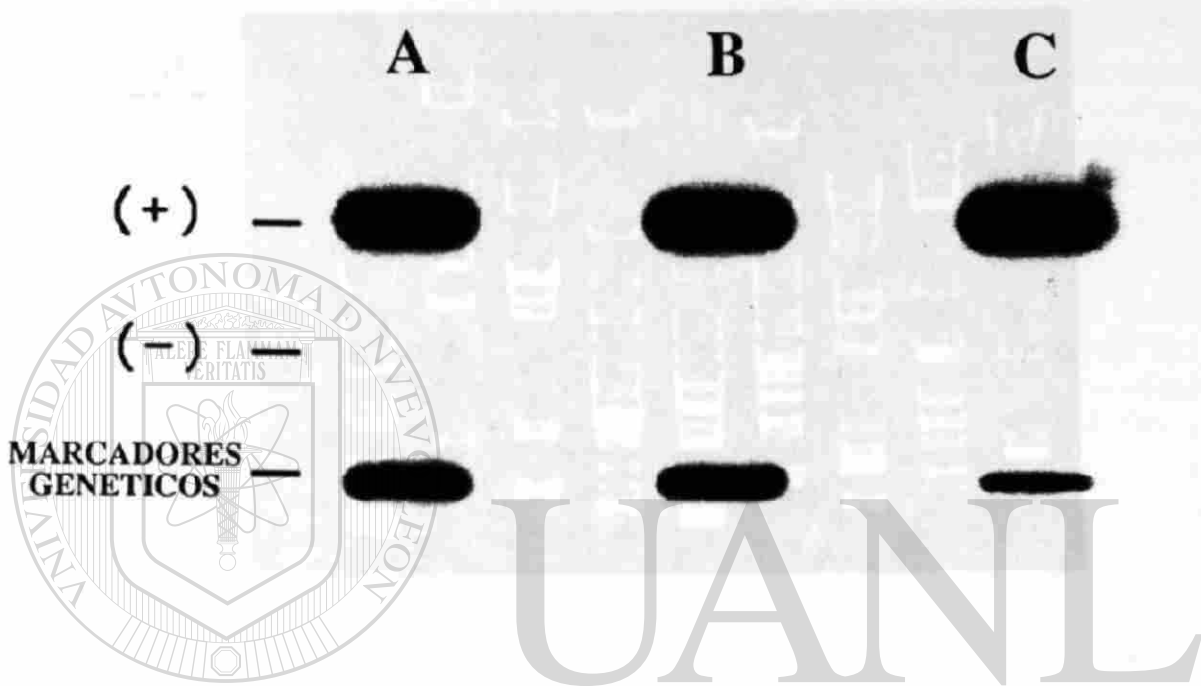


Figura 4. Identificación de marcadores genéticos que contienen una secuencia (GT)_n por hibridación en gota.

Autorradiografía de una hibridación utilizando como sonda radiactiva un copolímero bicatenario sintético del dinucleótido guanina y timina. Las señales en los tres carriles A, B, y C de la línea indicada (+), corresponden al testigo positivo del experimento, el cual consistió de un fragmento de DNA de 150 pb (20 ng) que contenía una secuencia repetida del dinucleótido guanina y timina. En los carriles de la línea indicada (-) se encuentran los testigos negativos del experimento, los cuales corresponden a 1 µg de los siguientes plásmidos: A) pTZ19R, B) pUC18 y C) pBR322. En la tercer línea se colocaron 1 µg de los siguientes marcadores genéticos: A) cH3, B) c1517 y C) c1516.

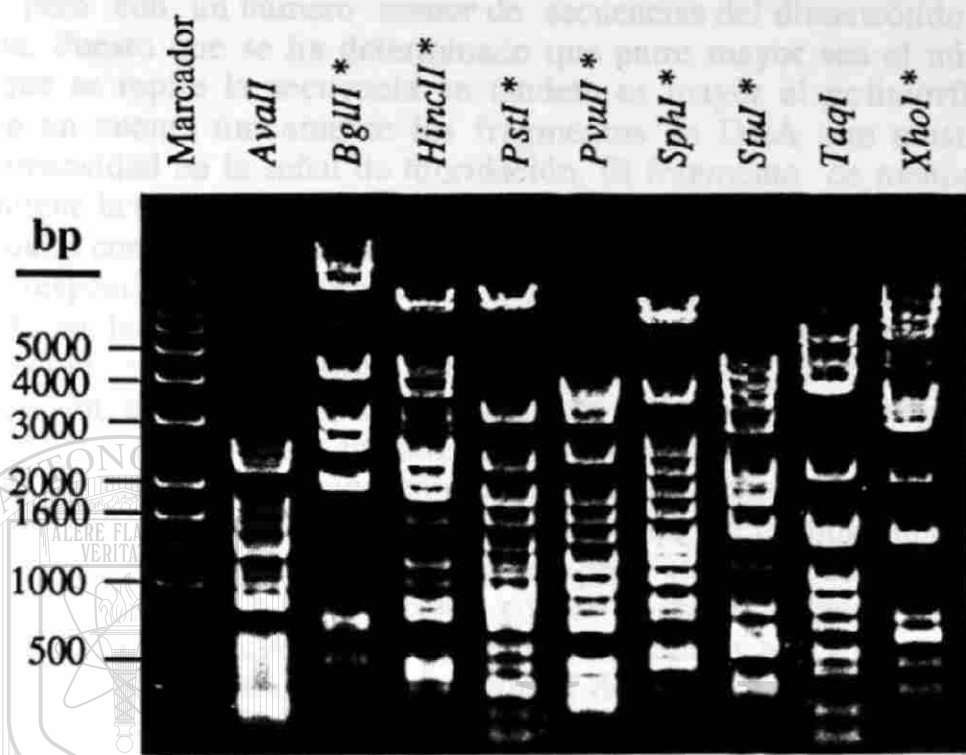


Figura 5. Patrón de restricción del marcador genético cH3.

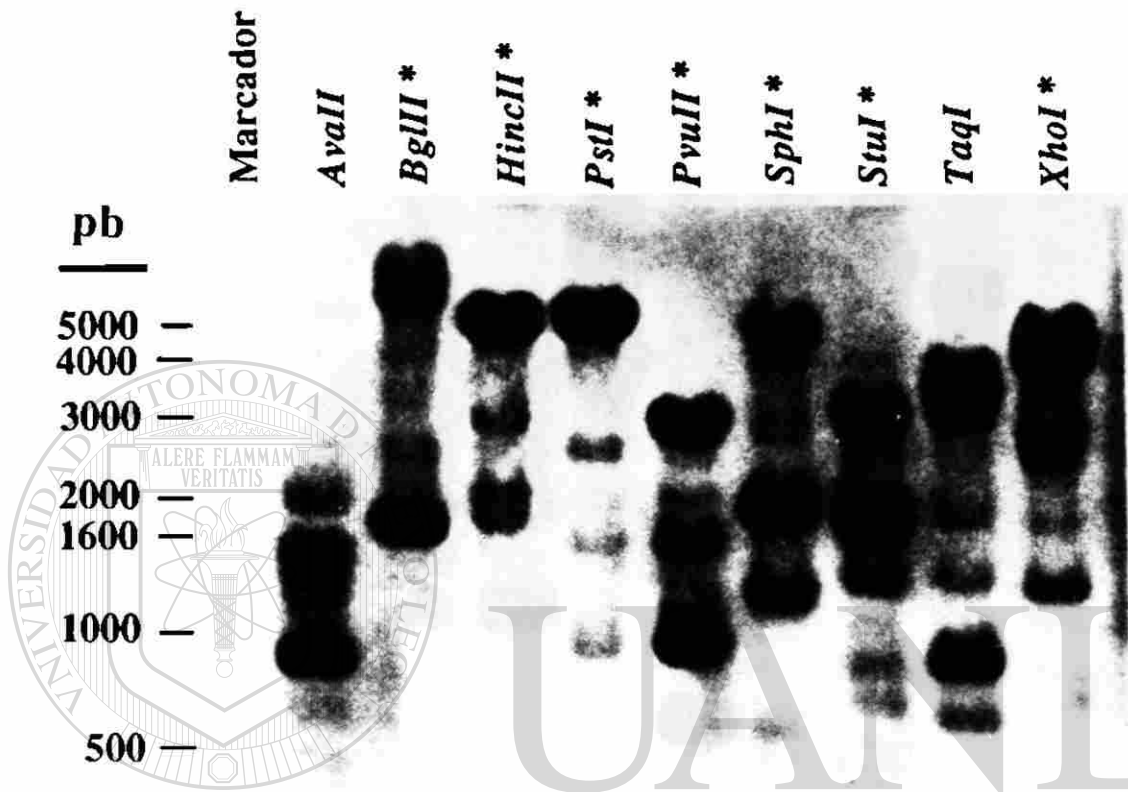
Fotografía de los fragmentos de restricción del marcador cH3. Cada carril representa la digestión de 1 µg de DNA del marcador cH3 con las diferentes enzimas de restricción indicadas en la parte superior de la figura, y se analizaron en un gel de agarosa al 1.5%. El gel se tiñó con bromuro de etidio y se visualizó por iluminación con luz ultravioleta. El gran número de bandas es debido a que este marcador corresponde a un cósmido con un fragmento de DNA humano de aproximadamente 40 kb. Este mismo gel se utilizó en el procedimiento tipo Southern para identificar por hibridación molecular cual fragmento de restricción contenía la secuencia (GT)_n (Figura 6). El * indica que las digestiones contenían además la enzima *EcoRI*.

observa en la Figura 6 prácticamente en todos los carriles se puede distinguir una banda principal con respecto a la señal de hibridación. Las otras señales de hibridación, de diferente intensidad deben corresponder también a secuencias (GT)_n, pero con un número menor de secuencias del dinucleótido guanina y timina. Puesto que se ha determinado que entre mayor sea el número de veces que se repite la secuencia en tandem es mayor el polimorfismo, se tomaron en cuenta únicamente los fragmentos de DNA que mostraron la mayor intensidad en la señal de hibridación. El fragmento de menor tamaño que contiene la secuencia (GT)_n del cósmido cH3 se observa en los carriles 2 y 9, los cuales corresponden a una digestión con las enzimas de restricción *AvaII* y *TaqI* respectivamente. De acuerdo con el patrón de digestión enzimática mostrado en la Figura 5, se observa que el cósmido cH3 fué fraccionado mayormente con la enzima *AvaII* que con *TaqI*. Tomando esto en consideración, es muy probable que en el área del gel de agarosa a nivel de la señal de hibridación exista un número mayor de fragmentos diferentes de DNA en el carril correspondiente a la enzima *AvaII*, que en el carril correspondiente a la enzima *TaqI*. Por esta razón decidimos subclonar del cósmido cH3 el fragmento *TaqI* positivo para una secuencia (GT)_n. Este fragmento se sometió a una reacción con el fragmento klenow de la DNA polimerasa I, para convertir sus extremos de cadena sencilla en extremos romos, y se subclonó en el sitio romo *HincII* de pTZ19R. De acuerdo con los marcadores de peso molecular utilizados, este fragmento *TaqI* correspondió a un tamaño de 900 pb.

En las Figuras 7 y 8 se muestran los resultados del Southern para la identificación de secuencias (GT)_n correspondientes respectivamente a los marcadores c1517 y c1541. En estos casos no se muestra el patrón electroforético de la restricción de cada uno de ellos, sin embargo al igual que para el marcador cH3 se siguieron los mismos criterios para la selección del fragmento a subclonar que contuviera la secuencia (GT)_n.

De acuerdo con el patrón de restricción y el resultado del Southern para el marcador c1517, mostrado en la Figura 7, se seleccionó el fragmento *PstI* de 450 pb presente en el sexto carril, y se subclonó en el mismo sitio del vector pTZ19R. Para el caso del marcador c1541, Figura 8, se seleccionó el fragmento *SphI* de 550 pb presente en el octavo carril, y se subclonó en el sitio correspondiente de pTZ19R.

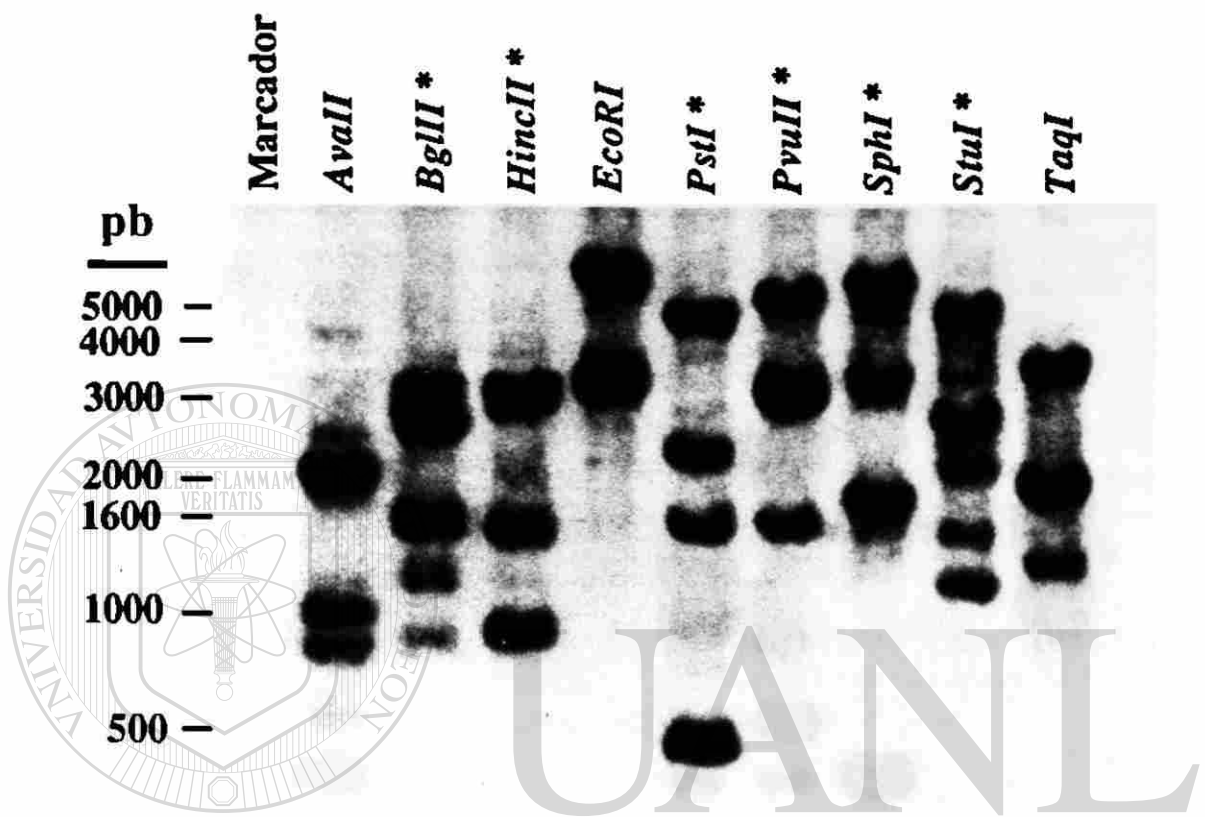
En el caso del marcador VAW409R1, inicialmente se identificó la secuencia (GT)_n en un fragmento *PstI* de 2000 pb (Figura 10). Sin embargo, debido a que era un fragmento muy grande para secuenciar, este



UNIVERSIDAD AUTÓNOMA DE NUEVO LEÓN
Figura 6. Detección de secuencias (GT)_n por hibridación tipo Southern en fragmentos de restricción del marcador genético cH3. [®]

DIRECCIÓN GENERAL DE BIBLIOTECAS

Autorradiografía de una hibridación tipo Southern utilizando como sonda radiactiva un copolímero sintético del dinucleótido guanina y timina. Para este experimento se utilizó el gel mostrado en la figura 5. En la parte superior de la figura se indican las diferentes enzimas de restricción utilizadas. El * indica que las digestiones contenían además la enzima *EcoRI*. De este marcador se escogió para subclonar en pTZ19R el fragmento correspondiente a la señal de hibridación en el carril *TaqI*, con un tamaño ≈ de 1000 pb.

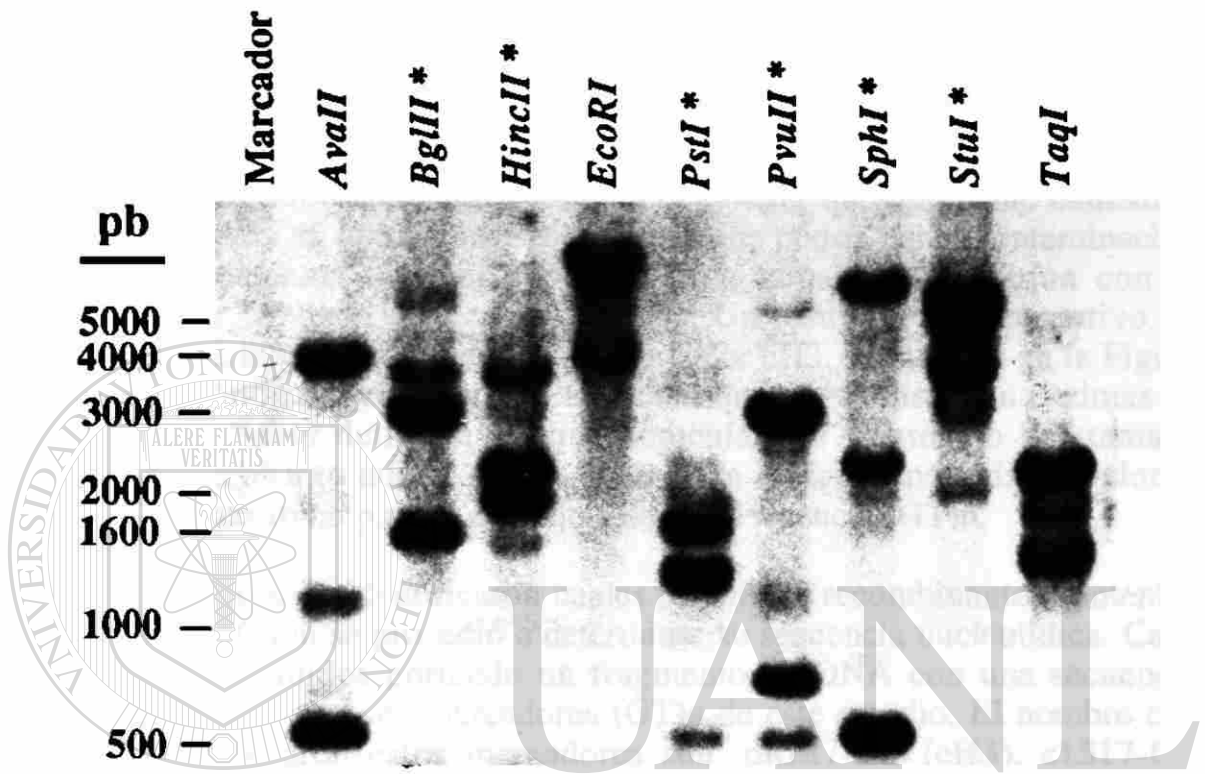


UNIVERSIDAD AUTÓNOMA DE NUEVO LEÓN

Figura 7. Detección de secuencias (GT)_n por hibridación tipo Southern en fragmentos de restricción del marcador genético c1517.

DIRECCIÓN GENERAL DE BIBLIOTECAS

Este es un experimento similar al descrito en la figura 6, excepto que corresponde al marcador c1517. El * indica que las digestiones contenían además la enzima *EcoRI*. De este marcador se escogió para subclonar en pTZ19R el fragmento correspondiente a la señal de hibridación del carril *PstI*, con un tamaño de ≈500 pb.



UNIVERSIDAD AUTÓNOMA DE NUEVO LEÓN

Figura 8. Detección de secuencias (GT)_n por hibridación tipo Southern en fragmentos de restricción del marcador genético c1541.

Este es un experimento similar al descrito en la figura 6, excepto que corresponde al marcador c1541. El * indica que las digestiones contenían además la enzima *EcoRI*. De este marcador se escogió para subclonar en pTZ19R el fragmento correspondiente a la señal de hibridación del carril *SphI*, con un tamaño de ~500 pb.

fragmento se digirió con las enzimas de restricción *AluI*, *HaeIII* y *RsaI*, y los fragmentos resultantes de la digestión se purificaron de un gel de agarosa al 1.5%. Estos fragmentos se sometieron a una Hibridación de DNA en gota para determinar cual de ellos contenía el fragmento más apropiado para la secuenciación. De acuerdo con el resultado (datos no mostrados) se seleccionó un fragmento *HaeIII* de 250 pb y se subclonó en el sitio *HincII* del vector pTZ19R.

Cada uno de los plásmidos recombinantes que se obtuvieron de la clonación de las secuencias (GT)_n de los diferentes marcadores, se analizaron por la técnica de hibridación en gota para demostrar que realmente contenían una secuencia (GT)_n. Esto se realizó debido a que podría haber contaminación con otros fragmentos que por ser del mismo tamaño comigrarían con el fragmento que contenía la secuencia (GT)_n. Un resultado representativo de este procedimiento, correspondiente al marcador cH3, se muestra en la Figura 10. En esta hibridación se utilizaron 13 clonas que por análisis con enzimas de restricción se había determinado que contenían un fragmento del tamaño esperado, sin embargo tal y como se observa en la figura, no todas las clonas dan una señal positiva para la presencia de una secuencia (GT)_n.

Una vez que se identificaron cuales plásmidos recombinantes contenían una secuencia (GT)_n se procedió a determinar su secuencia nucleotídica. Cada una de estos plásmidos portando un fragmento de DNA con una secuencia (GT)_n, corresponden a los marcadores (GT)_n de este estudio. El nombre con el cual se designaron estos marcadores son: pRM7-GT (cH3), c1517-GT (c1517), c1541-GT (c1541) y pRM11-GT (VAW409R1).

UNIVERSIDAD AUTÓNOMA DE NUEVO LEÓN

II. Caracterización de los marcadores (GT)_n.

DIRECCIÓN GENERAL DE BIBLIOTECAS

Con el propósito de caracterizar la secuencia repetida de los marcadores (GT)_n y sintetizar oligonucleótidos que flanquean esta región, para la obtención de los genotipos (GT)_n, se determinó la secuencia nucleotídica de estos marcadores por el método de Sanger (77). El tipo de la secuencia repetida de los marcadores (GT)_n es la siguiente:

pRM7-GT:	(GT) ₁₉
c1517-GT:	(GT) ₁₃ G (GT) ₂
c1541-GT:	(TGC) ₄ TTGC (GT) ₃ AT(GT) ₁₀
pRM11-GT:	(TA) ₅ N (GT) ₁₇ (AT) ₈

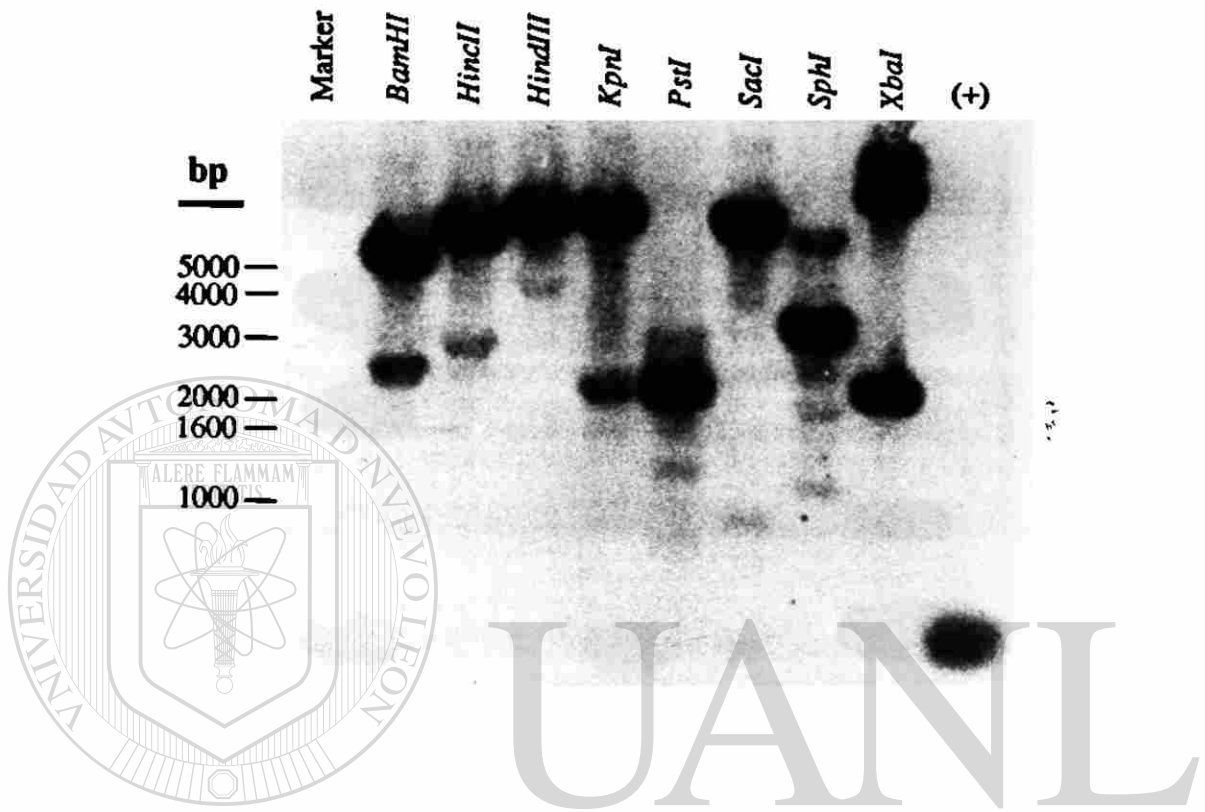


Figura 9. Detección de secuencias (GT)_n por hibridación tipo Southern en fragmentos de restricción del marcador genético VAW409R1.

Este es un experimento similar al descrito en la figura 6, excepto que corresponde al marcador VAW409R1. Del fragmento *PstI* de ~2000 pb positivo para una secuencia (GT)_n, se identificó y subclonó en pTZ19R un fragmento *HaeIII* de 250 pb que contenía la secuencia (GT)_n. El carril (+) representa un testigo positivo que corresponde a un fragmento de DNA de 150 pb con una secuencia (GT)_n.

Note que la secuencia (GT)_n del marcador c1541-GT está precedida por una secuencia repetida del trinucleótido TGC. Mientras que la secuencia (GT)_n del marcador pRM11-GT está flanqueada por secuencias repetidas del dinucleótido adenina y timina.

En la Figura 11 se muestra únicamente la secuencia nucleotídica completa del marcador pRM11-GT, el cual es el marcador más relevante de este estudio. En ella se señalan las secuencias utilizadas para la síntesis de oligonucleótidos, así como la región de la secuencia repetida. De esta figura se puede deducir que el producto de una reacción de PCR a partir de este marcador, utilizando sus respectivos oligonucleótidos, corresponde a un tamaño de 159 pb. En base al tamaño de este producto se determinó por comparación el tamaño de todos los alelos (GT)_n de las personas que se analizaron con este marcador.

Las secuencias nucleotídicas de los marcadores genéticos (GT)_n que se seleccionaron para la síntesis de oligonucleótidos, en dirección 5'-3', son las siguientes:

RM7-GT: ATTATTTATTTTGATGTCCTGAACAC y CTTGGTGAAACCGCTGTCTGTCA
c1517-GT: CAGGTGCACAGTCACACACTGG y GGCTTAGCTGCTGTTTCATGGC
c1541-GT: CTCCTCAACATGCTTTCTCTC y AATGGCTCCAAAAGGAGATATTG
RM11-GT: CAGAACCACAAAATGTCTTGCATTC y GGCCAGACAGACCAGGCTCTGC

La funcionalidad de estos oligonucleótidos se determinó mediante la reacción de PCR utilizando como plantados sus respectivos marcadores y DNA genómico humano. En el presente trabajo se describe el uso de los marcadores pRM11-GT y OS1-βGT; y en los artículos que se anexan de Greenberg y cols. (57) y Guzzetta y cols. (85) se describe el de los otros tres marcadores (GT)_n.

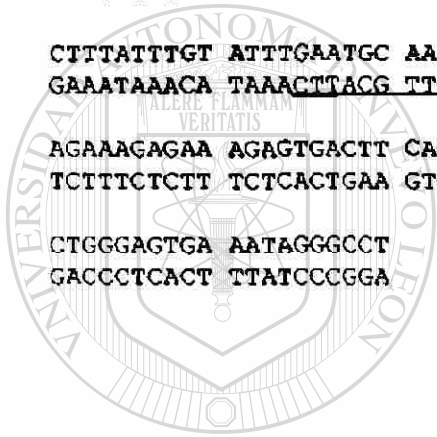
GGCCAGACAG ACCAGGCCT GCTCTACTTA AATATCTTT ATGTATATAT ATATGCACAC 60
CCGGTCTGTC TGGTCCGAGA CGAGATGAAT TTATTAGAAA IACATATATA TATACGTGTG

ACACACACAC ACACACACAC ACACACACAT ATATATATAT ATATAAATAA ACTGTGGTAG 120
TGTGTGTGTG TGTGTGTGTG TGTGTGTGTA TATATATATA TATATTTATT TGACACCTAC

CTTTATTTGT ATTTGAATGC AAGACATTTT GTGGTCTGT AATCCAAAA AAAAGAAGAA 180
GAAATAAACA TAACTTACG TTCTGTAAA CACCAAGACA TTAAGGTTTT TTTTCTTCTT

AGAAAGAGAA AGAGTGACTT CAGCAGAACA GCTCTCTGCA ATCAGCCAAG AACAAGGAAA 240
TCTTTCTCTT TCTCACTGAA GTCGCTTGT CGAGAGACGT TAGTCGGTTC TTGTTCCCTT

CTGGGAGTGA AATAGGGCCT
GACCCTCACT TTATCCCGGA



UANL

UNIVERSIDAD AUTÓNOMA DE NUEVO LEÓN



DIRECCIÓN GENERAL DE BIBLIOTECAS

Figura 11. Secuencia nucleotídica del marcador pRM11-GT.

En esta figura se muestra la secuencia de nucleótidos en cadena doble del marcador pRM11-GT. Esta secuencia nucleotídica se obtuvo de la autorradiografía del gel de secuenciamiento para este marcador mostrado en la figura 14. Se subrayan las secuencias de nucleótidos utilizadas para la síntesis de oligonucleótidos, mientras que la región repetida se delimita por los paréntesis.

III. Evidencia inicial de una duplicación del locus D17S122 en pacientes con CMT1A.

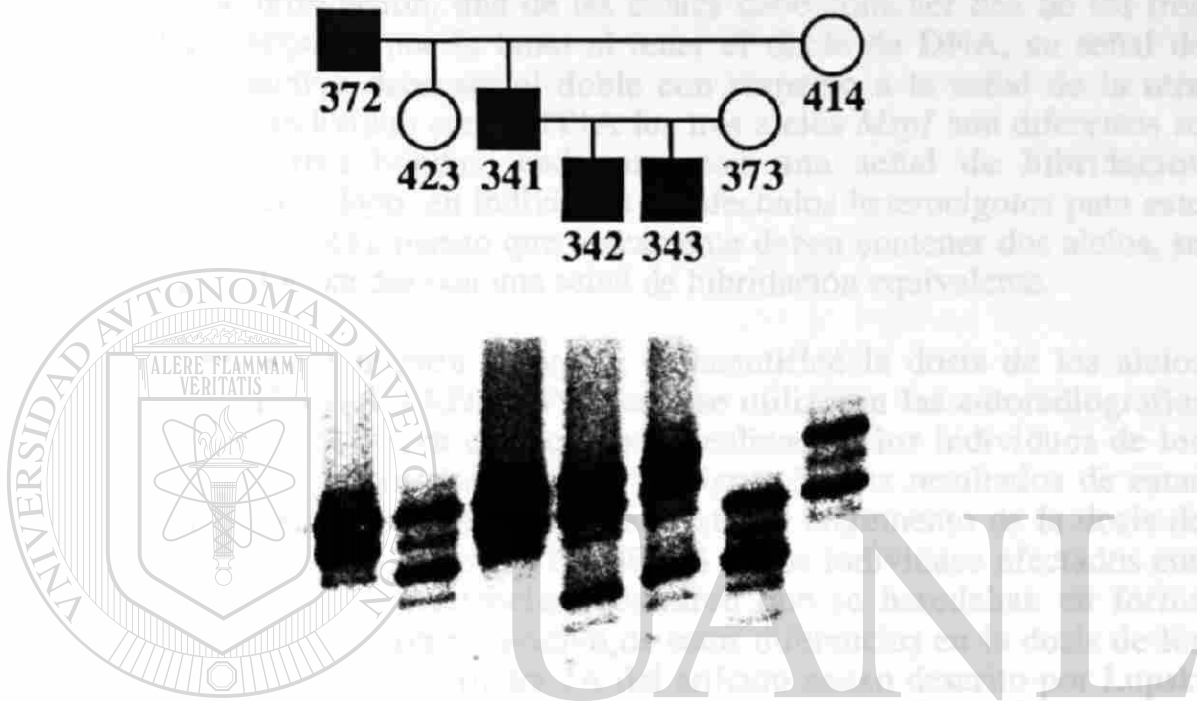
Con cada uno de estos marcadores se analizó inicialmente en todos los individuos del árbol genealógico HOU89 (éste se escogió por que es el árbol con un número mayor de generaciones). Para nuestra sorpresa, al analizar los genotipos (GT)_n que se obtuvieron con el marcador pRM11-GT, se observaron tres alelos (GT)_n únicamente en tres individuos afectados con CMT1A. Además los tres individuos eran hermanos. En la Figura 12 se presentan los genotipos (GT)_n de la familia del árbol genealógico HOU89 a la que pertenecen los pacientes con tres alelos (GT)_n. Como se observa en la figura los individuos 89-342 y 89-343 muestran tres alelos (GT)_n los cuales son similares a los alelos (GT)_n de sus padres (89-341 y 89-373). Este resultado indicó que podría existir una duplicación de la secuencia de DNA del marcador pRM11-GT (marcador del locus D17S122), en los individuos afectados con CMT1A. Sin embargo, puesto que ésto se observó únicamente en tres de los trece individuos afectados con CMT1A del árbol genealógico HOU89, también cabía la posibilidad de que fuera un artefacto técnico.

Como se mencionó en resultados anteriores, el marcador pRM11-GT fué subclonado del marcador VAW409R1 el cual pertenece al locus D17S122. Puesto que el marcador VAW409R3 pertenece también al mismo locus se pensó que posiblemente la duplicación podría ser observada utilizando el polimorfismo tipo RFLP que se ha descrito para este marcador VAW409R3 con la enzima *MspI*. Ya que a los individuos de los árboles genealógicos presentes en la Figura 3 se les había analizado dicho polimorfismo con el marcador VAW409R3, nos enfocamos a analizar las autoradiografías de los Southern para determinar si los individuos afectados tenían un alelo extra para este marcador. En la siguiente sección se describen los hallazgos de este análisis. DIRECCIÓN GENERAL DE BIBLIOTECAS

IV. Confirmación de la duplicación del locus D17S122 por RFLP usando el marcador VAW409R3.

La probable duplicación del locus D17S122 que se observó en tres pacientes del árbol genealógico HOU89, dió pauta al análisis de los alelos RFLP de este locus. Por análisis tipo Southern se detectan en el locus D17S122 hasta tres diferentes alelos *MspI* en la población humana (y hasta dos alelos por individuo) utilizando el marcador VAW409R3; y únicamente dos diferentes alelos con el marcador VAW409R1 (56,86). Teniendo en cuenta

HOU 89



UNIVERSIDAD AUTÓNOMA DE NUEVO LEÓN
Figure 12. Observación inicial de tres alelos (GT)_n en pacientes con CMT1A [®]
 del árbol genealógico HOU89.

DIRECCIÓN GENERAL DE BIBLIOTECAS

Alelos (GT)_n para el marcador pRM11-GT en una familia del árbol genealógico HOU89. Se utilizan símbolos estándar y los individuos afectados se representan en negro. Las bandas de menor tamaño y menos intensas que las bandas principales de los alelos, son invariablemente observadas en los polimorfismos de secuencias repetidas; sin embargo es posible leer los genotipos sin ambigüedad (72). Note que en los pacientes 89-342 y 89-343 se observan tres bandas principales: tres alelos (GT)_n (73).

que en individuos afectados con CMT1A hay tres copias del locus D17S122 y que el marcador VAW409R3 pertenece al mismo locus, cabe esperar que un individuo con CMT1A contenga tres alelos *MspI* para este marcador. Si el individuo con CMT1A es homocigoto para los alelos *MspI*, únicamente se observará una banda (alelo) de hibridación, la cual debe contener los tres alelos *MspI*; si el individuo con CMT1A es heterocigoto, se deben observar dos bandas de hibridación, una de las cuales debe contener dos de los tres posibles alelos *MspI*, y por lo tanto al tener el doble de DNA, su señal de hibridación radiactiva debe ser el doble con respecto a la señal de la otra banda; y si en el individuo con CMT1A los tres alelos *MspI* son diferentes se espera observar tres bandas, cada una con una señal de hibridación equivalente. Por otro lado, en individuos no afectados heterocigotos para este marcador (VAW409R3), puesto que únicamente deben contener dos alelos, se espera observar dos bandas con una señal de hibridación equivalente.

Para demostrar nuestra hipótesis, se cuantificó la dosis de los alelos *MspI* del marcador VAW409R3. Para esto se utilizaron las autoradiografías de los análisis tipo Southern que se habían realizado a los individuos de los siete árboles genealógicos presentes en la Figura 3. Los resultados de estas observaciones indican que efectivamente existe un incremento de la dosis de los alelos *MspI* para el marcador VAW409R3 en los individuos afectados con CMT1A. Además estas diferencias mostraron que se heredaban en forma mendeliana. Un ejemplo representativo de estas diferencias en la dosis de los alelos *MspI* se observa en la figura 3A del artículo anexo descrito por Lupski y cols. (73). Como se observa en esta familia, la señal de hibridación entre los dos alelos de un mismo individuo heterocigoto no afectado es equivalente (85-326 y 85-312) y por lo tanto les corresponde el genotipo AB. Por el contrario en los individuos heterocigotos afectados con CMT1A, aparentemente con el genotipo AB, la señal de hibridación entre sus dos alelos no es equivalente, y una de las bandas se observa con el doble de señal de hibridación con respecto a la otra (85-302, 85-303 y 85-304), por lo tanto, los genotipos reales para estos individuos afectados, 85-302, 85-303 y 85-304, deben ser respectivamente AAB, ABB y ABB. Si se analizan los genotipos de esta familia se deduce que el "cromosoma CMT1A" porta los alelos A y B, y que ambos se segregan en una forma Mendeliana con respecto a la enfermedad. Resultados similares se obtuvieron en todas las familias de los siete árboles genealógicos.

En la misma Figura (73) se observa también el resultado de una comparación por Southern utilizando el marcador VAW409R3, entre individuos heterocigotos afectados con CMT1A (panel C) y no afectados

(panel D). Nuevamente, se puede notar la presencia de un alelo extra al comparar la proporción de la señal de hibridación entre las bandas alélicas de un mismo individuo con CMT1A. Como se observa en el panel C, los individuos en los carriles 4-6 tienen el doble de señal para el alelo B, y por ende su genotipo es ABB, en tanto que los individuos en los carriles 7 y 8 muestran el doble de señal para el alelo A y por lo tanto su genotipo es AAB. La evidencia más concluyente de la presencia de tres alelos por RFLP, en pacientes con CMT1A, se observa en los individuos de los carriles 1 al 3 del panel C, los cuales tienen tres alelos polimórficos diferentes y además equivalentes en su señal de hibridación, y por ende les corresponde el genotipo ABC (este tercer alelo C, es raro en la población y únicamente se detectó en 3 de 131 pacientes con CMT1A).

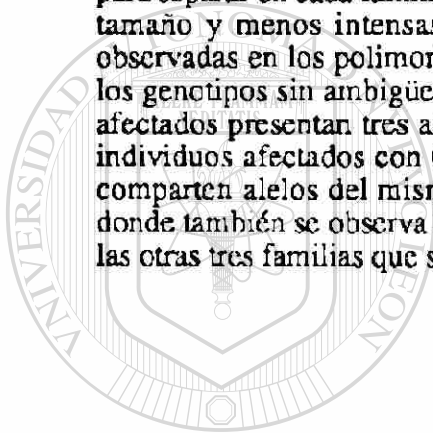
Para corroborar que los individuos con CMT1A presentan un alelo extra, se cuantificó de acuerdo con la metodología, la señal en las bandas de hibridación de 76 individuos afectados heterocigotos para el marcador VAW409R3. Los resultados de este análisis indicaron que todos estos individuos afectados tenían cuantitativamente tres copias del locus D17S122. Por el contrario, en ninguno de 63 individuos no afectados heterocigotos para este marcador, se observó una diferencia cuantitativa en la dosis de sus alelos. Estos resultados demuestran que el genotipo con dos copias alélicas en una de las dos bandas de hibridación de los individuos heterocigotos fué específico para pacientes con CMT1A, y por lo tanto confirman que existe una duplicación del locus D17S122 en individuos afectados con CMT1A.

V. Demostración de la duplicación del locus D17S122 mediante el uso de marcadores (GT)_n.

Con el marcador pRM11-GT se analizó el resto de los árboles genealógicos presentados en la Figura 3: cinco de ascendencia Acadio-Francés (HOU1, HOU2, HOU42, HOU85 y HOU89) y uno de ascendencia Judío Ashkenazic (HOU76) (73). En la Figura 13 se muestran los alelos correspondientes a cuatro familias de los árboles genealógicos HOU85, HOU1, HOU88 y HOU76. Como se observa en la figura, trece de quince individuos afectados con CMT1A tienen tres alelos (GT)_n (por ejemplo, individuos 88-340 y 76-352), mientras que todos los individuos no afectados son ya sea homocigotos o heterocigotos para el marcador RM11-GT. Esto indica que existe una asociación entre la presencia de tres alelos detectados aquí por la secuencia (GT)_n y la enfermedad de CMT1A. Los dos individuos afectados que no se les observa tres alelos (GT)_n (88-339, 88-380) se debe a

Figura 13. Detección de tres alelos (GT)_n en pacientes con CMT1A utilizando el marcador RM11-GT.

Los genotipos (GT)_n se obtuvieron por PCR como se describió en MATERIALES y MÉTODOS, y se indican debajo de cada individuo. La línea diagonal en el genotipo se utilizó para separar en cada familia, el par de alelos que segrega con el CMT1A. Las bandas de menor tamaño y menos intensas que las bandas principales de los alelos, son invariablemente observadas en los polimorfismos de secuencias repetidas (72); sin embargo, no evitan el leer los genotipos sin ambigüedad. **A** y **B** son muestras de familias en donde todos los individuos afectados presentan tres alelos. **C** representa una familia en donde se puede observar a dos individuos afectados con únicamente dos alelos (88-339 y 88-380), debido a que los padres comparten alelos del mismo tamaño. **D** representa una familia de origen Judío Ashkenazic, donde también se observa la herencia de tres alelos en pacientes con CMT1A. A diferencia de las otras tres familias que son de origen Acadio-Francés (73).

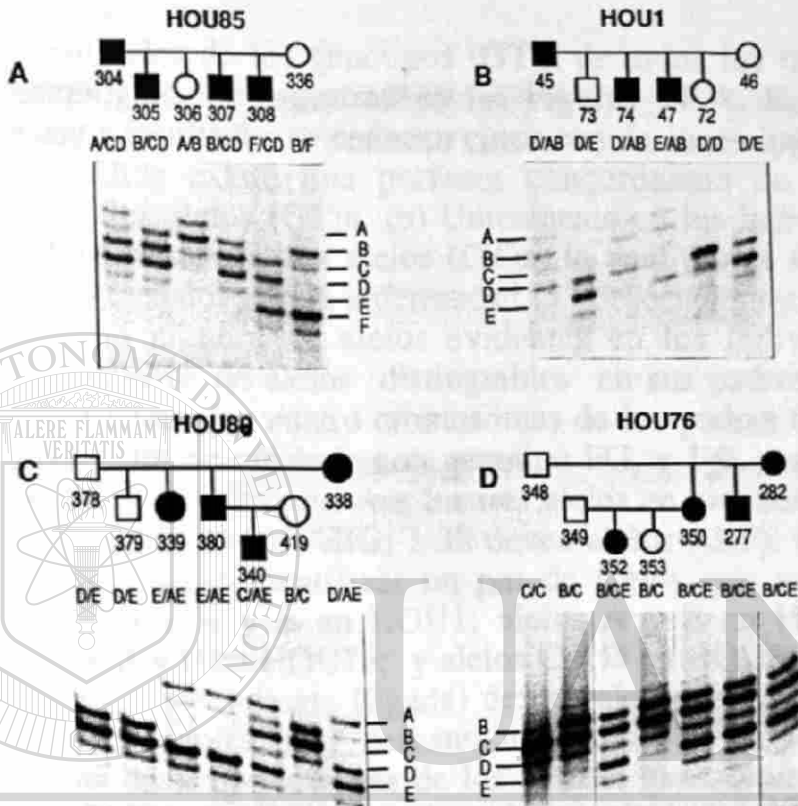


UANL

UNIVERSIDAD AUTÓNOMA DE NUEVO LEÓN



DIRECCIÓN GENERAL DE BIBLIOTECAS



UNIVERSIDAD AUTÓNOMA DE NUEVO LEÓN
 DIRECCIÓN GENERAL DE BIBLIOTECAS

Figura 13. Detección de tres alelos (GT)_n en pacientes con CMT1A utilizando el marcador pRM11-GT.

que heredaron de sus padres dos alelos del mismo tamaño (alelo E) los cuales migran en la misma posición y se observan como un solo alelo E (Figura 13 C). Estos resultados indican que pacientes con CMT1A tienen tres copias del locus D17S122 lo cual demuestra que existe una duplicación de este locus en pacientes con CMT1A.

Los resultados de los genotipos (GT)_n de todos los individuos de los árboles genealógicos se muestran en las Figuras 14 A, B, C, D y E. Del análisis de estos resultados se deducen cinco conclusiones importantes. (a) En todas las familias existe una perfecta concordancia en la segregación Mendeliana de los alelos (GT)_n. (b) Únicamente en los individuos afectados con CMT1A se observan tres alelos (GT)_n, lo cual nunca se observó en 84 individuos no afectados con la enfermedad (53 descendientes y 31 conyuges). Sin embargo, el número de alelos evidentes en los individuos afectados dependió del número de alelos distinguibles en sus padres. Así entonces, cuando se distinguen los cuatro cromosomas de los padres (por ejemplo, los padres: 1-49, padre no afectado con genotipo FG, y 1-9, madre afectada con genotipo ABC) se pueden observar los tres alelos en los individuos afectados (1-153 y 1-37 tienen alelos ABG; 1-38 tienen alelos ABF). (c) En cada árbol genealógico, se puede identificar un par de alelos que se segrega con la enfermedad (alelos A y B en HOU1; alelos A y E en HOU2, HOU42 y HOU88; alelos B y E en HOU76; y alelos C y D en HOU85 y HOU89). Esta segregación no independiente (ligada) de dos alelos indica que el segmento duplicado se encuentra muy cercano a su sitio de origen en el mismo cromosoma, es decir que a través de los árboles genealógicos se segrega un "cromosoma CMT1A", el cual basado en los resultados, debe portar dos copias del locus D17S122. (d) La transmisión de la duplicación es sumamente específica. Si tomamos en cuenta todas las familias donde las meiosis son informativas, se observan 45 casos donde el padre afectado transmite la duplicación (dos alelos) a los descendientes afectados y 18 casos donde el padre afectado transmitió únicamente un alelo a sus descendientes no afectados con CMT1A. En estos apareamientos, ninguno de los descendientes no afectados recibió el segmento duplicado y ninguno de los descendientes afectados recibió un alelo único del padre afectado. Esto es, en 63 meiosis informativas, la duplicación se transmitió correctamente a la descendencia afectada y sin recombinación con el cromosoma normal. Y finalmente (e), esta mutación de duplicación ocurre en dos poblaciones con CMT1A de diferente origen étnico. Estos resultados demuestran completamente que existe una duplicación de DNA del locus D17S122 asociada con la enfermedad de CMT1A (73).

Figuras 14 A, B, C, D y E. Genotipos (GT)_n del locus D17S122 de los árboles genealógicos segregando la enfermedad autosómica dominante CMT1A.

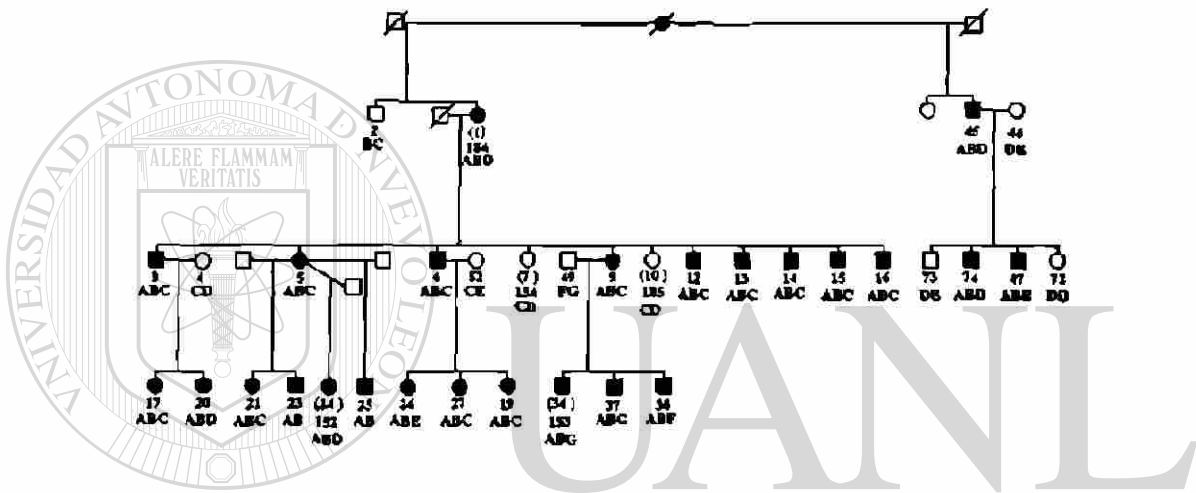
Los árboles genealógicos HOU1, HOU2, HOU42, HOU85, HOU88, y HOU89, son de origen Acadio-Francés, mientras que el HOU76 es de origen Judío Ashkenazic. Se utilizan símbolos estándar; con los individuos afectados representados en negro. Los números corresponden a la identificación del laboratorio, y el genotipo (GT)_n de cada individuo se indica debajo de su respectivo símbolo. Los genotipos (GT)_n se obtuvieron por PCR como descrito en los métodos, y se les designó de acuerdo a su tamaño en pares de bases: A= 165, B=163, C=161, D=159, E=157, F=155 y G=153. Los individuos donde se observó únicamente una banda se registraron como portadores de dos copias de un mismo alelo. Los genotipos para cada individuo se leyeron de las autorradiografías sin conocer su estatus con respecto a la enfermedad, y se confirmaron independientemente por otros dos investigadores. Con excepción de los individuos: 1-45, 1-46, 1-47, 1-72, 1-73 y 1-74, a todos se les determinó la VCN. Note que hay una familia en donde ambos padres están afectados con CMT1A: 42-331, 42-332 y 42-333. En cada árbol genealógico se puede distinguir un par de alelos que se segrega con la enfermedad; A y E en HOU2, HOU42, y HOU88; A y B en HOU1; C y D en HOU85 y HOU89; y con alelos B y E en HOU76 (73).

UNIVERSIDAD AUTÓNOMA DE NUEVO LEÓN



DIRECCIÓN GENERAL DE BIBLIOTECAS

HOU1

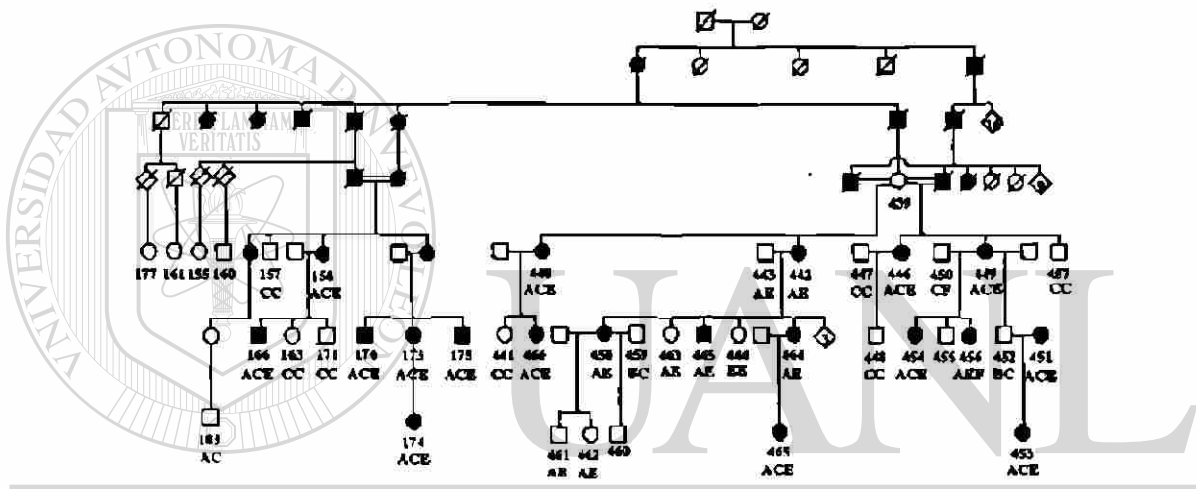


UNIVERSIDAD AUTÓNOMA DE NUEVO LEÓN

DIRECCIÓN GENERAL DE BIBLIOTECAS

Figura 14 A . Genotipos (GT)_n para el marcador pRM11-GT en el árbol genealógico HOU1, donde la enfermedad segrega con los alelos A y B (73).

HOU2

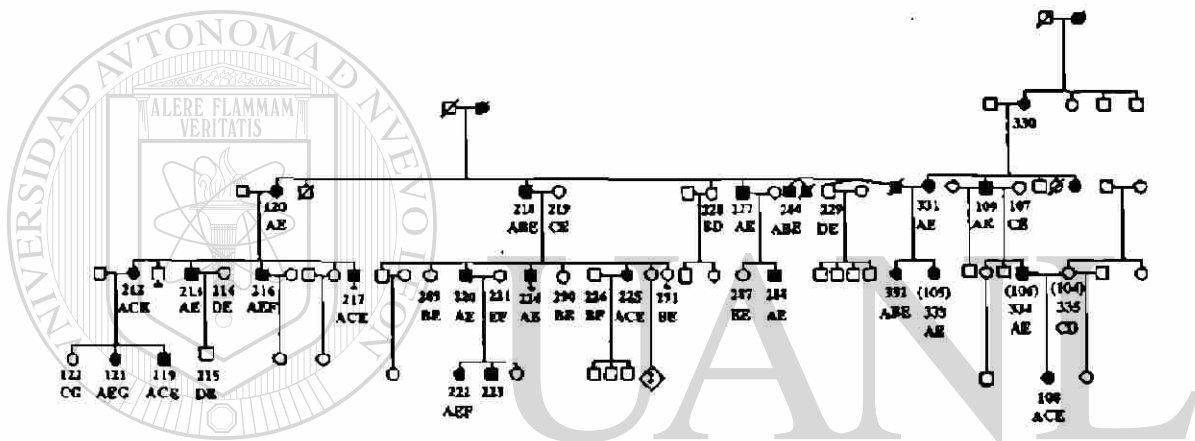


UNIVERSIDAD AUTÓNOMA DE NUEVO LEÓN
DIRECCIÓN GENERAL DE BIBLIOTECAS



Figura 14 B. Genotipos (GT)_n para el marcador pRM11-GT en el árbol genealógico HOU2, donde la enfermedad segrega con los alelos A y B (73).

HOU42



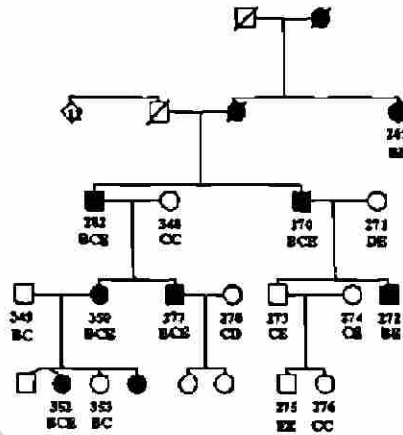
UNIVERSIDAD AUTÓNOMA DE NUEVO LEÓN



DIRECCIÓN GENERAL DE BIBLIOTECAS

Figura 14 C. Genotipos (GT)_n para el marcador pRM11-GT en el árbol genealógico HOU42, donde la enfermedad segrega con los alelos A y E. Note que hay una familia en donde ambos padres están afectados con CMT1A: 42-331, 42-332 y 42-333 (73).

HOU76



HOU85

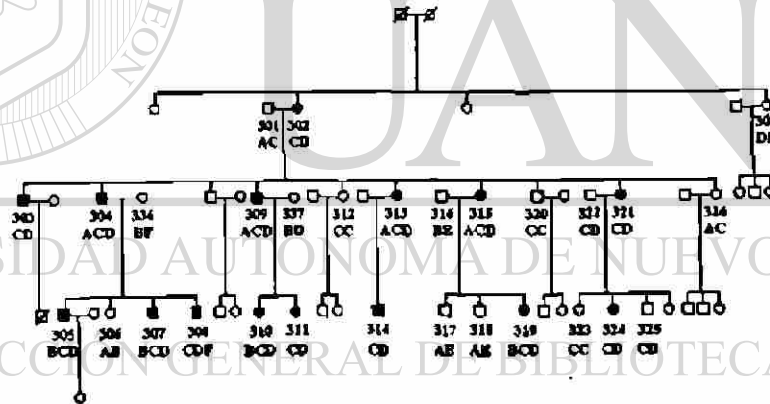


Figura 14 D. Genotipos (GT)_n para el marcador pRM11-GT en los árboles genealógicos HOU76 y HOU85, donde la enfermedad segrega con los alelos B y E, y C y D, respectivamente (73).

Además seguimos otra estrategia combinando los polimorfismos del marcador (GT)_n pRM11-GT y RFLP, para demostrar la presencia de un alelo extra en las bandas alélicas con una señal doble de hibridación en individuos heterocigotos con CMT1A.

VI. La duplicación de DNA es una mutación común en pacientes con CMT1.

Con el propósito de determinar si la duplicación de DNA es un mecanismo general asociado con la enfermedad de CMT1A, y no específica para los árboles genealógicos analizados, se analizó con el marcador pRM11-GT pacientes con un fenotipo equivalente a CMT1 los cuales tenían diferentes orígenes familiares. Varios de estos pacientes mostraron tres alelos (GT)_n, y en la mayoría de aquellos que mostraron dos alelos (GT)_n se pudo observar una mayor intensidad en uno de sus dos alelos (resultados no mostrados). Estos resultados indican que la duplicación de DNA es una mutación común asociada con el CMT1.

VII. Los alelos RFLP *MspI* con una señal doble de hibridación en los pacientes con CMT1A tienen dos alelos (GT)_n.

El propósito de este experimento fué demostrar, utilizando el marcador pRM11-GT, que las bandas alélicas *MspI* con una señal doble de hibridación efectivamente contienen dos alelos. Como se mencionó, el marcador pRM11-GT corresponde a un fragmento *HaeIII* de 250 pb que se aisló del marcador VAW40R1; esto significa que cada alelo *MspI* identificado por el marcador VAW409R1 debe de contener un alelo (GT)_n correspondiente al marcador pRM11-GT. De acuerdo con ésto, en un individuo con CMT1A heterocigoto para el marcador VAW409R1, la banda de su alelo *MspI* que muestra una señal doble de hibridación debe de contener dos alelos (GT)_n, y la banda de su alelo *MspI* con una señal sencilla debe de contener un alelo (GT)_n.

Tal y como se describió en METODOS, se obtuvieron los fragmentos de DNA correspondientes a las bandas de los alelos *MspI* de diferentes individuos heterocigotos de una familia del árbol genealógico HOU42, detectadas con el marcador VAW409R1, y se sometieron a una reacción de PCR para determinar el número de alelos (GT)_n utilizando el marcador RM11-GT.

En la Figura 15A se muestra la familia utilizada en este experimento, así

como sus correspondientes alelos *MspI* detectados por Southern con el marcador VAW409R1. Como se puede apreciar los individuos afectados (42-218 y 42-225) tienen el alelo A con una señal doble de hibridación (dos copias) con relación al alelo A de los individuos no afectados (42-289 y 42-219), mientras que el alelo B en los cuatro familiares se observan con una señal de hibridación similar (una copia). Al DNA de cada uno de estos alelos *MspI*, se le determinó por PCR el número de alelos (GT)_n utilizando el marcador pRM11-GT.

En la figura 15B se muestra el resultado de la determinación del número de alelos (GT)_n para cada uno de los alelos *MspI*, así como del DNA genómico total de cada uno de los individuos. Como se observa en la figura, en los carriles 218, 289, 225 y 219 se observan los alelos (GT)_n obtenidos al utilizar el DNA genómico de estos individuos a los cuales les corresponde respectivamente los genotipos ABE, BE, ACE y CE (estos genotipos son exactamente iguales a los resultados obtenidos previamente y mostrados en la Figura 17C). En el segundo y octavo carril se observa que los alelos A de los pacientes afectados (42-218 y 42-225) que tenían una señal doble de hibridación, tienen dos alelos (GT)_n (218-A y 225-A); en tanto que el resto de los alelos A y B, con una señal sencilla de hibridación, tienen únicamente un alelo (GT)_n. Si se comparan los alelos (GT)_n que se obtuvieron de las diferentes bandas alélicas (carriles A y B) con las presentes en sus respectivos DNA genómicos, se puede observar una perfecta concordancia entre ellos.

Estos resultados demuestran que efectivamente las bandas de los alelos *MspI* con doble señal de hibridación, característico de los individuos heterocigotos afectados con CMT1A, contienen dos alelos (73); y además indican que este tipo característico de RFLP es un procedimiento confiable para determinar cuales otros marcadores genéticos están duplicados en los pacientes con CMT1A.

VIII. El tamaño de la duplicación es al menos de 100 kb.

Tomando en cuenta el característico RFLP que se observa en los individuos afectados con CMT1A, heterocigotos para un marcador de la región duplicada, se analizaron las autorradiografías de los RFLP correspondientes a diversos marcadores genéticos de la región 17p11.2-12 (resultados ya preexistentes en el laboratorio), y se encontró que los individuos con CMT1A, heterocigotos para los marcadores VAW412 y VAW401, tenían una banda alélica con una señal doble de hibridación (73)

Figura 15. Demostración de que en alelos polimórficos *MspI* del Locus D17S122 con doble señal de hibridación, hay dos alelos (GT)_n.

A, análisis tipo Southern utilizando el DNA genómico de una familia del árbol HOU42, digerido con la enzima *MspI* e hibridado contra un fragmento de restricción *MspI* de 5.3 Kb del marcador VAW409R1 (locus D17S122). El genotipo de cada individuo se obtuvo tomando en cuenta la dosis de cada alelo. Note que los individuos afectados 42-218 y 42-225 tienen dos copias del alelo A y una del alelo B. El examen de la herencia de los alelos de este árbol genealógico, indicó que la enfermedad se segrega con los alelos A y B. En este polimorfismo el alelo A es cortado en dos bandas (alelo B) las cuales son reconocidas por la sonda radiactiva

B, alelos (GT)_n presentes en cada uno de las bandas alélicas de cada individuo del panel A. El DNA correspondiente a cada una de las bandas alélicas polimórficas *MspI* se purificó de un gel de agarosa similar al utilizado en el panel A y se le determinó por PCR el número de alelos (GT)_n. En los carriles 218, 289, 225 y 219 se observan los alelos (GT)_n que se obtuvieron utilizando directamente su DNA genómico, y en los carriles marcados con A y B, se observan los alelos (GT)_n correspondientes a cada una de sus dos bandas alélicas polimórficas *MspI*. Note que el alelo A de los individuos 42-218 y 42-225, el cual está presente con una señal doble de hibridación, tiene dos alelos (GT)_n, mientras que el resto de los alelos presentes en una sola copia presentan un solo alelo (GT)_n (73).

UNIVERSIDAD AUTÓNOMA DE NUEVO LEÓN



DIRECCIÓN GENERAL DE BIBLIOTECAS

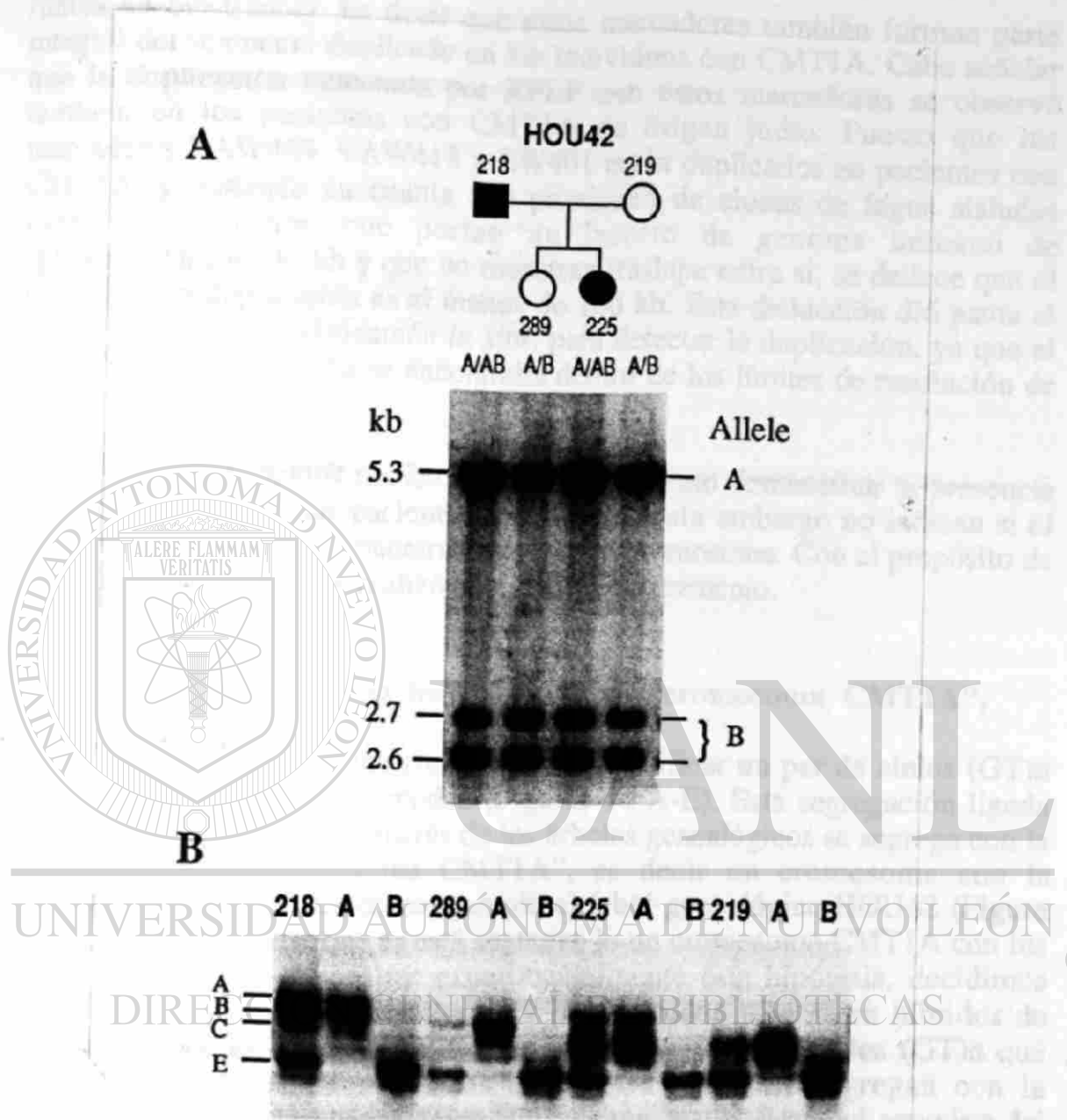


Figura 15. Demostración de dos alelos (GT)_n en alelos polimórficos *MspI* del locus D17S122 (73).

(datos no mostrados). Es decir que estos marcadores también forman parte integral del segmento duplicado en los individuos con CMT1A. Cabe señalar que la duplicación detectada por RFLP con estos marcadores se observó también en los pacientes con CMT1A de origen judío. Puesto que los marcadores VAW409, VAW412 y EW401 están duplicados en pacientes con CMT1A, y tomando en cuenta que provienen de clonas de fagos aisladas independientemente, que portan un inserto de genoma humano de aproximadamente 40 kb y que no muestran traslape entre si, se deduce que el tamaño de la duplicación es al menos de 100 kb. Esta deducción dió pauta al hecho de utilizar la hibridación *in situ*, para detectar la duplicación, ya que el tamaño de la duplicación se encontraba dentro de los límites de resolución de este procedimiento.

Los experimentos mencionados anteriormente demuestran la presencia de un alelo extra en los pacientes con CMT1A, sin embargo no indican si el segmento duplicado se encuentra en el mismo cromosoma. Con el propósito de dilucidar esta cuestión se realizó el siguiente experimento.

IX. Demostración de la herencia de un “cromosoma CMT1A”.

En cada árbol genealógico se puede identificar un par de alelos (GT)_n que se segrega con la enfermedad (Figuras 14 A-E). Esta segregación ligada de dos alelos sugiere que a través de los árboles genealógicos se segrega con la enfermedad un “cromosoma CMT1A”, es decir un cromosoma con la duplicación. Si tomamos como ejemplo el árbol genealógico HOU42 (Figura 14C) podemos deducir que se está segregando un cromosoma CMT1A con los alelos A y E. Para demostrar experimentalmente esta hipótesis, decidimos separar los cromosomas homólogos 17 del individuo 42-332 en híbridos de células somáticas y determinar en cada uno de ellos los alelos (GT)_n que contienen. Si postulamos que los alelos (GT)_n que se segregan con la enfermedad en el árbol genealógico HOU42 son A y E, y que el genotipo del individuo 42-332 es ABE, esperamos que un cromosoma 17 porte el alelo B y que su homólogo, en este caso el “cromosoma CMT1A” porte los alelos A y E. El resultado de este experimento se observa en la Figura 16.

Tal y como se describió en METODOS, se obtuvieron los híbridos celulares, y se utilizó el marcador OS1-βGT para identificar dos híbridos celulares que contuvieran por separado cada uno de los dos cromosomas homólogos 17. El resultado se muestra en los tres primeros carriles del panel B de la Figura 20. En el primer carril (332) se observan los alelos (GT)_n que

se obtuvieron al utilizar el DNA genómico de este individuo como templado, y como se aprecia el individuo es heterocigoto para este marcador ya que tiene dos alelos (GT)_n, designados arbitrariamente A y B. En otras palabras, el individuo 332 posee un cromosoma 17 con el alelo A y su otro cromosoma 17 porta el alelo B. En los siguientes dos carriles (a y b), se encuentran los productos de amplificación que corresponden a dos híbridos celulares que se derivaron de este individuo. Como se puede observar el híbrido celular del carril a retuvo el cromosoma 17 con el alelo A y el híbrido celular del carril b retuvo el cromosoma 17 con el alelo B. Esto demuestra que efectivamente se logró separar los cromosomas homólogos 17 del individuo 332.

Una vez que se demostró la separación de los cromosomas homólogos 17 del individuo 332 en híbridos celulares se procedió a determinar cuales alelos (GT)_n, para el marcador pRM11-GT, contenían cada uno de ellos. En la figura 16A se muestra el resultado de este análisis. En el carril 332 correspondiente al DNA genómico se observa que este individuo afectado posee tres alelos (GT)_n: A, B y E (tal y como se había determinado anteriormente en la Figura 14C). En los siguientes dos carriles (a y b) se encuentra el resultado de los dos híbridos celulares. Como se puede observar uno de ellos (carril a) porta el alelo B, mientras que el otro híbrido (carril b) porta los alelos A y E. Esto indica que el individuo afectado con CMT1A heredó un cromosoma 17 con la duplicación portando los alelos A y E. Este resultado está acorde con la segregación ligada de los alelos A y E con la enfermedad en el árbol genealógico HOU42, y confirma nuestra hipótesis de la segregación de la enfermedad con un cromosoma portando una duplicación ("cromosoma CMT1A") en los diferentes árboles genealógicos (73).

Este mismo experimento se realizó para el árbol genealógico HOU1[®] utilizando el individuo afectado 1-14, en donde se pudo demostrar que uno de sus dos cromosomas homólogos 17 separados en híbridos celulares portaba los alelos (GT)_n A y B para el marcador pRM11-GT. Lo cual es congruente con la segregación de un "cromosoma CMT1A" con los alelos A y B en los individuos afectados de este árbol genealógico (datos no mostrados).

Los resultados hasta aquí obtenidos, aun y cuando concluyen que existe una perfecta correlación entre una duplicación de DNA y la enfermedad de CMT1A, no demuestran si la duplicación es responsable de la aparición del fenotipo. Con el propósito de obtener una evidencia que indicara una relación entre el genotipo y el fenotipo, se determinó si un individuo con un fenotipo de CMT1A muy severo era homocigoto para la duplicación de DNA, es decir si portaba dos "cromosomas CMT1A".

Figura 16. Demostración de un individuo homocigoto para el CMT1A, por análisis de (GT)_n de sus dos cromosomas homólogos separados en híbridos de células somáticas.

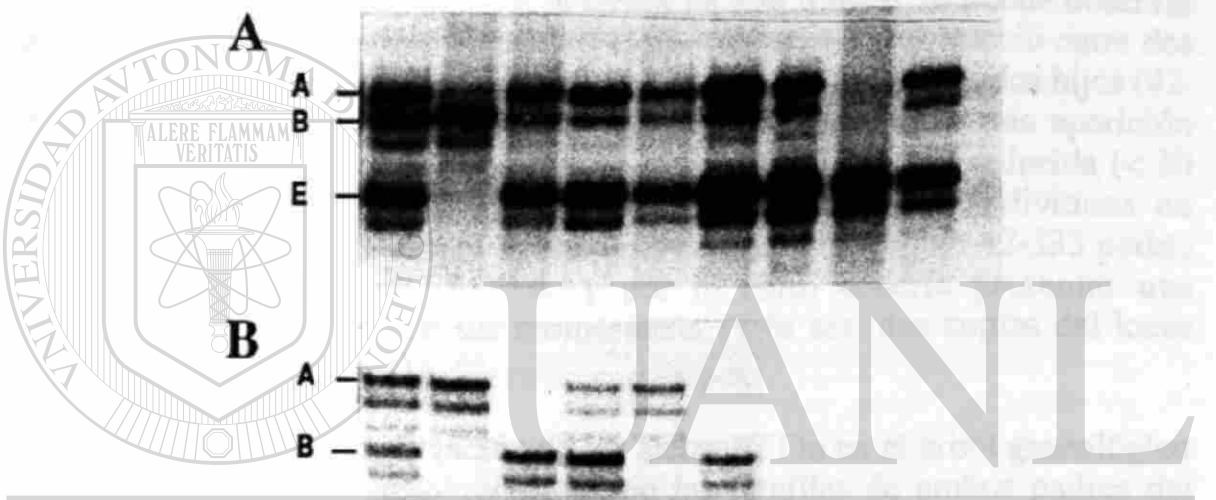
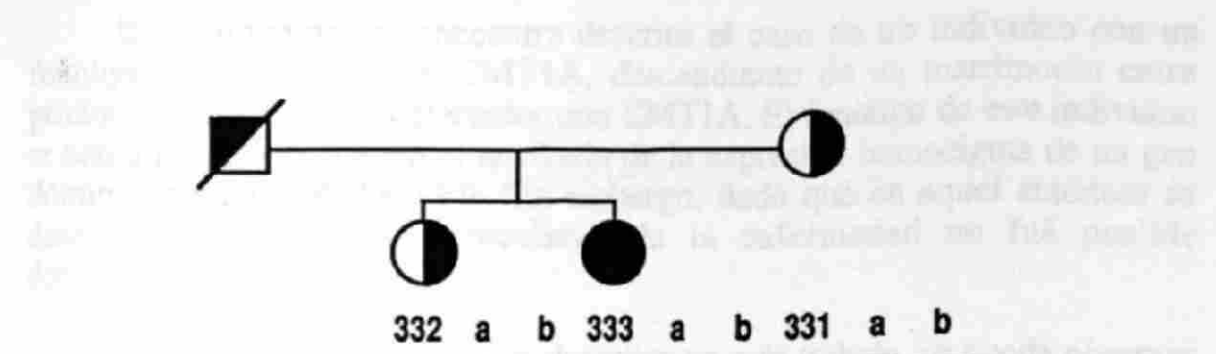
A los individuos 42-332 y 42-333, hijos de un matrimonio entre dos personas afectadas con CMT, y a su madre afectada (42-331), se les separaron sus cromosomas 17 homólogos en híbridos de células somáticas, tal y como se describió en METODOS. Lisados de los híbridos seleccionados se sometieron a otra reacción de PCR para determinar el número de alelos (GT)_n del locus D17S122. En el panel A se muestran los resultados de este análisis, en donde los carriles 332, 333 y 331 corresponden a los alelos (GT)_n presentes en estos individuos, y en los carriles con las letras a y b se encuentran los productos de amplificación correspondientes a los cromosomas 17 homólogos separados en los híbridos de células somáticas. En el panel B se muestran los productos de amplificación del marcador OS1-βGT de los individuos 42-332 y 42-333, y sus correspondientes híbridos, en donde se muestra el éxito en la separación de los cromosomas 17 homólogos. La enfermedad segrega con los alelos (GT)_n A y E en las familias de ambos padres del individuo 42-333, quien como se observa en el panel A, es homocigoto para los cromosomas que contienen la duplicación. Los símbolos en la familia reflejan el genotipo con respecto a los alelos de la enfermedad.

UNIVERSIDAD AUTÓNOMA DE NUEVO LEÓN

®

DIRECCIÓN GENERAL DE BIBLIOTECAS

entre famílias e grupos de indivíduos com o mesmo tipo de CMT1A, demonstrando de tal maneira que...



UNIVERSIDAD AUTÓNOMA DE NUEVO LEÓN
 DIRECCIÓN GENERAL DE BIBLIOTECAS

Figura 16. Demostración de un individuo homocigoto para el CMT1A, por análisis de (GT)n de sus dos cromosomas homólogos separados en híbridos de células somáticas.

X. Correlación entre fenotipo y genotipo: un individuo severamente afectado con CMT1A es homocigoto para la duplicación.

En la literatura se encuentra descrito el caso de un individuo con un fenotipo clínico severo de CMT1A, descendiente de un matrimonio entre primos hermanos, ambos afectados con CMT1A. El fenotipo de este individuo se había propuesto que era el resultado de la expresión homocigota de un gen dominante para CMT1A (30). Sin embargo, dado que en aquel entonces se desconocían las bases moleculares de la enfermedad no fué posible demostrarlo.

En los árboles genealógicos descritos en este trabajo, se puede observar que en una familia del árbol HOU42 (Figura 3A) hay un matrimonio entre dos individuos afectados con dos descendientes afectados. Uno de sus dos hijos (42-333) tiene un fenotipo clínico severo de CMT1A, incluyendo una aparición temprana de los síntomas (< 1 año) y una VCN marcadamente reducida (< 10 m/s, vs. 20-40 m/s en individuos afectados y >60 m/s en individuos no afectados). Nuevamente surgió la hipótesis de que el individuo 42-333 podría ser homocigoto para el CMT1A, y por lo tanto debería presentar una duplicación en cada uno de sus cromosomas 17 (o sea, dos copias del locus D17S122 por cromosoma).

Si se analiza la segregación de los alelos (GT)_n en el árbol genealógico HOU42 (Figura 17C), se observa que en las familias de ambos padres del individuo 42-333, los alelos A y E están segregando con la enfermedad de CMT1A (esto indica que el genotipo del "cromosoma CMT1A", en ambos padres debe ser AE). Esto está congruente con el genotipo AE de la madre[®] (42-331), aunque desafortunadamente como el padre ya había fallecido, no se pudo determinar su genotipo. Sin embargo, tomando en cuenta que el genotipo de su hija (42-332) es ABE, y que la madre no posee ningún alelo B, se deduce que el genotipo del padre debería ser ABE. Por lo tanto, puesto que el genotipo del individuo en cuestión (42-333) es AE, se deduce que ha heredado dos "cromosomas CMT1A", siendo homocigoto para la duplicación. Cabe recalcar que su hermana (42-332), por tener el genotipo ABE, ha heredado únicamente un cromosoma con la duplicación y por ello tiene un fenotipo menos severo.

Para confirmar la observación de que el individuo 42-333 posee dos cromosomas CMT1A, cada uno con dos alelos (GT)_n, A y E, se siguió la misma estrategia del experimento anterior de separar sus cromosomas

homólogos 17 en híbridos de células somáticas, y determinar a cada uno de ellos los alelos (GT)_n que contienen. Además se incluyeron en este experimento tanto células de su madre (42-331) como de su hermana (42-332), que también están afectadas.

Una vez obtenidos los híbridos celulares se utilizó el marcador OS1-βGT para identificar dos híbridos celulares de cada persona que llevaran por separado cada uno de los dos cromosomas homólogos 17 (Figura 16B). El DNA de estos híbridos celulares, se analizó por PCR con el marcador polimórfico pRM11-GT, para determinar el número de alelos (GT)_n por cromosoma. En la Figura 16A se muestra el resultado de este análisis. Como se observa, el individuo 42-332 tiene un cromosoma con el alelo B y otro cromosoma con los alelos A y E, lo cual es congruente con su genotipo ABE (primer carril); el individuo 42-331 tiene un cromosoma con el alelo E y otro cromosoma con los alelos A y E, de aquí que su genotipo se observe únicamente como AE (séptimo carril); y el individuo 42-333 tiene dos cromosomas, cada uno con los dos alelos del mismo tamaño, A y E, por lo que su genotipo se observa simplemente como AE (cuarto carril). En resumen, los individuos 42-331 y 42-332 son heterocigotos para el cromosoma portando la duplicación, mientras que el individuo 42-333 es homocigoto para la duplicación, con cada uno de sus dos cromosomas homólogos conteniendo dos copias del locus D17S122 (73). Estos resultados apoyan la hipótesis de que la duplicación es la responsable del fenotipo clínico de la enfermedad de CMT1A, y que el CMT1A es una mutación semidominante ya que la homocigosis para la duplicación resulta en un fenotipo clínico más severo.

UNIVERSIDAD AUTÓNOMA DE NUEVO LEÓN

XI. Identificación de un nuevo marcador genético para la duplicación de DNA en pacientes con CMT1A.

Los marcadores genéticos tipo RFLP que detectaron la duplicación (VAW409R3, VAW409R1, VAW412 y EW401) corresponden a marcadores genéticos que ya habían sido descritos en la literatura. Con la finalidad de determinar el área duplicada en los pacientes con CMT1A se inició el aislamiento de YAC (cromosomas artificiales de levadura), los cuales pueden portar insertos de DNA hasta de 1000 kb. Utilizando los oligonucleótidos del marcador pRM11-GT se aisló un YAC, y de uno de los extremos del inserto se amplificó por PCR un fragmento de 500 bp. A su vez, utilizando este fragmento como sonda se aisló un cósmido el cual fué designado como cO8H4. Puesto que los fragmentos genómicos de DNA humano se clonaron en el sitio *EcoRI* del cósmido, éste se digirió con la enzima *EcoRI* para separar el

inserto. La digestión produjo al menos 8 fragmentos de 8.5, 7.5, 6.5, 4.8, 2.5, 2, 1.8 y 1.3 kb, y varios fragmentos de menor tamaño. Los cinco fragmentos de mayor tamaño se purificaron independientemente de un gel de agarosa, y se utilizaron para buscar RFLP de las enzimas *BglIII*, *TaqI* y *MspI*, mediante hibridaciones tipo Southern, en los individuos de los árboles genealógicos segregando la enfermedad de CMT1A. El fragmento *EcoRI* de 7.5kb denominado c08H4-R2 detectó un RFLP para la enzima *TaqI* compuesto por dos diferentes alelos *TaqI* de 2.2 y 2.1 kb designados como alelos A y B, respectivamente. En la Figura 17 se presenta un resultado representativo de este experimento y como se observa, los tres individuos afectados con CMT1A, heterocigotos para este marcador, muestran el característico RFLP de los individuos con CMT1A: un alelo en doble dosis con respecto al otro. De estos resultados se deduce que uno de los extremos del YAC contiene DNA que se localiza en la región duplicada. De acuerdo con esto se podría utilizar el fragmento de 500 pb anteriormente mencionado para aislar un nuevo YAC y aplicar la misma estrategia para determinar hasta donde se extiende la región duplicada.

XII. Diagnóstico molecular del CMT1A con el marcador pRM11-GT.

Tanto el hecho de que existe una perfecta correlación entre la enfermedad de CMT1A y la presencia de tres alelos, así como la observación de que el individuo homocigoto estaba severamente afectado, indican que en los individuos con CMT1A la duplicación del locus D17S122 es la característica más definida y asociada con la enfermedad. Por lo tanto, la detección de la duplicación se puede considerar como un medio de diagnóstico de la enfermedad de CMT1A. Con este propósito, se evaluó el potencial del marcador pRM11-GT, como una herramienta para el diagnóstico del CMT1A. Para este estudio, a treinta y tres individuos con fenotipo clínicamente de CMT1 se les determinó el número de alelos (GT)_n utilizando el marcador pRM11-GT. Cabe señalar que estos individuos no forman parte de los árboles genealógicos descritos anteriormente, ni son familiares entre sí. Los resultados se muestran en la Figura 18, y como se observa, 11 de los 33 pacientes tienen tres alelos (GT)_n. Esto significa que la probabilidad de detectar la duplicación con el marcador pRM11-GT es de un 33%. Cabe señalar que aproximadamente la mitad de estos individuos, incluyendo sus padres (no mostrados en la Figura 18), se habían analizado previamente para llevar a cabo el experimento de la sección VI.

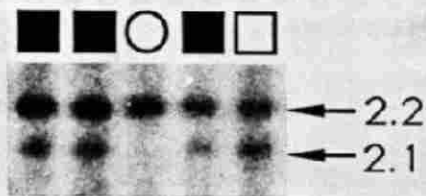


Figura 17. Identificación de un nuevo marcador para la región duplicada de los pacientes con CMT1A.

Análisis tipo Southern del DNA genómico de una familia representativa del árbol genealógico HOU85. El DNA se digirió con *TaqI* y se hibridó con la sonda cO8H4-R2. Note la diferencia característica que existe entre los dos alelos de individuos con CMT1A (símbolos negros) versus individuos no afectados.

DIRECCIÓN GENERAL DE BIBLIOTECAS

DISCUSION

En el presente trabajo se demostró que la enfermedad de CMT1A se asocia con una duplicación de DNA. Esta duplicación se detectó con el 100% de sensibilidad utilizando los marcadores pRM11-GT, y pRM11-GT. Con base en la base de datos se observó que personas con esta enfermedad tienen un número de alelos (GT)_n que varía entre 10 y 30. En el presente estudio se analizaron treinta y tres individuos que no tenían nexos familiares entre sí y que se diagnosticaron clínicamente como afectados con CMT1. A cada uno de ellos se le determinó por PCR el número de alelos (GT)_n utilizando los oligonucleótidos para el marcador pRM11-GT. Con asteriscos se indican aquellos individuos que mostraron tres alelos (GT)_n, y que por lo tanto son diagnosticados genéticamente como CMT1A.

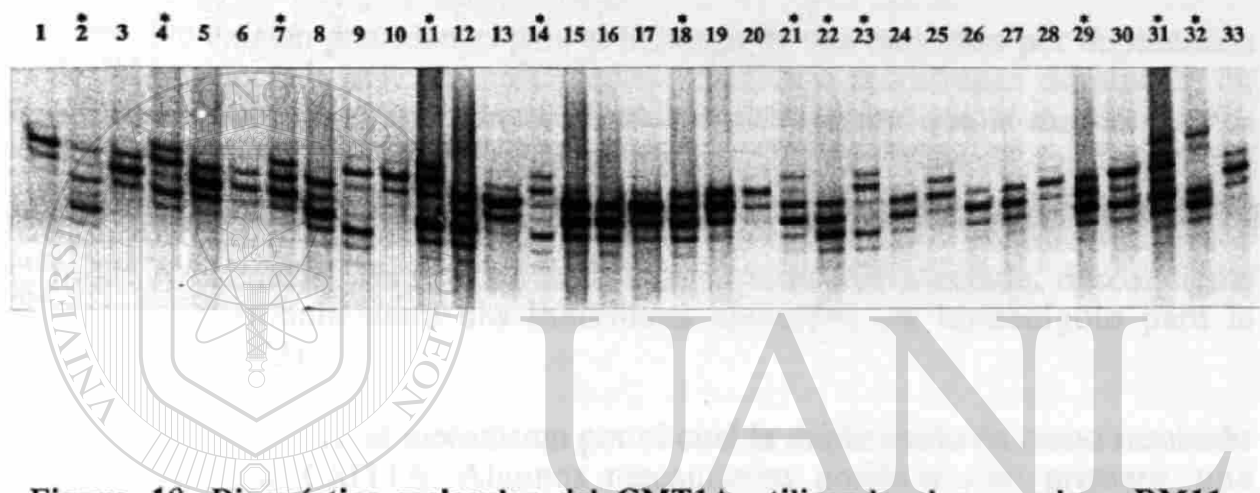


Figura 18. Diagnóstico molecular del CMT1A utilizando el marcador pRM11-GT.

En este estudio se analizaron treinta y tres individuos que no tenían nexos familiares entre sí y que se diagnosticaron clínicamente como afectados con CMT1. A cada uno de ellos se le determinó por PCR el número de alelos (GT)_n utilizando los oligonucleótidos para el marcador pRM11-GT. Con asteriscos se indican aquellos individuos que mostraron tres alelos (GT)_n, y que por lo tanto son diagnosticados genéticamente como CMT1A.

DISCUSION

En el presente trabajo se demostró que la enfermedad de CMT1A se asocia con una duplicación de DNA. Esta duplicación se detectó con un total de tres marcadores polimórficos del locus D17S122 (VAW409R3, VAW409R1, y RM11-GT). Con cada uno de ellos se observó una perfecta correlación entre el genotipo de la duplicación y el fenotipo de la enfermedad CMT1A. Análisis por RFLP usando los marcadores VAW412R3 (D17S125) y VAW401 (D17S61) (86), indican que estos también están duplicados en pacientes con CMT1A.

No existen precedentes para el hallazgo de una mutación por duplicación de DNA asociada con una enfermedad hereditaria autosómica dominante en múltiples familias. Varias líneas de evidencia sugieren que la duplicación es responsable del fenotipo de CMT1A. Primero, la mutación de duplicación se observó únicamente en pacientes con CMT1A. Segundo, la duplicación se demostró en pacientes con CMT1A de diferente origen, Acadio-Francés y Judío Ashkenazic. Tercero, un individuo severamente afectado, descendiente de un matrimonio entre dos individuos afectados, es homocigoto para la duplicación (73).

Se desconoce el mecanismo por el cual la duplicación da como resultado el fenotipo de CMT1A. Algunos mecanismos posibles son: primero, una sobreexpresión de algun(os) gen(es) en la región duplicada; segundo, si la duplicación se origina en la parte interna de un gen puede inhibir la expresión del mismo o expresar un producto génico alterado con un efecto dominante deletereo; tercero, la duplicación puede cambiar la posición física de un gen y ocasionar que se altere la regulación de su expresión.

Un posible gen candidato del CMT1A era el gen para la subunidad β del receptor muscular de la acetilcolina, localizado en la región 17p11.2-p12 (87). Este gen juega un papel muy importante en la transducción de la señal a nivel de la unión neuromuscular (88). Nos propusimos determinar si este gen se encontraba en la región duplicada. Puesto que la demostración más concluyente de la presencia de una duplicación fué mediante el uso de un marcador genético (GT)_n (pRM11-GT), se decidió obtener un marcador similar para este gen. Para lo cual utilizando como sonda radiactiva el DNA complementario al RNA mensajero de este gen se aisló un cósmido y de éste se clonó una repetición (GT)_n. De acuerdo con la secuencia nucleotídica se determinó que esta secuencia repetida estaba incluida en el primer intrón del

gen. Afortunadamente este marcador (OS1-βGT) resultó más polimórfico que el pRM11-GT (se detectaron hasta 14 diferentes alelos en únicamente 17 individuos), sin embargo al analizar los individuos de los árboles genealógicos no se detectó la presencia de tres alelos característico de un marcador en la región duplicada asociada con el CMT1A. Claro que este experimento no descarta la posibilidad de que la duplicación se origine en otra parte del gen. Por otro lado, una evidencia que excluye este gen como un posible candidato, es que de acuerdo con nuestros resultados acerca de la posición de este gen, demostramos que no se localiza en la región 17p11.2-p12 como se había descrito (87); su ubicación real es en la banda 17p13 (58), la cual está considerablemente muy alejada de la región involucrada en la duplicación. La importancia de este experimento, además de eliminar este gen como un posible candidato, es que se identificó dentro de el un marcador genético (GT) sumamente polimórfico, el cual se puede utilizar en estudios moleculares o estudios de ligamiento genético de este gen con respecto a otras enfermedades donde se sospeche que sea el gen responsable (58).

Se desconoce también el mecanismo de origen de la mutación que conduce a la duplicación. Se han descrito mutaciones *de novo*, como deleciones y duplicaciones, en la región proximal del brazo corto del cromosoma 17 (89-91). Es posible que los mismos mecanismos de recombinación que originan una deleción de un cromosoma pueden ocasionar una duplicación en su cromosoma homólogo. Estudios recientes acerca de duplicaciones en *Escherichia coli* y humanos, han demostrado que las duplicaciones ocurren en regiones que contienen secuencias repetidas, por ejemplo las secuencias REP en bacterias (92) y las secuencias Alu en el humano (93,94). Otro ejemplo de duplicación cromosómica ocurre en el locus *Bar* de *Drosophila melanogaster* (95). Esta mutación es semidominante y frecuentemente se asocia con una duplicación en tandem de las bandas cromosómicas 16A1-A7 (96). Análisis moleculares recientes sugieren que existe un elemento transposón (B104) en la unión de la duplicación, por lo que se propuso que este transposón está involucrado en la producción de la duplicación. Basados en el análisis de la secuencia de DNA del elemento B104 que se encuentra en las uniones de la duplicación, se propuso como modelo que la duplicación se genera por un evento de recombinación entre dos elementos B104, localizados uno en la banda 16 A1 y el otro en 16 A7 (97). Un análisis detallado de la región duplicada en pacientes con CMT1A de diferente origen étnico, especialmente a nivel de las uniones, podría dilucidar el mecanismo responsable de la generación de esta duplicación (73).

Existen dos posibles modelos animales para el CMT los cuales

corresponden a los ratones mutantes designados como “trembler” (tr, el cual es dominante) (98) y trembler-J (tr^J, el cual es semidominante) (99). Estos ratones presentan una neuropatía desmielinizante con un decremento en la VCN similar al observado en el CMT1; además, su mutación se localiza en el cromosoma 11 el cual es el homólogo del cromosoma 17 en el humano. Las bases moleculares del CMT1A se podrán delucidar más claramente al definir y caracterizar los límites de la región duplicada mediante el aislamiento de clonas genómicas humanas que se sobrepongan, así como al identificar genes que se localicen en esta región duplicada.

Otra cuestión interesante es si la duplicación ocurrió dentro del mismo cromosoma, o si el segmento duplicado proviene de su cromosoma homólogo por un evento de recombinación. El hecho de que en todos los árboles genealógicos analizados los dos alelos (GT)_n en el “cromosoma CMT1A” (es decir los dos alelos ligados que se segregan con la enfermedad) sean diferentes, apoyan la hipótesis de que el segmento duplicado se haya originado del cromosoma homólogo.

Otro punto importante de discutir es el hecho de haber detectado la duplicación en pacientes con CMT1A de dos orígenes diferentes: Acadio-Francés y Judío-Ashkenazic, además de varios individuos con CMT1A de diferente ascendencia, sin nexos familiares entre sí. Esto sugiere que el evento de la mutación de duplicación sea un mecanismo inherente a esta enfermedad, es decir independiente del origen de los individuos.

I. Implicaciones en el diagnóstico molecular del CMT1A.

Una implicación sumamente importante de este trabajo es el uso del marcador RM11-GT para el diagnóstico molecular del CMT1A, y de cualquier otro marcador que se localice en la región duplicada. Como se demostró en este trabajo existe una perfecta correlación entre la presencia de una duplicación de DNA y la enfermedad de CMT1A. Por esta razón se evaluó el potencial del marcador pRM11-GT, como una herramienta para el diagnóstico del CMT1A. Desafortunadamente cuando los padres tienen alelos (GT)_n similares no es posible observar tres alelos en los pacientes con CMT1A, de aquí que únicamente se pudieron diagnosticar el 33% de los 33 pacientes analizados con el marcador pRM11-GT. Puesto que un sólo marcador no detecta la duplicación de cualquier individuo afectado, se puede combinar el uso de ellos para incrementar el potencial de diagnóstico. Teniendo en cuenta que la presencia de un alelo extra se detecta también por

RFLP en los individuos heterocigotos, se puede utilizar el polimorfismo *MspI* de los marcadores VAW409R1 y VAW409R3 para detectar la duplicación. Resultados preeliminares indican que usando en combinación los tres marcadores anteriormente mencionados se incrementó al doble el porcentaje de individuos diagnosticados (67%). Sin tomar en cuenta los marcadores VAW401 y VAW412R3, que también detectan la duplicación. Un procedimiento alternativo muy efectivo es el uso de la Hibridación Fluorescente *in situ*, ya que para esto no se requieren polimorfismos (73). El uso de marcadores genéticos muy polimórficos, como el utilizado en este estudio, incrementa la posibilidad de detectar trisomías submicroscópicas parciales que pudiesen ser la alteración molecular para otras enfermedades genéticas humanas (73).

II. Consecuencias de la duplicación en los estudios de ligamiento genético para la localización del gen del CMT1A.

Diferentes grupos de investigación, tanto en Estados Unidos como en Europa, estaban aún enfocados hasta mediados del año 1991, a la localización del locus del CMT básicamente por estudios de ligamiento genético. El objetivo de estos estudios es el de encontrar un marcador genético que se cosegrege con la enfermedad, es decir que esté ligado a ella. En un estudio de ligamiento genético, las recombinaciones que ocurran entre la enfermedad y el marcador, son los datos más importantes que van a determinar la distancia que existe entre ellos. Así por ejemplo, un marcador con 1% de recombinación (un evento de recombinación por cada 100 meiosis informativas) equivale a un Centimorgan y esto indica que el marcador se encuentra a una distancia de un millón de bases nucleotídicas con respecto al locus de la enfermedad. ®

El número de recombinantes entre el marcador VAW409 y el locus del CMT1A se sabía que era más frecuente en comparación con otros marcadores localizados fuera de la región duplicada. **¿Cómo es posible que un marcador genético localizado en el locus de la enfermedad, tal y como es el caso para el marcador VAW409, muestre un mayor número de recombinantes y por ende un menor grado de ligación con la enfermedad, con respecto a otros marcadores fuera del locus de la enfermedad?** Este resultado obviamente es incorrecto, puesto que el marcador VAW409 forma parte del locus del CMT1A. Sin embargo, el hecho de existir una duplicación, implica la presencia de un alelo extra para este marcador y da como consecuencia la observación de falsos recombinantes (73).

CONCLUSIONES

La heterocidad de Charcot-Marie-Tooth tipo 1A está asociada con una duplicación de DPM1, e involucra al locus en los loci D17S122, D17S125 y D17S126.

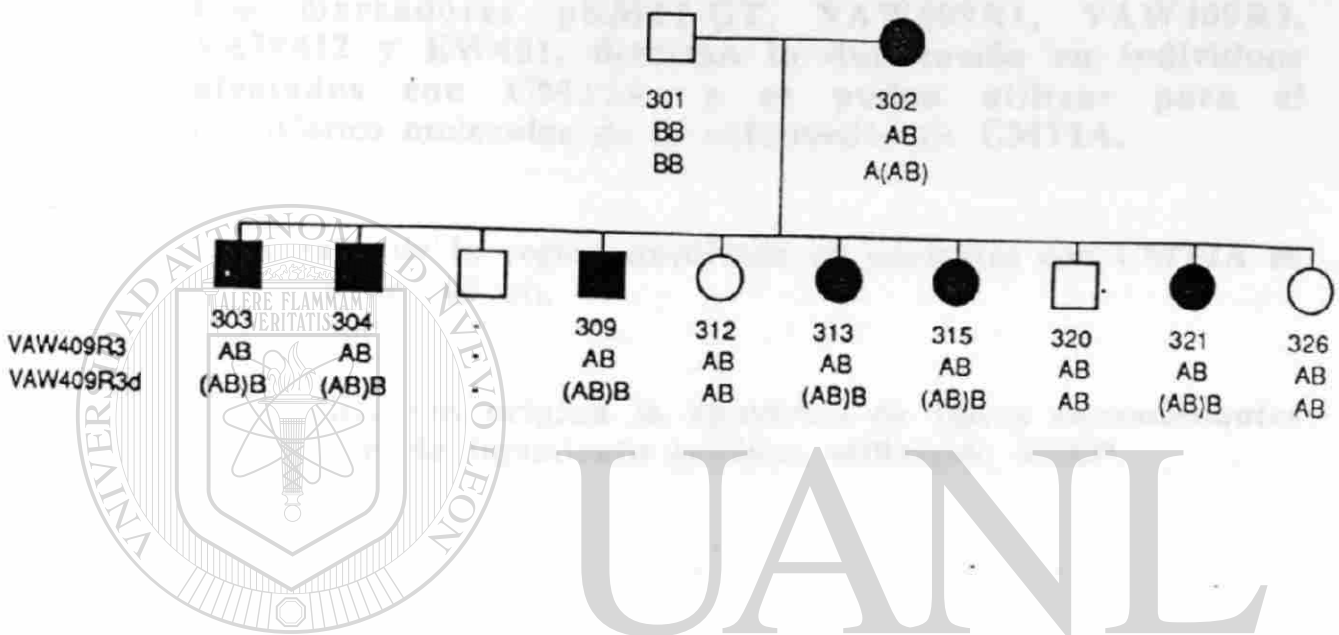
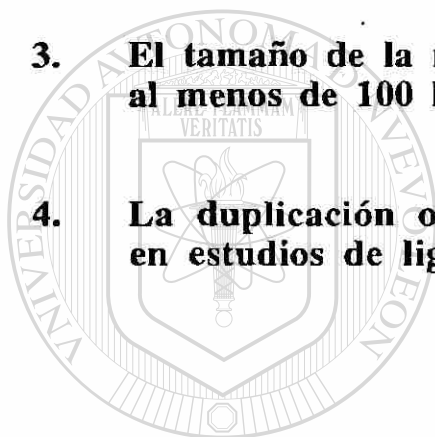


Figura 19. Observación de falsos recombinantes debido a la duplicación.

En esta figura se muestra una familia del árbol genealógico HOU85 mostrando la clasificación incorrecta de los alelos *MspI* del marcador VAW409R3. Debajo del símbolo de cada individuo, se encuentra en orden descendiente primero el número de identificación del individuo, segundo el genotipo para el marcador VAW409R3 sin tomar en cuenta la dosis de los alelos, y tercero el genotipo considerando la diferente dosis alélica. La segregación de los alelos del marcador demuestra que sin considerar la dosis alélica, el cromosoma CMT1A del individuo 85-302 porta el alelo A, en tanto que si se considera la dosis, el cromosoma CMT1A porta los alelos A y B. Los individuos 85-312, 85-20 y 85-326 aparecen como recombinantes para el marcador VAW409R3; sin embargo, considerando la dosis alélica, se demuestra que los recombinantes son debidos a una interpretación incorrecta (73).

CONCLUSIONES

1. **La enfermedad de Charcot-Marie-Tooth tipo 1A está asociada con una duplicación de DNA, e involucra al menos los loci D17S122, D17S125 y D17S61.**
2. **Los marcadores pRM11-GT, VAW409R1, VAW409R3, VAW412 y EW401, detectan la duplicación en individuos afectados con CMT1A, y se pueden utilizar para el diagnóstico molecular de la enfermedad de CMT1A.**
3. **El tamaño de la región duplicada en pacientes con CMT1A es al menos de 100 kb.**
4. **La duplicación origina la aparición de falsos recombinantes en estudios de ligamiento genético utilizando RFLP.**



UANL

UNIVERSIDAD AUTÓNOMA DE NUEVO LEÓN®
DIRECCIÓN GENERAL DE BIBLIOTECAS

PERSPECTIVAS

I. Desde el punto de vista científico.

Esta es la primera vez que se encuentra una duplicación, como un mecanismo molecular generalizado responsable de una enfermedad autosómica dominante. Este hecho se debe tomar en cuenta como un posible mecanismo para otras enfermedades genéticas humanas.

Diversos grupos de investigación en Estados Unidos y Europa, habían dirigido sus esfuerzos para identificar el gen responsable de producir la enfermedad de CMT1A mediante estudios de ligamiento genético. Este tipo de estudios son bastante laboriosos e implican métodos muy sofisticados para "caminar en los cromosomas" hasta encontrar el gen responsable. El hecho de haber encontrado una duplicación asociada con el CMT1A, define el locus de esta enfermedad y hace que ahora los esfuerzos se concentren específicamente en esta región para identificar el gen(es) responsable del CMT1A.

Estudios de ligamiento genético se utilizan actualmente para la identificación del gen responsable de diferentes enfermedades genéticas humanas. En este trabajo se demostró que por una duplicación de DNA se originan falsos recombinantes en los análisis por RFLP, y como consecuencia, un resultado erróneo en el grado de ligamiento de un marcador genético con el locus de una enfermedad.

II. Desde el punto de vista de desarrollo tecnológico.

En el presente trabajo se sentaron las bases para el establecimiento de una nueva prueba para el diagnóstico molecular de la enfermedad de CMT1A.

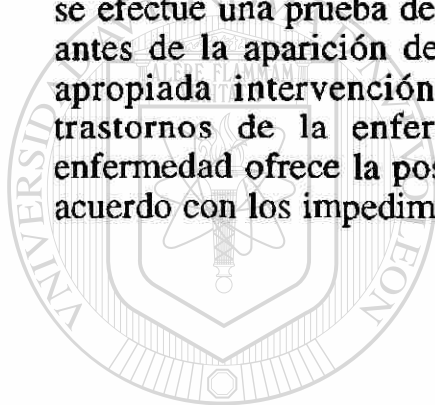
¿Qué tan importante sería esta prueba de diagnóstico molecular de la enfermedad de CMT1A? Actualmente los métodos de diagnóstico del CMT incluyen métodos electrofisiológicos para medir la conducción nerviosa, histometría para cuantificar las fibras nerviosas en biopsias de nervio y microscopía electrónica para examinar la ultraestructura de los nervios involucrados. Estos métodos son dolorosos, tienen cierto riesgo para el paciente y son caros (el costo estimado de este tipo de diagnóstico, en los Estados Unidos, es alrededor de \$5000.00 dólares).

Por el contrario una prueba de diagnóstico molecular, como la descrita

en el presente trabajo, origina la posibilidad de que se obtenga el diagnóstico utilizando simplemente el DNA genómico aislado de sangre periférica. Este método de diagnóstico molecular tendría las ventajas de que es más económico, sin ningún riesgo para el paciente y el diagnóstico se puede realizar desde el primer día de nacimiento. Otra ventaja sumamente importante es la posibilidad de realizar un diagnóstico prenatal, lo cual no era posible con los métodos actuales.

III. Desde el punto de vista de beneficios sociales y de salud.

Como se mencionó en la Introducción, la edad promedio de aparición de los síntomas del CMT1A es a los 12 años de vida. El establecimiento de un método más económico, dará posibilidad a que un mayor número de personas se efectúe una prueba de diagnóstico. El hecho de diagnosticar la enfermedad antes de la aparición de los síntomas, brinda la oportunidad a realizar una apropiada intervención farmacológica y quirúrgica para disminuir los trastornos de la enfermedad. Además una detección temprana de la enfermedad ofrece la posibilidad al individuo afectado de orientar su vida de acuerdo con los impedimentos que va a manifestar.



UANL

UNIVERSIDAD AUTÓNOMA DE NUEVO LEÓN[®]
DIRECCIÓN GENERAL DE BIBLIOTECAS

BIBLIOGRAFIA

1. McKusick, V.A. (1992). **Mendelian Inheritance in Man**. 10th Edition. Johns Hopkins University Press.
2. Orkin, S.H., and Kazazian, H.H. (1984). Mutation and polymorphism of the human β -globin gene and its surrounding DNA. *Ann. Rev. Genet.* 18:131-171.
3. Brown, M.S., and Goldstein, J.L. (1986). A receptor-mediated pathway for cholesterol homeostasis. *Science* 232: 34-47.
4. Rommens, J.H., Iannuzzi, M.C., Kerem, B.S., Drumm, M.L., Melmer, G., et al. (1989). Identification of the cystic fibrosis gene: chromosome walking and jumping. *Science* 245:1059-1065.
5. Riordan, J., Rommens, J.M., Kerem, B.S., Alon, N., Rozmahel, R., et al. (1989). Identification of the cystic fibrosis gene: cloning and characterization of the complementary DNA. *Science* 245:1066-1073.
6. Kerem, B.S., Zielenski, J., Markiewicz, D., Bozon, D., Gazit, E., et al. (1989). Identification of the cystic fibrosis gene: genetic analysis. *Science* 245:1073-1080.
7. Gusella, J.F., Wexler, N.S., Conneally, P.M., Naylor, S.L., Anderson, M.A., et al. (1983). A polymorphic DNA marker genetically linked to Huntington's disease. *Nature* 306:234-238.
8. Reeders, S.T., Breuning, M.H., Davies, K.E., Nicholls, R.D., Jarman, A.P., et al. (1985). A highly polymorphic DNA marker linked to adult polycystic kidney disease on chromosome 16. *Nature* 317:542-544.
9. Francke, U., Ochs, H.D., de Martinville, B., Giacalone, J., Lindgren, V., et al. (1985). Minor Xp21 chromosome deletion in a male associated with expression of Duchenne Muscular Dystrophy, chronic granulomatous disease, retinitis pigmentosa, and McLeod syndrome. *Am. J. Hum. Genet.* 37: 250-267.
10. Worton, R.G., Duff, C., Sylvester, J.E., Schmickel, R.D., and Willard, H.F. (1984). Duchenne muscular dystrophy involving translocation of the *dmd* gene next to ribosomal RNA genes. *Science* 224: 1447-1449.

11. Friend, S.H., Bernards, R., Rogelj, S., Weinberg, R.A., Rapaport, J.M., et al. (1986). A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. *Nature* 323: 643-646.
12. Royer-Pokora, B., Kunkel, L.M., Monaco, A.P., Goff, S.C., Newburger, P.E., et al. (1986). Cloning the gene for an inherited human disorder -chronic granulomatous disease- on the basis of its chromosomal location. *Nature* 322: 32-38.
13. Cawthon, R.M., Weiss, R., Xu, G., Viskochil, D., Culver, M., et al. (1990). A major segment of the neurofibromatosis type 1 gene: cDNA sequence, genomic structure, and point mutations. *Cell* 62:193-201.
14. Wallace, M.R., Marchuk, D.A., Andersen, L.B., Letcher, R., Odeh, H.M., et al. (1990). Type 1 neurofibromatosis gene: identification of a large transcript disrupted in three NF1 patients. *Science* 249:181-186.
15. Charcot, J.M., Marie, P. (1886). Sur une forme particuliere d'atrophie musculaire progressive, souvent familiale, debutant par les pieds et les jambes et atteignant plus tard les mains. *Rev. Med.* 6:97-138.
16. Tooth, H.H. (1886). The peroneal type of progressive muscular atrophy. London: HK Lewis.
17. Henriksen, J.D. (1956). Conduction velocity of motor nerves in normal subjects and patients with neuromuscular disorders. Thesis, University of Minnesota.
18. Gilliat, R.W. and Thomas, P.K. (1957). Extreme slowing of nerve conduction in peroneal muscular atrophy. *Ann. Phys. Med.* 4:104-106.
19. Dyck, P.J., and Lambert, E.H. (1968). Lower motor primary sensory neuron diseases with peroneal muscular atrophy. I. Neurologic, genetic, and electrophysiologic findings in hereditary polyneuropathies. *Arch. Neurol.* 18:603-618.
20. Dyck, P.J., and Lambert, E.H. (1968). Lower motor and primary sensory neuron diseases with peroneal muscular atrophy. II. Neurologic genetic, and electrophysiologic findings in various neuronal degenerations. *Arch. Neurol.* 18:619-625.

21. Brust, J.C.M., Lovelace, R.E., and Devi, S. (1978). Clinical and electrodiagnostic features of Charcot-Marie-Tooth syndrome. *Acta Neurol. Scand. Suppl.* 58:1-42.
22. Davis, C.J.F., Bradley, W.G., and Madrid, R. (1978). The peroneal muscular atrophy syndrome clinical, genetic, electrophysiologic, and nerve biopsy studies. *J. Genet. Hum.* 26:311-349.
23. Bird, T.D. (1989). Hereditary motor-sensory neuropathies. Charcot-Marie-Tooth syndrome. *Neurol. Clin.* 7:9-23.
24. Dyck, P.J. (1984). Inherited Neuronal Degeneration and atrophy affecting peripheral motor, sensory, and autonomic neurons in Dyck, Thoma, Lambert, and Bunge (2nd Ed.). *Peripheral Neuropathy*. Philadelphia, WB Saunders Co., pp. 1600-1655.
25. Lupski, J.R., Garcia, C.A., Parry, G.J., and Patel, P.I. (1991). Charcot-Marie-Tooth Polyneuropathy Syndrome: Clinical, Electrophysiological, and Genetic Aspects. In *Current Neurology*. S. Appel, ed. (Chicago: Mosby-Yearbook), pp. 1-25.
26. Berciano J., Combarros O., Calleja J., Polo J.M., and Leno C. (1989). The application of nerve conduction and clinical studies to genetic counseling in hereditary motor and sensory neuropathy type I. *Muscle Nerve* 12:302-306.

27. Gutmann L., Fakadej A., and Riggs J.E. (1983). Evolution of nerve conduction abnormalities in children with dominant hypertrophic neuropathy of the Charcot-Marie-Tooth type. *Muscle Nerve* 6:515-519.
28. Dyck, P.J., Karnes, J.L., and Lambert, E.H. (1989). Longitudinal study of neuropathic deficits and nerve conduction abnormalities in hereditary motor and sensory neuropathy type I. *Neurology* 39:1302-1308.
29. Allan W. (1939). Relation of hereditary pattern to clinical severity as illustrated by peroneal atrophy. *Arch. Intern. Med.* 63:1123-1131.
30. Killian J.M., Kloepfer H.W. (1979). Homozygous expression of a dominant gene for Charcot-Marie-Tooth neuropathy. *Ann. Neurol.* 5:515-522.

31. Bird, T.D., and Kraft, G.H. (1978). Charcot-Marie-Tooth disease: Data for genetic counseling relating age to risk. *Clin. Genet.* 14:43-49.
32. Patel, P.I., Franco, B., Garcia, C., Slaugenhaupt, S.A., Nakamura, Y., et al. (1990). Genetic mapping of autosomal dominant Charcot-Marie-Tooth disease in a large French-Acadian kindred: Identification of new linked markers on chromosome 17. *Am. J. Hum. Genet.* 46:801-809.
33. Garcia, C.A., Malamut, R., Parry, G., Patel, P.I., and Lupski, J.R. (1991). Clinical heterogeneity in two pairs of identical twins. In preparation.
34. Weiden P.L., and Wright S.E. (1972). Vincristine neurotoxicity. *New Engl. J. Med.* 286:1369-1370.
35. Hogan-Dann C.M., Fellmeth W.G., McGuire S.A., and Kiley, V.A. (1984). Polyneuropathy following vincristine therapy in two patients with Charcot-Marie-Tooth syndrome. *J. Am. Med. Assoc.* 252:2862-2863.
36. Griffiths J.D., Stark R.J., Ding J.C., and Cooper, L.A. (1985). Vincristine neurotoxicity in Charcot-Marie-Tooth syndrome. *Med. J. Aust.* 143:305-306.
37. Rosen S.A., Wang H., Cornblath D.R., Uematsu, S., and Hurko, O. (1989). Compression syndromes due to hypertrophic nerve roots in hereditary motor sensory neuropathy type I. *Neurology* 39:1173-1177.
38. Botstein, D., White, R.L., Skolnick, M.H., and Davis, R.W. (1980). Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *Am. J. Hum. Genet.* 32:314-331.
39. Southern, E.M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98: 503-517.
40. Morton, N.E. (1955). Sequential tests for the detection of linkage. *Am. J. Hum. Genet.* 8:80-96.
41. Ott, J. (1985). Analysis of human genetic linkage. The Johns Hopkins University Press.

42. Bird, T.D., Ott, J., and Giblett, E.R. (1980). Linkage of Charcot-Marie-Tooth neuropathy to the Duffy locus on chromosome 1. *Am. J. Hum. Genet.* 32:99A.
43. Bird, T.D., Ott, J., and Giblett, E.R. (1982). Evidence for linkage of Charcot-Marie-Tooth neuropathy to the Duffy locus on chromosome 1. *Am. J. Hum. Genet.* 34:388-394.
44. Guiloff, R.J., Thomas, P.K., Contreras, M., Armitage, S., Schwartz, G., and Sedgwick, E.M. (1982). Linkage of autosomal dominant type I hereditary motor and sensory neuropathy to the Duffy locus on chromosome 1. *J. Neurol. Neurosurg. Psych.* 45: 69-674.
45. Stebbins, N.B., and Conneally, P.M. (1982). Linkage of dominantly inherited Charcot-Marie-Tooth neuropathy to the Duffy locus in an Indiana family. *Am. J. Hum. Genet.* 34:195 A.
46. Lebo, R.V., Dyck, P.J., Chance, P.F., King, M.C., Lynch, E., et al. (1989). Charcot- Marie-Tooth locus in FCγRII gene region. *Am. J. Hum. Genet.* 45:A148
47. Chance, P.F., Murray, J.C., Bird, T.D., and Kochin, R.S. (1987). Genetic linkage relationships of Charcot-Marie-Tooth disease (HMSN-Ib) to chromosome 1 markers. *Neurology* 37:325-329.
48. Bird, T.D., Ott, J., Giblett, E.R., Chance, P.F., Sumi, S.M., et al. (1983). Genetic linkage evidence for heterogeneity in Charcot-Marie-Tooth neuropathy (HMSN Type I). *Ann. Neurol.* 14:679-684. ®
49. Dyck, P.J., Ott, J., Moore, S.B., Swanson, C.J., and Lambert, E.H. (1983). Linkage evidence for genetic heterogeneity among kinships with hereditary motor and sensory neuropathy. Type 1. *Mayo. Clin. Proc.* 58:430-435.
50. Griffiths, L.R., Zwi, M.B., McLeod, J.G., and Nicholson, G.A. (1988). Chromosome 1 linkage studies in Charcot-Marie-Tooth neuropathy type I. *Am. J. Hum. Genet.* 42:756-771.
51. Vance, J.M., Nicholson, G.A., Yamaoka, L.H., Stajich, J., Stewart, C.S., et al. (1989). Linkage of Charcot-Marie-Tooth neuropathy Type 1a to chromosome 17. *Experimental Neurol.* 104:186-189.

52. Raeymakers, P., Timmerman, V., DeJonghe, P., Swerts, L., Gheuens, J., et al. (1989). Localization of the mutation in an extended family with Charcot-Marie-Tooth neuropathy (HMSNI). *Am. J. Hum. Genet.* 45:953-958.
53. McAlpine, P.J., Feasby, T.E., Hahn, A.F., Komarnicki, L., James, S., et al. (1990). Localization of a locus for Charcot-Marie-Tooth neuropathy type Ia (CMT 1A) to chromosome 17. *Genomics* 7:408-415.
54. Middleton-Price, H.R., Harding, A.E., Monteiro, C., Berciano, J., and Malcolm, S. (1990). Linkage of hereditary motor and sensory neuropathy type 1 to the pericentromeric region of chromosome 17. *Am. J. Hum. Genet.* 46:92-94.
55. Patel, P.I., Garcia, C.A., Montes de Oca-Luna, R., Malamut, R.I., Franco, B., et al. (1990). Isolation of a marker linked to the Charcot-Marie-Tooth disease type 1A gene by differential *Alu*-PCR of human chromosome 17 retaining hybrids. *Am. J. Hum. Genet.* 47:926-934.
56. Vance, J.M., Barker, D., Yamaoka, L.H., Stajich, J.M., Loprest, L., et al. (1991). Localization of Charcot-Marie-Tooth disease type 1a (CMT 1a) to chromosome 17p11.2. *Genomics* 9:623-628.
57. Greenberg, F., Guzzetta, V., Montes de Oca-Luna, R., Ellen-Magenis, R., Smith, A.C.M., et al. (1991). Molecular analysis of the Smith-Magenis syndrome: a possible contiguous gene syndrome associated with del(17)(p11.2). *Am. J. Hum. Genet.*, 49:1207-1218.
58. Guzzetta, V., Franco, B., Zhang H., Saucedo-Cárdenas, O., Montes de Oca-Luna, R., et al. (1992). Somatic cell hybrids, sequence tagged sites, simple repeat polymorphisms and yeast artificial chromosomes for physical and genetic mapping of proximal 17p. *Genomics*, 13:551-559.
59. Hamada, H., Petrino, M.G., and Kakunaga, T. (1982). A novel repeated element with Z-DNA-forming potential is widely found in evolutionarily diverse eukaryotic genomes. *Proc. Natl. Acad. Sci.* 79:6465-6469.
60. Hamada, H., Petrino, M.G., Kakunaga, T., Seidman, M., and Stollar, B.D. (1984). Characterization of genomic Poly(dT-dG).Poly(dC-dA) sequences; structure, organization, and conformation. *Mol. Cell. Biol.*

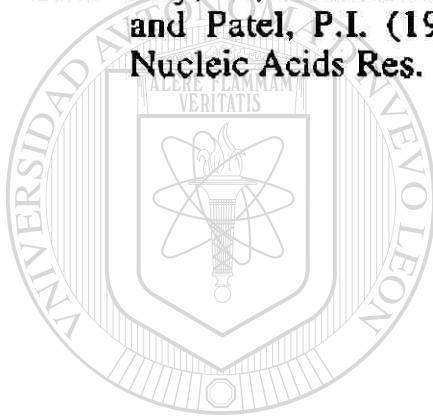
4:2610-2621.

61. Sun, L., Paulson, K.E., Schmid, C.W., Kadyk, L., and Leinwand, L. (1984). Non-Alu family interspersed repeats in human DNA and their transcriptional activity. *Nucleic Acids Res.* 12:2669-2690.
62. Tautz, D., and Renz, M. (1984). Simple sequences are ubiquitous repetitive components of eukaryotic genomes. *Nucleic Acids Res.* 12:4127-4138.
63. Jeffreys, A.J., Wilson, V., and Thein, S.L. (1985). Hypervariable 'minisatellite' regions in human DNA. *Nature* 314:67-73.
64. Nakamura, Y., Leppert, M., O'Connell, P., Wolff, R., Holm, T., et al. (1987). Variable number of tandem repeat (VNTR) markers for human gene mapping. *Science* 235, 1616-1622.
65. Tyler-Smith, C., and Brown, W.R.A. (1987). Structure of the major block of alphoid satellite DNA on the human Y chromosome. *J. Mol. Biol.* 195:457-470.
66. Wayne, J.S., Durfy, S.J., Pinkel, D., Kenwrick, S., Patterson, M., et al. (1987). Chromosome-specific alpha satellite DNA from human chromosome 1: hierarchical structure and genomic organization of a polymorphic domain spanning several hundred kilobase pairs of centromeric DNA. *Genomics* 1:43-51.
67. Motulsky, A.G. (1988). Normal and abnormal color-vision genes. *Am. J. Hum. Genet.* 42:405-407.
68. Kemp, D.J., Coppel, R.L., and Anders, R.F. (1987). Repetitive proteins and genes of malaria. *Annu. Rev. Microbiol.* 41:181-208.
69. Weber, J.L., and May, P.E. (1989). Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. *Am. J. Hum. Genet.* 44:388-396.
70. Dracopoli, N.C., and Meisler, M.H. (1990). Mapping the human amylase gene cluster on the proximal short arm of chromosome 1 using a highly informative (CA)_n repeat. *Genomics* 7:97-102.

71. Litt, M., and Luty, J.A. (1989). A hypervariable microsatellite revealed by in vitro amplification of a dinucleotide repeat within the cardiac muscle actin gene. *Am. J. Hum. Genet.* 44:397-401.
72. Weber, J.L. (1990). Human DNA polymorphisms based on length variation in simple-sequence tandem repeats. *Genome Analysis vol. 1 Genetic and Physical Mapping.* K.E. Davies, S.M. Tilghman (Eds.) Cold Spring Harbor Press, Cold Spring Harbor, New York pp. 157-181.
73. Lupski, J.R., Montes de Oca-Luna, R., Slaugenhaupt, S., Pentao, L., Guzzetta, V., et al. (1991). DNA duplication associated with Charcot-Marie-Tooth disease type 1A. *Cell*, 66:219-232.
74. Miller, S.A., Dykes, D.D., and Polesky, H.F. (1988). A simple salting out procedure for extracting DNA from human nucleated cells. *Nucl Acids Res.* 16:1215.
75. Nakamura, Y., Gillilan, S., O'Connell, P., Leppert, M., Lathrop, G.M., Lalouel, J.-M., and White, R. (1987). Isolation and mapping of a polymorphic DNA sequence pYNH24 on chromosome 2 (D2S44). *Nucleic Acids Res.* 15:10073.
76. Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). *Molecular Cloning: A laboratory manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
-
77. Davis, L.G., Dibner, M.D., and Battey, J.F. (1986). *Basic Methods in Molecular Biology.* Elsevier Science Publishing Co. New York, pp 147-149.
78. Sanger, F., Nicklen, S., and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci.* 74:5463-5467.
79. Kraft, R., Tardifs, J., Krauter, K.S., and Leinwand, L.A. (1988). Using mini-prep plasmid DNA for sequencing double stranded templates with sequenase. *Biotechniques* 6:544-547.
80. Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., et al. (1988). Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239:487-491.

81. Feinberg, A.P., and Vogelstein, B. (1983). A technique for radiolabeling fragments to high specific activity. *Anal. Biochem.* 132:6-13.
82. Wallace, D.M. (1987). Precipitation of nucleic acids. *Methods Enzymol* 152:41-48.
83. Bedford, M.T. and van Helden, P.D. (1990). A method to analyze allele-specific methylation. *BioTechniques* 9:744-748.
84. Zoghbi, H.Y., Sandkuijl, L.A., Ott, J., Daiger, S.P., Pollack, M., et al. (1989). Assignment of autosomal dominant spinocerebellar ataxia (SCAI) centromeric to the HLA region on the short arm of chromosome 6, using multilocus linkage analysis. *Am. J. Hum. Genet.* 44:255-263.
85. Westerveld, A., Visser, R.P.L.S., Khan, P.M., and Bootsma, D. (1971). Loss of human genetic markers in man-Chinese hamster somatic cell hybrids. *Nature New Biol.* 234:20-24.
86. Wright, E.C., Goldgar, D.E., Fain, P.R., Barker, D.F., and Skolnick, M.H. (1990). A genetic map of human chromosome 17p. *Genomics* 7: 103-109.
87. Beeson, D., Jeremiah, S., West, L.F., Povey, S., and Newsom-Davis, J. (1990). Assignment of the human nicotinic acetylcholine receptor genes: the alpha and delta subunit genes to chromosome 2 and the beta subunit gene to chromosome 17. *Ann. Hum. Genet.* 54:199-208.
88. Boulter, J., O'Shea-Greenfield, A., Duvoisin, R.M., Connolly, J.G., Wada, E., et al. (1990). Three members of the rat neuronal nicotinic acetylcholine receptor-related gene family form a gene cluster. *J. Biol. Chem.* 265:4472-4482.
89. Smith, A.C.M., McGavran, L., Robinson, J., Waldstein, G., Macfarlane, J., et al. (1986). Interstitial deletion of (17)(p11.2p11.2) in nine patients. *Am. J. Med. Genet.* 24:393-414.
90. Stratton, R.F., Dobyns, W.B., Greenberg, F., De Sana, J.B., Moore, C., et al. (1986). Interstitial deletion of (17)(p11.2p11.2): Report of six additional patients with a new chromosome deletion syndrome. *Am. J.*

101. Franco, B., Rincon-Limas, D., Nakamura, Y., Patel, P.I., and Lupski, J.R. (1990). An *MspI* RFLP at the D17S258 locus. *Nucleic Acids Res.* 18: 7196.
102. Nakamura, Y., Lathrop, M., O'Connell, P., Leppert, M., Barker, D., et al. (1988). A mapped set of DNA markers for human chromosome 17. *Genomics* 2, 302-309.
103. Guzzetta, V., Montes de Oca-Luna, R., Lupski, J.R., and Patel, P.I. (1991). Isolation of region-specific and polymorphic markers from chromosome 17 by restricted Alu polymerase chain reaction. *Genomics* 9:31-36.
104. Ray, R., Rincon-Limas, D., Wright, R.A., Davis, S.N., Lupski, J.R., and Patel, P.I. (1990). Three polymorphisms at the D17S29 locus. *Nucleic Acids Res.* 18: 4958.

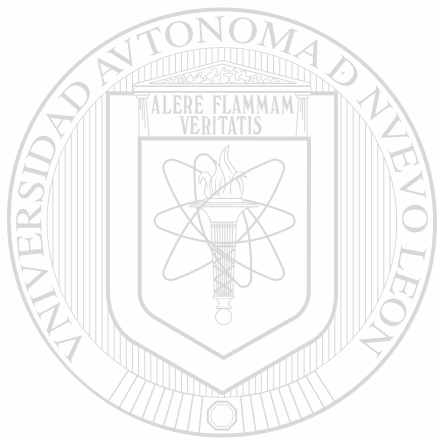


UANL

UNIVERSIDAD AUTÓNOMA DE NUEVO LEÓN



DIRECCIÓN GENERAL DE BIBLIOTECAS



ANEXO

UANL

UNIVERSIDAD AUTÓNOMA DE NUEVO LEÓN

®

DIRECCIÓN GENERAL DE BIBLIOTECAS

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 *Ssr*I RFLPs associated with S6.1 were identified and used in linkage analysis of both a previously reported and a newly identified French-Canadian 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-Canadian 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 dysmorphism, 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*

Received March 1, 1990; final revision received August 6, 1990.

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

© 1990 by The American Society of Human Genetics. All rights reserved. 0002-9297/90/4706-0007\$02.00

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, H10-11, 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 μ M.

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 *Hind*III and OX174 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 cm² 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 *Hind*III 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 *Hinc*II-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 DH5 α competent cells were transformed with the ligation products and plated on LB plates containing 100 μ g 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-Canadian 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-Canadian 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.

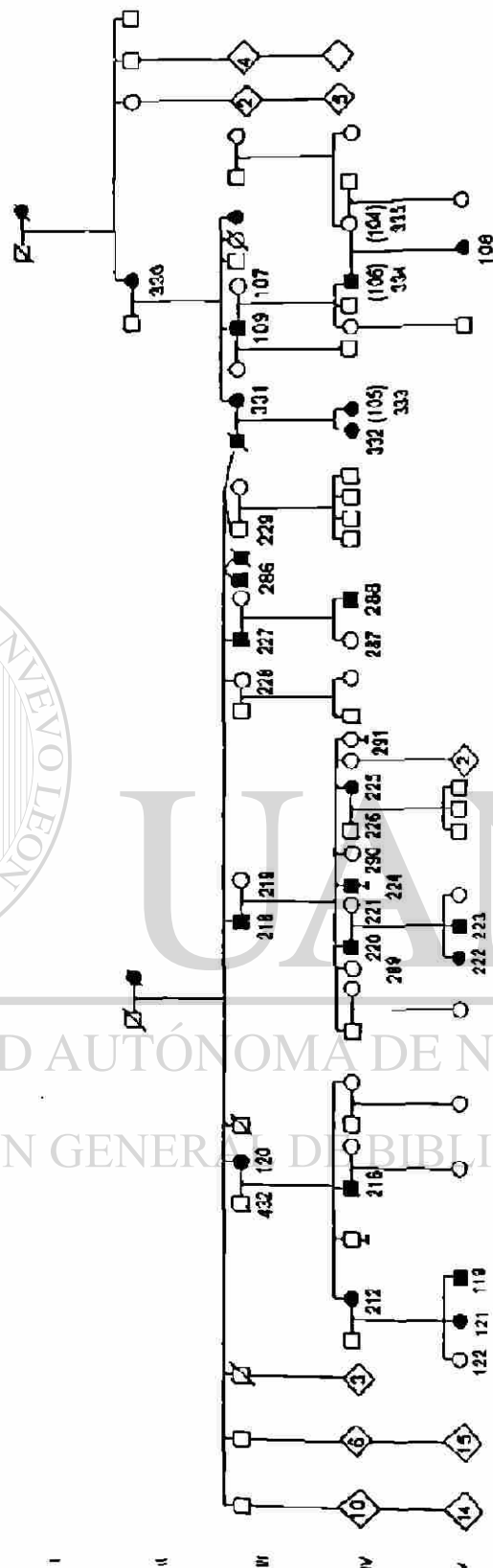
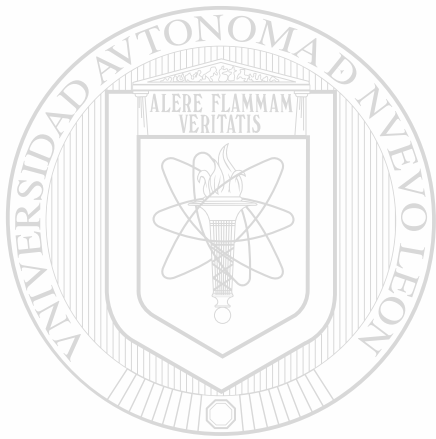


Figure 1 French-Canadian pedigree 42, segregating CMT in an autosomal dominant fashion. □ and ○ = unaffected male and female, respectively; ■ and ● = affected male and female, respectively; ◇ = unaffected progeny, whose the enclosed number is the number of individuals. The numbers under the symbols refer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

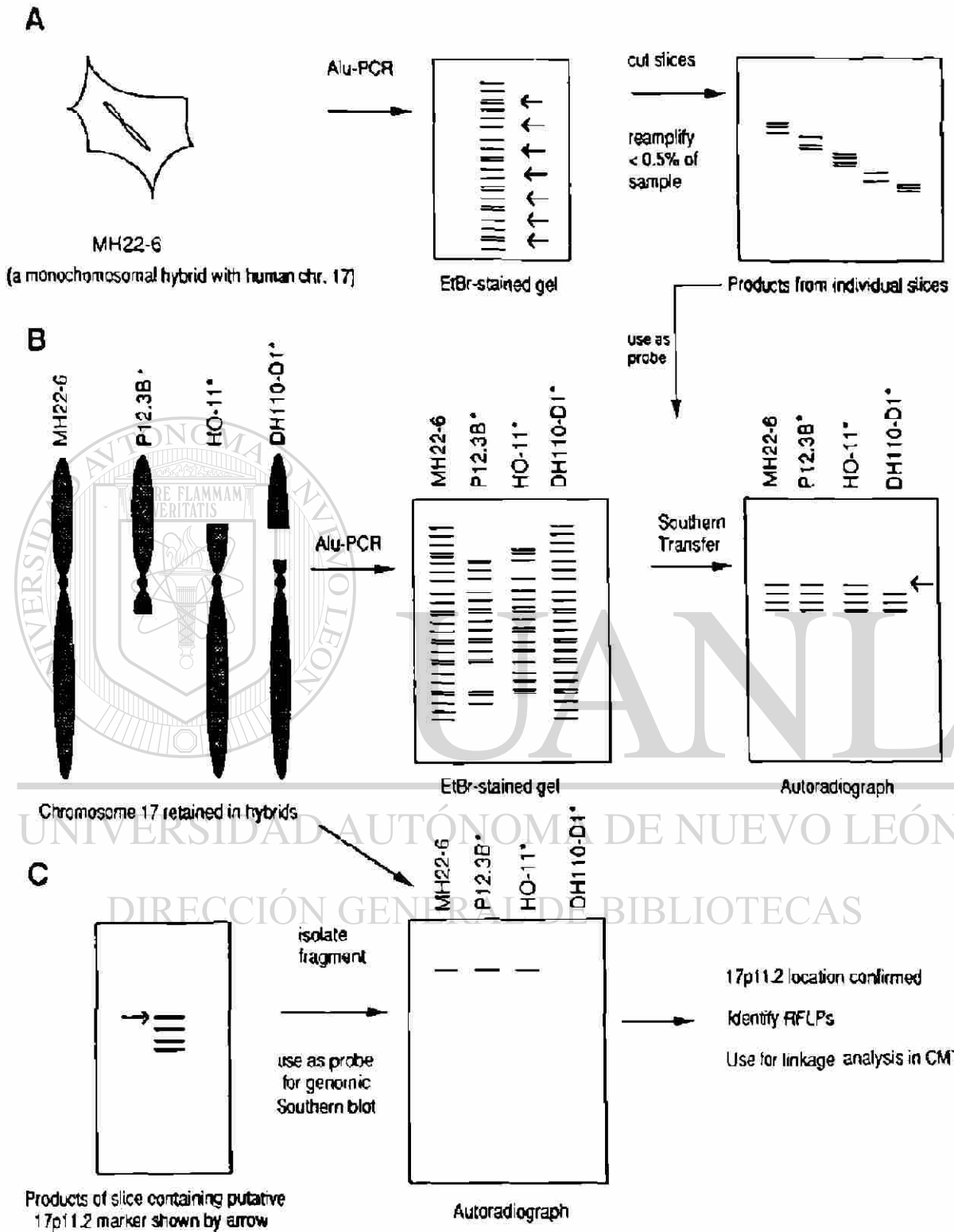


Figure 2 Schematic diagram of method used for isolation of markers from 17p11.2. The hybrids are described in Material and Methods. Hybrids marked with an asterisk retain the indicated derivative chromosome 17 in addition to other human chromosomes.

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 μ l (<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 3A, 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 3B. 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.

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 *Ap*I, *Av*II, *Bam*HI, *Ban*I, *Ban*II, *Bcl*I, *Bgl*I, *Bgl*II, *Dra*I, *Eco*RI, *Eco*RV, *Hin*CI, *Hind*III, *Hin*FI, *Msp*I, *Mbo*I, *Pst*I, *Pvu*II, *Rsa*I, *Sc*AI, *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

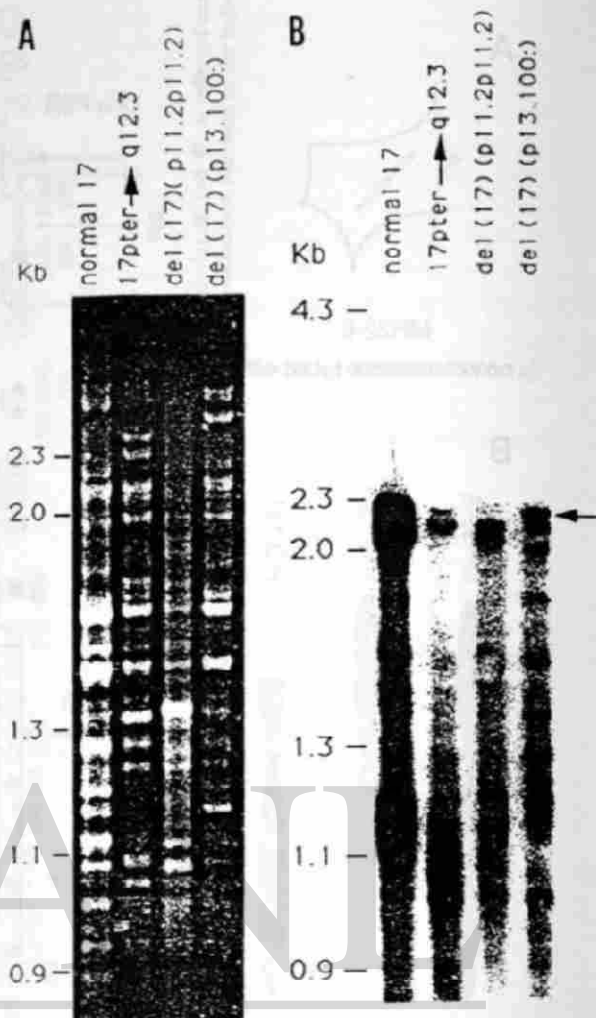


Figure 3 Southern analysis of *Alu*-PCR products from hybrids MH22-6, P12.3B, DH110-D1, and HO-11. One-half microgram from each of these hybrids was amplified with primer TC-65 as described in Material and Methods. Ten percent of the products were electrophoresed in a 1.2% agarose gel, and a Southern 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.

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

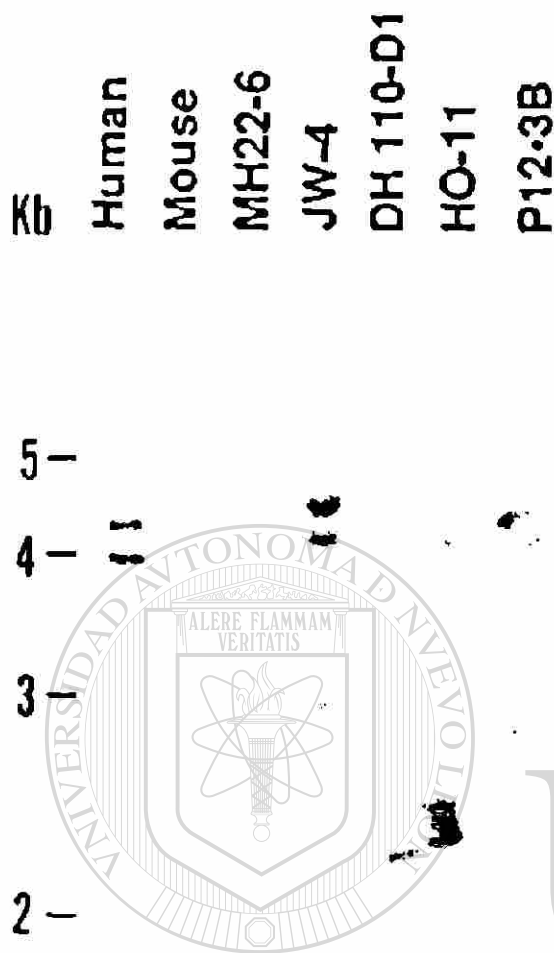


Figure 4 Southern analysis of genomic DNA with *Alu*-PCR product, S6.1. Two and one-half micrograms of human DNA, 5 μ g of mouse (clone 1D) DNA, and 10 μ g of DNA from the somatic cell hybrids MH22-6, JW-4, DH110-D1, HO-11, and P12.3B were digested with *Eco*RI and hybridized with S6.1, which is the fragment identified as missing in the hybrid DH110-D1 as shown in fig. 3B. 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-Canadian Kindred Which Segregates CMT 1A

We have previously reported a French-Canadian 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-Canadian 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 $Z = 2.33$ at $\theta = 0$, suggesting that pedigree 42 segregates CMT 1A.

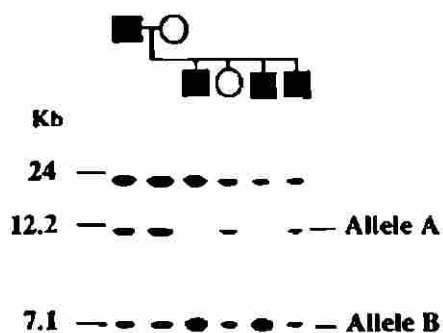
Linkage Analysis of Pedigrees 1 and 42 with Marker S6.1

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 ($\theta = .12$) 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)

A *Sst*I Polymorphism



B *Msp*I Polymorphism

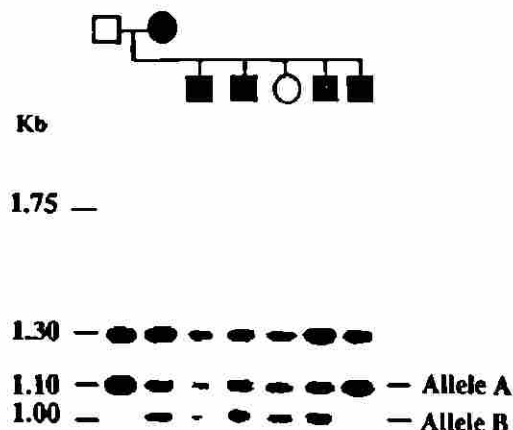


Figure 5 Mendelian segregation of RFLPs revealed by enzymes *Sst*I (A) and *Msp*I (B). A cloned subfragment of the marker S6.1 designated S6.1-HB2 (see Material and Methods) was used for Southern analysis. The variant fragments are indicated as allele A and allele B, respectively. The *Sst*I pattern obtained with S6.1-HB2 is identical to that seen with S6.1. The *Msp*I pattern obtained with S6.1-HB2 is simpler and allows distinction of overlapping *Msp*I fragments. Occasionally a strongly hybridizing 1.75-kb *Msp*I fragment was seen with this probe, but it could never be conclusively established whether this represented a variant allele of the 1.3-kb *Msp*I 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	0	2
A10-41 (<i>Msp</i>I):									
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: <i>Hind</i>III and <i>Msp</i>I:									
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 (<i>Taq</i>I):									
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 (<i>Sst</i>I and <i>Msp</i>I):									
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 (*Hind*III), and EW301 in pedigree 1 were published by Patel et al. (1990); the remaining results are from the present study.

^b *Hind*III 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, *Av*alI, *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.

Acknowledgments

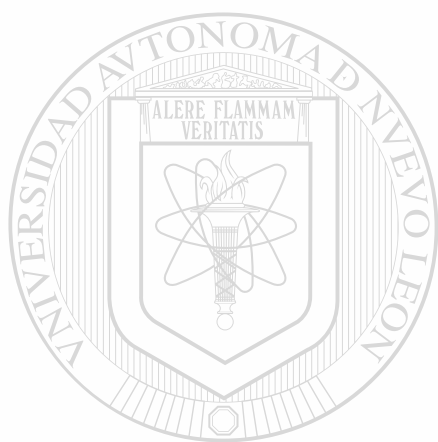
We thank Susie Ledbetter for valuable discussions and for sharing unpublished data which enabled us to devise the probe isolation method. We are indebted to David Ledbetter for providing all somatic cell hybrids used in this study. We thank Robert Wright, Stewart Davis, and Velma Holiday for technical assistance. We thank David Nelson, Rick Myers, and David and Susie Ledbetter for critically reading the manuscript. We thank Linda Haway for preparation of the manuscript. This work was supported in part by a Muscular Dystrophy Association (MDA) Task Force on Genetics grant, an Advanced

Technology Program grant from the state of Texas, Baylor Meatal Retardation Center grant HD24064-02, NIH grant R01 NS-27042 to J.R.L. and P.I.P., and by NIH grants GM33771 and HD00774 (R.C.D.A.) to A.C. B.F. is a recipient of an MDA postdoctoral fellowship.

References

- Anderson MA, Gusella JF (1984) The use of cyclosporin A in establishing EBV-transformed human lymphoblastoid cell lines. *In Vitro* 20:856-858
- Ballabio A, Bardoni B, Guioli S, Basler E, Camerino G. Two families of low copy number repeats are interspersed on Xp22.3: implications for the high frequency of deletions in this region. *Genomics* (in press)
- Bird TD, Ott J, Giblett ER (1982) Evidence for linkage of Charcot-Marie-Tooth neuropathy to the Duffy locus on chromosome 1. *Am J Hum Genet* 34:388-394
- Charcot JM, Marie P (1886) Sur une forme particulière d'atrophie musculaire progressive souvent familiale debutant par les pieds et les jauntes et atteignant plus tard les mains. *Rev Med* 6:97-138
- Franco B, Rincon-Limas D, Nakamura Y, Patel PI, Lupski JR. Identification of an *Msp*I RFLP at the D17258 locus on 17p11.2 (submitted)
- Ledbetter SA, Nelson KL, Warren ST, Ledbetter DH (1990) Rapid isolation of DNA probes within specific chromosome regions by interspersed repetitive sequence (IRS) PCR. *Genomics* 6:475-481
- McKusick VA (1988) Mendelian inheritance in man, 8th ed. Johns Hopkins University Press, Baltimore and London
- Mead K, Szczesna-Skorupa E, Kenyer B (1986) Single-stranded DNA "blue" T7 promoter plasmids: a versatile tandem promoter system for cloning and protein engineering. *Protein Eng* 1:67-74
- Middleton-Price HR, Harding AE, Montiero C, Berciano J, Malcolm S (1990) Linkage of hereditary motor and sensory neuropathy type I to the pericentromeric region of chromosome 17. *Am J Hum Genet* 46:92-94
- Miller SA, Dykes DD, Polesky HF (1988) A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 16:1215
- Nelson KL, Ledbetter SA, Corbo L, Victoria MF, Ramirez-Solis R, Webster TD, Ledbetter DH, et al (1989) *Alu* polymerase chain reaction: a method for rapid isolation of human-specific sequences from complex DNA sources. *Proc Natl Acad Sci USA* 86:6686-6690
- Patel PI, Franco B, Garcia C, Slaugenhaupt SA, Nakamura Y, Ledbetter DH, Chakravarti A, et al (1990) Genetic mapping of autosomal dominant Charcot-Marie-Tooth disease in a large French-Canadian kindred: identification of new linked markers on chromosome 17. *Am J Hum Genet* 46:801-809
- Raeymaekers P, Timmerman V, De Jonghe P, Swerts L, Cheuens J, Martin J-J, Muylle L, et al (1989) Localization

- of the mutation in an extended family with Charcot-Marie-Tooth neuropathy (HMSN I). *Am J Hum Genet* 45:953-958
- Smith ACM, McGavran L, Robinson J, Waldstein G, Macfarlane J, Zonona J, Reiss M, et al (1986) Interstitial deletion of (17)(p11.2p11.2) in nine patients. *Am J Med Genet* 24:393-414
- Stratton RF, Dobyns WB, Greenberg F, DeSana JB, Moore C, Fidone G, Runge GH, et al (1986) Interstitial deletion of (17)(p11.2p11.2): report of six additional patients with a new chromosome deletion syndrome. *Am J Med Genet* 24:421-432
- Vance JM, Nicholson GA, Yamaoka LH, Stajich J, Stewart CS, Speer MC, Hung W-Y, et al (1989) Linkage of Charcot-Marie-Tooth neuropathy type 1a to chromosome 17. *Exp Neurol* 104:186-189
- vanTuinen P, Dobyns WB, Rich DC, Summers KM, Robinson TJ, Nakamura Y, Ledbetter DH (1988) Molecular detection of microscopic and submicroscopic deletions associated with Miller-Dieker syndrome. *Am J Hum Genet* 43:587-596
- vanTuinen P, Rich DC, Summers KM, Ledbetter DH (1987) Regional mapping panel for human chromosome 17: application to neurofibromatosis type 1. *Genomics* 1:374-381



UANL

UNIVERSIDAD AUTÓNOMA DE NUEVO LEÓN

DIRECCIÓN GENERAL DE BIBLIOTECAS



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 J. PATEL*¹

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

Received June 11, 1990; revised August 24, 1990

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. © 1991 Academic Press, Inc.

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 L1s (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

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

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 μ g 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 μ l 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 μ l with 1 μ g of DNA, primer TC-65 at 1 μ M, or primer 517 at 0.1 μ M in 50 mM KCl/10 mM Tris-HCl, pH 8.0/1.5 mM MgCl₂/0.01% 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 thermalcycler (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 μ l 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 DH5 α competent cells were transformed with the ligation products and plated on LB plates containing 100 μ g/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 μ g of DNA from each hybrid and 5 μ g of human and mouse DNA were digested for >4 h with 3–4 units/ μ g 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.

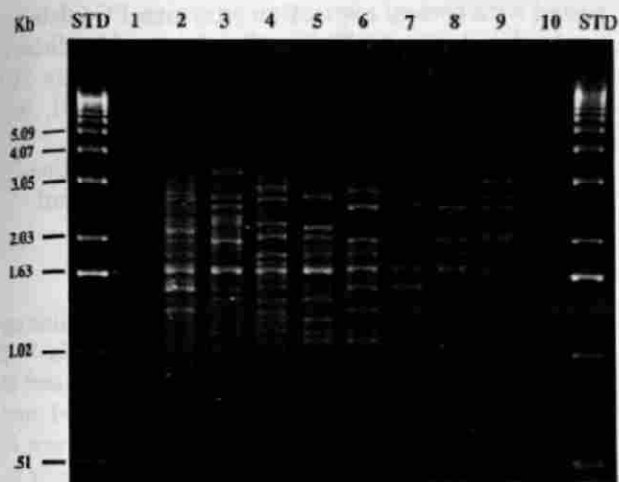


FIG. 1. Simplification of the *Alu*-PCR pattern of the hybrid DH110-D1. One microgram of DNA from the rodent cell line clone 1D and from the hybrid DH110-D1, either uncut or digested with several restriction endonucleases, was subjected to PCR with the *Alu* primer TC-65 and electrophoresed as described 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 *EcoRI* + *BglI*, *TaqI*, *AvaII*, *EcoRI* + *PstI*, *EcoRI* + *PstI* + *BamHI*, *BclI* + *PvuII*, *BclI* + *BglII*, and *RsaI*, 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 *BamHI*, *BclI*, *BglII*, *DraI*, *HindIII*, *PstI*, *RsaI*, *SstI*, or *TaqI*. FG3 detected an insertion-deletion polymorphism discernible with the enzymes *BclI*, *TaqI*, *PstI*, and *SstI* 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

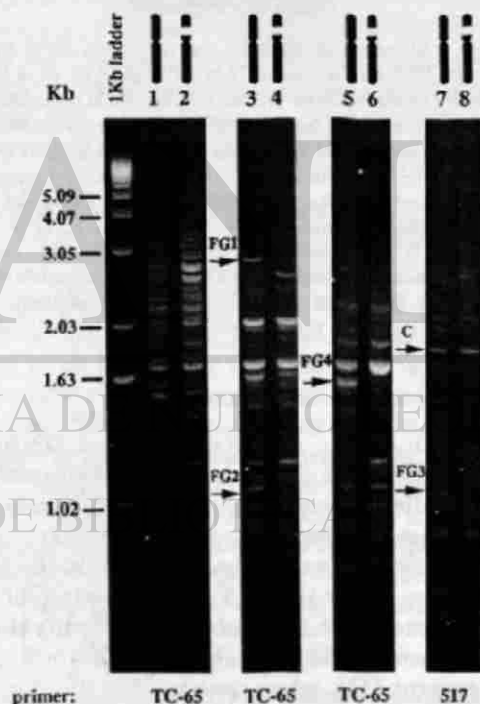


FIG. 2. Comparative restricted *Alu*-PCR of the chromosome 17-retaining hybrids MH22-6 and DH110-D1. Lanes 1, 3, 5, 7 and 2, 4, 6, 8 represent the amplification products from hybrids MH22-6 and DH110-D1, respectively. The status of the template in each lane is: lanes 1 and 2, uncut; lanes 3 and 4, digested with the enzymes *BclI*, *PstI*, and *EcoRI*; lanes 5 and 6, digested with the enzyme *AvaII*; lanes 7 and 8, digested with the enzymes *PstI* and *EcoRI*. 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.

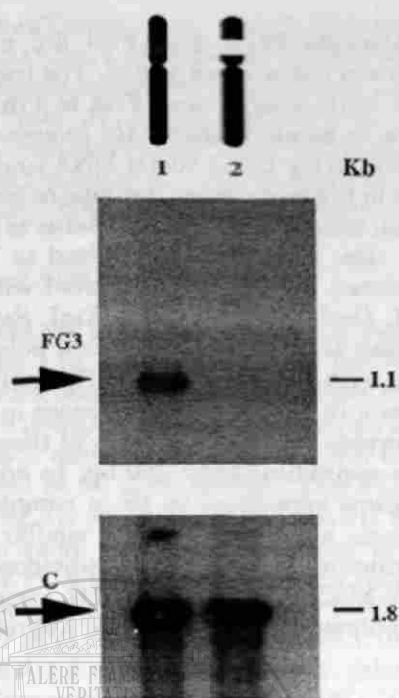


FIG. 3. Absence of FG3 in the total *Alu*-PCR product of DH110-D1. FG3 and a control *Alu*-PCR product were hybridized sequentially to a Southern blot of the *Alu*-PCR products obtained with 1 μ g of undigested template DNAs from hybrids MH22-6 and DH110-D1. Lanes 1 and 2 represent the total *Alu*-PCR 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 di-

gested 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, *Hind*III, *Hinf*I, *Msp*I, *Pst*I, *Pvu*II, *Rsa*I, or *Sst*I. FG2 was not polymorphic for *Bam*HI, *Bcl*I, *Bgl*II, *Eco*RI, *Hind*III, *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

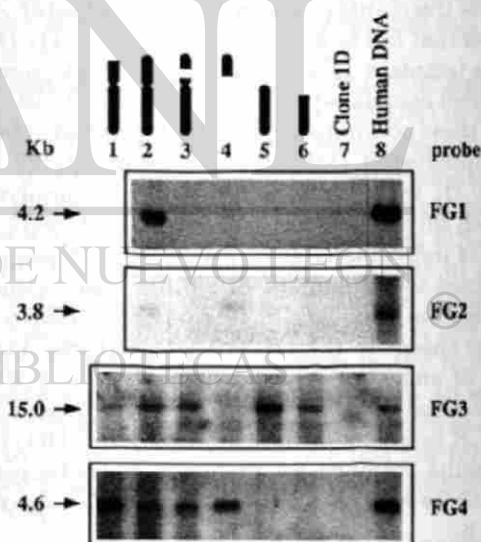


FIG. 4. Hybridization of fragments FG1, FG2, FG3, and FG4 to Southern blots of a somatic cell hybrid mapping panel. For ease of interpretation, hybrid lanes are identified by schematic idiograms representing the portions of chromosome 17 retained in the hybrids. Lanes 1-6, 10 μ g of genomic DNA from the hybrids HO-11, MH22-6, DH110-D1, 88H5, LS-1, and SP3, respectively; lane 7, 10 μ g of DNA from the parental mouse cell line, Clone 1D; lane 8, 5 μ g 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 *Hind*III-digested DNAs. FG1 and FG2 map to 17p11.2, FG3 maps to 17q11.2-qter, and FG4 maps to 17p12.

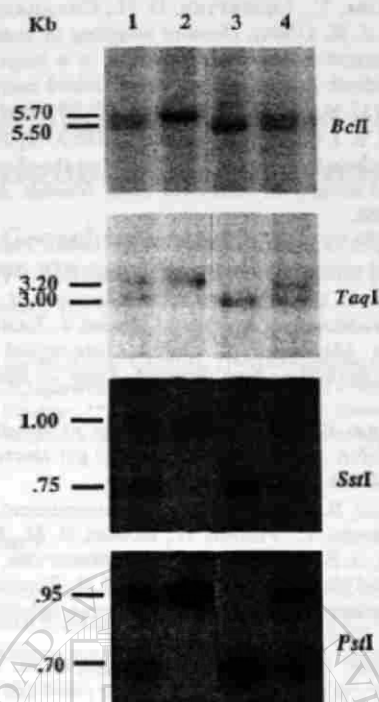


FIG. 5. Insertion-deletion polymorphism detected by FG3. Lanes 1-4 represent DNA from each of the same four unrelated individuals digested with the enzymes indicated on the right. The size of the alleles in kilobases (kb) is shown on the left and indicates that the polymorphic alleles in each case differ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

morphic 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, *Hinf*I, *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; Zuliiani 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.

ACKNOWLEDGMENTS

We thank David Ledbetter for providing all hybrids used in this study. We thank David Nelson, Huda Zoghbi, Andrea Ballabio, Richard Gibbs, Susie Ledbetter, and Robert Wright for critically reviewing the manuscript. We thank Velma Holliday for technical assistance. This research was supported by a Muscular Dystrophy Association Task Force on Genetics grant, Baylor Mental Retardation Research Center Grant HD 24064-02, an Advanced Technology Program grant from the state of Texas, and NIH Grant RO1 NS-27042 to J.R.L. and P.I.P. V.G. is a recipient of a postdoctoral fellowship from the Muscular Dystrophy Association.

REFERENCES

1. ANAGNOU, N. P., O'BRIEN, S. J., SHIMADA, T., NASH, W. G., MANN-JY, C., AND NIENHUIS, A. W. (1984). Chromosomal

- organization of the human dihydrofolate reductase genes: Dispersion, selective amplification, and a novel form of polymorphism. *Proc. Natl. Acad. Sci. USA* 81: 5170-5174.
2. COTTER, F. E., HAMPTON, G. M., NASIPURI, S., BODMER, W. F., AND YOUNG, B. D. (1990). Rapid isolation of human chromosome-specific DNA probes from a somatic cell hybrid. *Genomics* 7:257-263.
 3. DRMANAC, R., PETROVIC, N., GLISIN, V., AND CRKVENJAKOV, R. (1986). A calculation of fragment lengths obtainable from human DNA with 78 restriction enzymes: An aid for cloning and mapping. *Nucleic Acids Res.* 14: 4691.
 4. ECONOMOU, E. P., BERGEN, A. W., WARREN, A. C., AND ANTONARAKIS, S. E. (1990). The polydeoxyadenylate tract of Alu repetitive elements is polymorphic in the human genome. *Proc. Natl. Acad. Sci. USA* 87: 2951-2954.
 5. ELDER, F. F. B., NICHOLS, M. M., HOOD, O. J., AND HARRISON, W. R. (1985). Unbalanced translocation (15;17)(q13;p13.3) with apparent Prader-Willi syndrome but without Miller-Dieker syndrome. *Amer. J. Med. Genet.* 20: 519-524.
 6. FROSSARD, P. M., COLEMAN, R. T., PROTTER, A. A., SEILHAMER, J. J., FUNKE, H., AND ASSMANN, G. (1986). Deletion polymorphism 5' to the human apolipoprotein AI (apo AI) gene. *Nucleic Acids Res.* 14: 8694.
 7. LEDBETTER, S. A., NELSON, D. L., WARREN, S. T., AND LEDBETTER, D. H. (1990). Rapid isolation of DNA probes within specific chromosome regions by interspersed repetitive sequence polymerase chain reaction. *Genomics* 6: 475-481.
 8. MANIATIS, T., FRITSCH, E. F., AND SAMBROOK, J. (1982). "Molecular Cloning: A Laboratory Manual," Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
 9. MRAD, K., SZCZESNA-SKORUPA, E., AND KENYER, B. (1986). Single-stranded DNA "blue" T7 promoter plasmids: A versatile tandem promoter system for cloning and protein engineering. *Protein Eng.* 1: 67-74.
 10. NELSON, D. L., LEDBETTER, S. A., CORBO, L., VICTORIA, M. F., RAMIREZ-SOLIS, R., WEBSTER, T. D., LEDBETTER, D. H., AND CASKEY, C. T. (1989). Alu polymerase chain reaction: A method for rapid isolation of human-specific sequences from complex DNA sources. *Proc. Natl. Acad. Sci. USA* 86: 6686-6690.
 11. PATEL, P. I., FRANCO, B., GARCIA, C., SLAUGENHAUPT, A., NAKAMURA, Y., LEDBETTER, D. H., CHAKRAVARTI, A., AND LUTPSKI, J. R. (1990). Genetic mapping of autosomal dominant Charcot-Marie-Tooth disease in a large French-Canadian kindred: Identification of new linked markers on chromosome 17. *Amer. J. Hum. Genet.* 48: 801-809.
 12. ROOBY, E. I. (1989). Simple human DNA-repeats associated with genomic hypervariability, flanking the genomic retroposons and similar to retroviral sites. *Nucleic Acids Res.* 18: 1873-1885.
 13. SCHMID, C. W., AND JELINEK, W. R. (1982). The Alu family of dispersed repetitive sequences. *Science* 216: 1066-1070.
 14. SMITH, A. C. M., MCGAVRAN, L., ROBINSON, J., WALDBER, G., MACFARLANE, J., ZONONA, J., REISS, J., LAHR, M., ALLEN, L., AND MAGENIS, E. (1986). Interstitial deletion of (17)(p11.2p11.2) in nine patients. *Amer. J. Med. Genet.* 24: 393-414.
 15. SOUTHERN, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98: 503-517.
 16. STRATTON, R. F., DOBYNS, W. B., GREENBERG, F., DESANA, J. B., MOORE, C., FIDONE, G., RUNGE, G. H., FELDMAN, P., SEKHON, G. S., PAULI, R. M., AND LEDBETTER, D. H. (1986). Interstitial deletion of (17)(p11.1p11.2): Report of six additional patients with a new chromosome deletion syndrome. *Amer. J. Med. Genet.* 24: 421-432.
 17. VAN TUUNEN, P., RICH, D. C., SUMMERS, K. M., AND LEDBETTER, D. H. (1987). Regional mapping panel for human chromosome 17: Application to neurofibromatosis type I. *Genomics* 1: 374-381.
 18. VAN TUUNEN, P., DOBYNS, W. B., RICH, D. C., SUMMERS, K. M., ROBINSON, T. J., NAKAMURA, Y., AND LEDBETTER, D. H. (1988). Molecular detection of microscopic and submicroscopic deletions associated with Miller-Dieker syndrome. *Amer. J. Hum. Genet.* 43: 587-596.
 19. WOODS-SAMUELS, P., WONG, C., MATHIAS, S. L., SCOTT, A. F., KAZAZIAN, H. H., JR., AND ANTONARAKIS, S. E. (1989). Characterization of a nondeleterious L1 insertion in an intron of the human factor VIII gene and further evidence of open reading frames in functional L1 elements. *Genomics* 4: 290-296.
 20. ZULLANI, G., AND HOBBS, H. H. (1990). A high frequency of length polymorphisms in repeated sequences adjacent to Alu sequences. *Amer. J. Hum. Genet.* 46: 963-969.

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 polyembolokoilomania, 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 segregat-

ing 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-

Received May 30, 1991; revision received August 9, 1991.
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.
© 1991 by The American Society of Human Genetics. All rights reserved.
0002-9297/91/4906-0008\$02.00

cardation (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. 1990a, 1990b). 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. 1990a, 1990b). 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 TaqI fragment containing a (GT)₁₅ 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	<i>TaqI</i>	4.5/3.1	Barker et al. 1987
		<i>BglII</i>	10.0/8.0	Barker et al. 1987
FG1 (D17S446)	17p11.2	<i>ApaI</i>	12.0/7.5	Guzzetta et al. 1990
pYNM67-R5 (D17S29)	17p11.2	<i>TaqI</i>	3.4/2.0 + 1.3	Ray et al. 1990
		<i>BglII</i>	8.1/6.7	Ray et al. 1990
c1516 (D17S258)	17p11.2	<i>HindIII</i>	22.0/12 + 10	Patel et al. 1990a
p1516-R4 (D17S258)	17p11.2	<i>MspI</i>	3.3/2.4	Franco et al. 1990
pA10-41 (D17S71)	17p11.2	<i>MspI</i>	2.4/1.9	Barker et al. 1987
		<i>PvuII</i>	3.2/3.0	Barker et al. 1987
pS6.1-HB2 (D17S445)	17p11.2	<i>MspI</i>	1.7/1.3	Present study
			1.1/1.0	Patel et al. 1990b
		<i>SstI</i>	12.2/7.1	Patel et al. 1990b
VAW409R1 (D17S122)	17p11.2-p12	<i>MspI</i>	5.3/2.7 + 2.6	Wright et al. 1990
VAW409R3 (D17S122)	17p11.2-p12	<i>MspI</i>	2.8/2.7/1.9	Wright et al. 1990
VAW412R3 (D17S125)	17p11.2-p12	<i>MspI</i>	10.5/5.4	Wright et al. 1990
EW401 (D17S61)	17p11.2-p12	<i>MspI</i>	5.2/4.4	Wright et al. 1990
c1517 (D17S259)	17p11.2-p12	<i>MspI</i>	6.2/4.0/2.4	Patel et al. 1990a
VAW410R1 (D17S123)	17p11.2-p12	<i>BglII</i>	2.1/2.0	Wright et al. 1990
		<i>TaqI</i>	10.0/9.4	Wright et al. 1990
EW405 (D17S121)	17p11.2-p12	<i>MspI</i>	2.0/1.5	Wright et al. 1990
VAW411R2 (D17S124)	17p11.2-p12	<i>MspI</i>	10.5/6.1	Wright et al. 1990
		<i>BglII</i>	11.0/10.7	Wright et al. 1990
EW403 (D17S63)	17p11.2-p12	<i>MspI</i>	13.5/6.8	Wright et al. 1990
EW503 (D17S67)	17p11.2-p12	<i>MspI</i>	6.9/5.7	Wright et al. 1990
EW502 (D17S66)	17p11.2-p12	<i>BglII</i>	2.2/1.4	Wright et al. 1990

als). 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-ATGCTGAACAC-3', while that of the priming oligodeoxynucleotide on the CA strand of pRM7-GT is 5'-CTTGGTGAAACGCTGCTGTAC-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. 1990a; 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 chro-

mosome 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 [^γ³²P]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 μ l 10 \times PCR buffer (500 mM KCl, 120 mM Tris HCl [pH 8.0], 1.5 mM MgCl₂, and 0.01% gelatin), 0.63 units of AmpliTaq (Cetus) DNA polymerase, and 0.4 μ l 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 μ l) were mixed with 2 μ l 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 \pm 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 polyembolokoilomania (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. Al-

though 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* (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	13/23 (65)	51
Possible peripheral neuropathy ...	NE	4/5	13/26 (50)	55
Self-destructive behavior	73	5/6	15/24 (63)	70

* NE = not specifically examined for in earlier studies (i.e., Smith et al. 1982, 1986; Patil and Bartley 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)(p25.3;p11.1) 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-trans-

formed 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

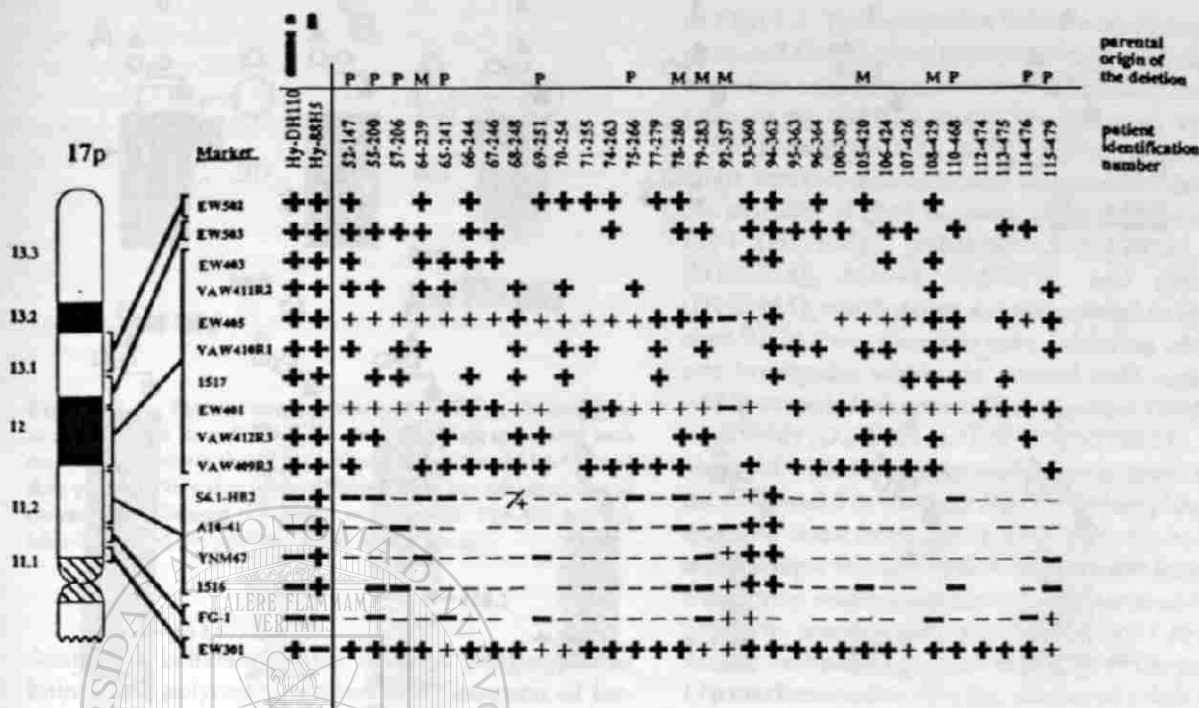


Figure 1 Deletion status of 17p DNA markers in SMS patients. An idiogram illustrating both the short arm of chromosome 17 and the relative position of individual markers is shown on the left. Marker order, from VAW409R3 distal, is taken from the published genetic map of Wright *et al.* (1990), except 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 S6.1-HB2, 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-

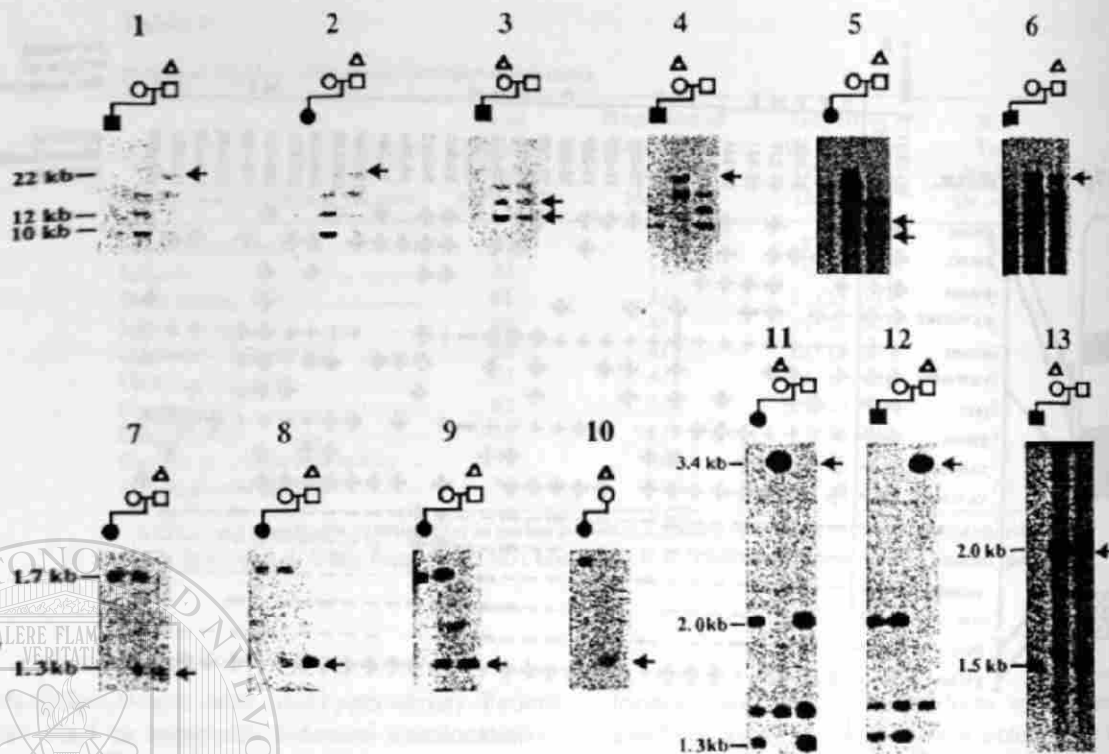


Figure 2 Parental origin of deletion in SMS patients. Genomic DNA from 13 SMS patients and their parent(s) was digested with the indicated restriction endonuclease and subjected to Southern analysis with the indicated probe. Panels 1–6, *Hind*III-digested DNA from patients 55-200, 57-206, 79-283, 105-420, 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-

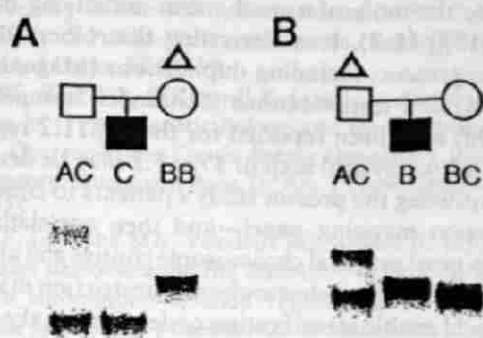


Figure 3 Parental origin determined by $(GT)_n$ polymorphism at the D17S446 locus. The $(GT)_n$ polymorphism associated with the D17S446 locus is shown for patients 108-429 and 114-476 and their parents. Note that patient 108-429 has not inherited one of the maternal alleles at this locus, while patient 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. 1990a, 1990b). 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. 1991a). CMT1A is characterized electrophysiologically by decreased nerve-conduction

velocity (Kimura 1989; Lupski et al. 1991a). 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 17p, a duplication which appears to encompass VAW409R3, VAW-412R3, and EW401 (Lupski et al. 1991b). 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 (Schrandner-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.

Acknowledgments

We are grateful to the patients and families for their cooperation in this study. We are indebted to the following individuals for evaluation and referral of individual patients and specimens: J. Allanson, J. Carey, P. Chance, E. Hoyme, R. Laxova, M. E. Pierpont, L. Stampien, T. Tarby, B. Woodis, and J. Zonana. We thank the following cytogeneticists for cytogenetic analysis on individual patients: H. Boyer, D. H. Ledbetter, D. Lockwood, L. McGarran, and C. G. Palmer. We thank D. Barker and Y. Nakamura for providing probes. We are grateful to Professor J. Ambrose of Bowdoin College and Dr. R. A. Lewis of Baylor College of Medicine for the derivation of the term "polyembolukoilomania." We also thank L. G. Haway and R. L. Tanagho for manuscript preparation. This work was supported in part by a Muscular Dystrophy Association (MDA) Task Force on Genetics grant, by an Advanced Technology Program grant from the State of Texas, by NIH grant R01 NS-27042 (all to J.R.L. and P.I.P.), by Baylor Mental Retardation Research grant HD24064-02, and by the Texas Children's Hospital General Clinical Research Center (NIH grant M01 RR-00188). V.G. is a recipient of an MDA postdoctoral fellowship. J.R.L. also acknowledges support from the PEW Scholars Program in Biomedical Sciences.

References

- Ballabio A, Bardoni B, Carrozzo R, Andria G, Bick D, Campbell L, Hamel B, et al (1989) Contiguous gene syndromes due to deletions in the distal short arm of the human X-chromosome. *Proc Natl Acad Sci USA* 86: 10001-10005
- Barker D, Wright E, Nguyen K, Cannon L, Fain P, Goldgar D, Bishop DT, et al (1987) Gene for von Recklinghausen neurofibromatosis is in the pericentromeric region of chromosome 17. *Science* 236:1100-1102

- Burd L, Veseley B, Martsolf J, Kerbeshian J (1990) Prevalence study of Prader-Willi syndrome in North Dakota. *Am J Med Genet* 37:97-99
- Chance PF, Bird TD, O'Connell P, Lipe H, Lalouel J-M, Leppert M (1990) Genetic linkage and heterogeneity in type I Charcot-Marie-Tooth disease (hereditary motor and sensory neuropathy type I). *Am J Hum Genet* 47:915-925
- Colley AF, Leversha MA, Voullaire LE, Rogers JG (1990) Five cases demonstrating the distinctive behavioral features of chromosome deletion 17(p11.2p11.2) (Smith-Magenis syndrome). *J Paediatr Child Health* 26:17-21
- Colver GB (1987) Onychotillomania. *Br J Dermatol* 117:397-399
- Dobyns WB, Curry CJR, Hayme HE, Turlington L, Ledbetter DH (1991) Clinical and molecular diagnosis of Miller-Dieker syndrome. *Am J Hum Genet* 48:584-594
- Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* 132:6-13
- Francke U, Harper JF, Darras BT, Cowan JM, McCabe ERB, Kohlschütter A, Seltzer WK, et al (1987) Congenital adrenal hypoplasia, myopathy, and glycerol kinase deficiency: molecular genetic evidence for deletions. *Am J Hum Genet* 40:212-227
- Franco B, Lai LW, Patterson D, Ledbetter DH, Trask BJ, van den Engh G, Iannaccone S, et al (1991) Molecular characterization of a patient with del(1)(q23-q25). *Hum Genet* 87:269-277
- Franco B, Rincon-Limas D, Nakamura Y, Patel PI, Lupski JR (1990) An *MspI* RFLP at the D17S258 locus. *Nucleic Acids Res* 18:7196
- Guzzetta V, Montes de Oca-Luna R, Lupski JR, Patel PI (1991) Isolation of region-specific and polymorphic markers from chromosome 17 by restricted *Alu*-polymerase chain reaction. *Genomics* 9:31-36
- Hall JG (1990) Genomic imprinting: review and relevance to human diseases. *Am J Hum Genet* 46:857-873
- Hamill MA, Roberts SH, Maguire MJ, Laurence KM (1990) Interstitial deletion of 17p11.2: case report and review. *Ann Genet* 31:36-38
- Hobson JA (1990) Sleep and dreaming. *J Neurosci* 10:371-382
- Ivens A, Havin N, Williamson R, Dixon M, Bates G, Buckingham M, Robert B (1990) The human homeobox gene *Hox7* maps to chromosome 4p16.1 and may be implicated in Wolf-Hirschhorn syndrome. *Hum Genet* 84:473-476
- Kimura J (1989) *Electrodiagnosis in diseases of nerves and muscle: principles and practice*, 2d ed. FA Davis, Philadelphia
- Ledbetter DH, Cavenee WK (1989) Molecular cytogenetics: interface of cytogenetics and monogenic disorders. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) *The metabolic basis of inherited diseases*, 6th ed. McGraw Hill, New York, pp 343-371
- Lockwood D, Hecht F, Dowman C, Hecht BK, Rizkallah TH, Goodwin TM, Allanson J (1988) Chromosome sub-band 17p11.2 deletion: a minute deletion syndrome. *J Med Genet* 25:732-737
- Lupski JR, Garcia CA, Parry GJ, Patel PI (1991a) Charcot-Marie-Tooth polyneuropathy syndrome: clinical, electrophysiological, and genetic aspects. In: Appel S (ed) *Current neurology*. Mosby-Yearbook, Chicago, pp 1-25
- Lupski JR, Montes de Oca-Luna R, Slaugenhaupt S, Pentao L, Guzzetta V, Trask BJ, Saucedo-Cardenas O, et al (1991b) DNA duplication associated with Charcot-Marie-Tooth disease type 1A. *Cell* 66:219-232
- McAlpine PJ, Feasby TE, Hahn AF, Komarnicki L, James S, Guy C, Dixon M, et al (1990) Localization of a locus for Charcot-Marie-Tooth neuropathy type 1a (CMT 1A) to chromosome 17. *Genomics* 7:408-415
- Magenis RE, Brown MG, Allen L, Reiss J (1986) De novo partial duplication of 17p [dup(17)(p12→p11.2)]: clinical report. *Am J Med Genet* 24:415-420
- Middleton-Price HR, Harding AE, Monteiro C, Berciano J, Malcolm S (1990) Linkage of hereditary motor and sensory neuropathy type I to the pericentromeric region of chromosome 17. *Am J Hum Genet* 46:92-94
- Nakamura Y, Leppert M, O'Connell P, Wolff R, Holm T, Culver M, Martin C, et al (1987) Variable number of tandem repeat (VNTR) markers for human gene mapping. *Science* 235:1616-1622
- Nicholls RD, Knoll JHM, Butler MG, Karam S, Lalande M (1989) Genetic imprinting suggested by maternal heterodisomy in non-deletion Prader-Willi syndrome. *Nature* 342:281-285
- Niebuhr E (1978) The cri-du-chat syndrome: epidemiology, cytogenetics and clinical features. *Hum Genet* 44:227-275
- Overhauser J, Bengtsson U, McMahon J, Ulm J, Butler MG, Santiago L, Wasmuth JJ (1989) Prenatal diagnosis and carrier detection of a cryptic translocation by using DNA markers from the short arm of chromosome 5. *Am J Hum Genet* 45:296-303
- Patel PI, Franco B, Garcia C, Slaugenhaupt SA, Nakamura Y, Ledbetter DH, Chakravarti A, et al (1990a) Genetic mapping of autosomal dominant Charcot-Marie-Tooth disease in a large French-Canadian kindred: identification of new linked markers on chromosome 17. *Am J Hum Genet* 46:801-809
- Patel PI, Garcia C, Montes de Oca-Luna R, Malamut RI, Franco B, Slaugenhaupt S, Chakravarti A, et al (1990b) Isolation of a marker linked to the Charcot-Marie-Tooth disease type 1A gene by differential *Alu*-PCR of human chromosome 17-retaining hybrids. *Am J Hum Genet* 47:926-934
- Patil SR, Bartley JA (1984) Interstitial deletion of the short arm of chromosome 17. *Hum Genet* 67:237-238
- Popp DW, Johnson CP, Stratton RF (1987) An additional case of deletion 17p11.2. *Am J Med Genet* 26:493-495
- Raeymaekers P, Timmerman V, De Jonghe P, Swerts L,

- Gheuens J, Martin J-J, Muylle L, et al (1989) Localization of the mutation in an extended family with Charcot-Marie-Tooth neuropathy (HMSN I). *Am J Hum Genet* 45:953-958
- Ray R, Rincon-Limas D, Wright RA, Davis SN, Lupski JR, Patel FI (1990) Three polymorphisms at the D17S29 locus. *Nucleic Acids Res* 18:4958
- Sait MA, Reddy BSN, Gars BR (1985) Onychotillomania. *Dermatologica* 171:200-202
- Schmickel RD (1986) Contiguous gene syndromes: a component of recognizable syndromes. *J Pediatr* 109:231-241
- Schrander-Stumpel C, Schrander J, Frys JP, Hamers G (1990) Trisomy 17p due to a t(8;17)(p23;p11.2) pat translocation: case report and review of the literature. *Clin Genet* 37:148-152
- Smith ACM, McGavran L, Robinson J, Waldstein G, Macfarlane J, Zonona J, Reiss J, et al (1986) Interstitial deletion of (17)(p11.2p11.2) in nine patients. *Am J Med Genet* 24:393-414
- Smith ACM, McGavran L, Waldstein G (1982) Deletion of the 17 short arm in two patients with facial clefts. *Am J Hum Genet* 34 [Suppl]: A410
- Stratton RF, Dobyns WB, Greenberg F, DeSana JB, Moore C, Fidone G, Runge GH, et al (1986) Interstitial deletion of (17)(p11.2p11.2): report of six additional patients with a new chromosome deletion syndrome. *Am J Med Genet* 24:421-432
- Tandan R, Taylor R, Adesina A, Sharma K, Fries T, Pendlebury W (1990) Benign autosomal dominant syndrome of neuronal Charcot-Marie-Tooth disease, ptosis, parkinsonism, and dementia. *Neurology* 40:773-779
- Vance JM, Barker D, Yamaoka LH, Stajich JM, Loprest L, Hung WY, Fischbeck K, et al (1991) Localization of Charcot-Marie-Tooth disease type 1A (CMT1A) to chromosome 17p11.2. *Genomics* 9:623-628
- Vance JM, Nicholson GA, Yamaoka LH, Stajich J, Stewart CS, Speer MC, Hung WY, et al (1989) Linkage of Charcot-Marie-Tooth neuropathy type 1a to chromosome 17. *Exp Neurol* 104:186-189
- vanTuinen P, Rich DC, Summers KM, Ledbetter DH (1987) Regional mapping panel for human chromosome 17: application to neurofibromatosis type 1. *Genomics* 1:374-381
- Weber JL, May PE (1989) Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. *Am J Hum Genet* 44:388-396
- Williams CA, Zori RT, Stone JW, Gray BA, Cantu ES, Ostrer H (1990) Maternal origin of 15q11-13 deletions in Angelman syndrome suggests a role for genomic imprinting. *Am J Med Genet* 35:350-353
- Wright EC, Goldgar DE, Fain PR, Barker DF, Skolnick MH (1990) A genetic map of human chromosome 17p. *Genomics* 7:103-109

UNIVERSIDAD AUTÓNOMA DE NUEVO LEÓN



DIRECCIÓN GENERAL DE BIBLIOTECAS

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

James R. Lupski,*¹ Roberto Montes de Oca-Luna,*
Susan Slaugenhaupt,² Liu Pentao,* Vito Guzzetta,*
Barbara J. Trask,³ Odila Saucedo-Cardenas,*
David F. Barker,⁴ James M. Killian,⁵
Carlos A. Garcia,** Aravinda Chakravarti,⁶
and Pragna I. Patel^{1*}

¹Institute for Molecular Genetics

²Human Genome Center

³Department of Pediatrics

⁴Department of Neurology

Baylor College of Medicine
Houston, Texas 77030

⁵Departments of Human Genetics and Psychiatry
University of Pittsburgh

Pittsburgh, Pennsylvania 15261

⁶Biomedical Sciences Division

Lawrence Livermore National Laboratories
Livermore, California 94550

⁷Department of Medical Informatics
University of Utah School of Medicine
Salt Lake City, Utah 84130

⁸Departments 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 *SacI* 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,

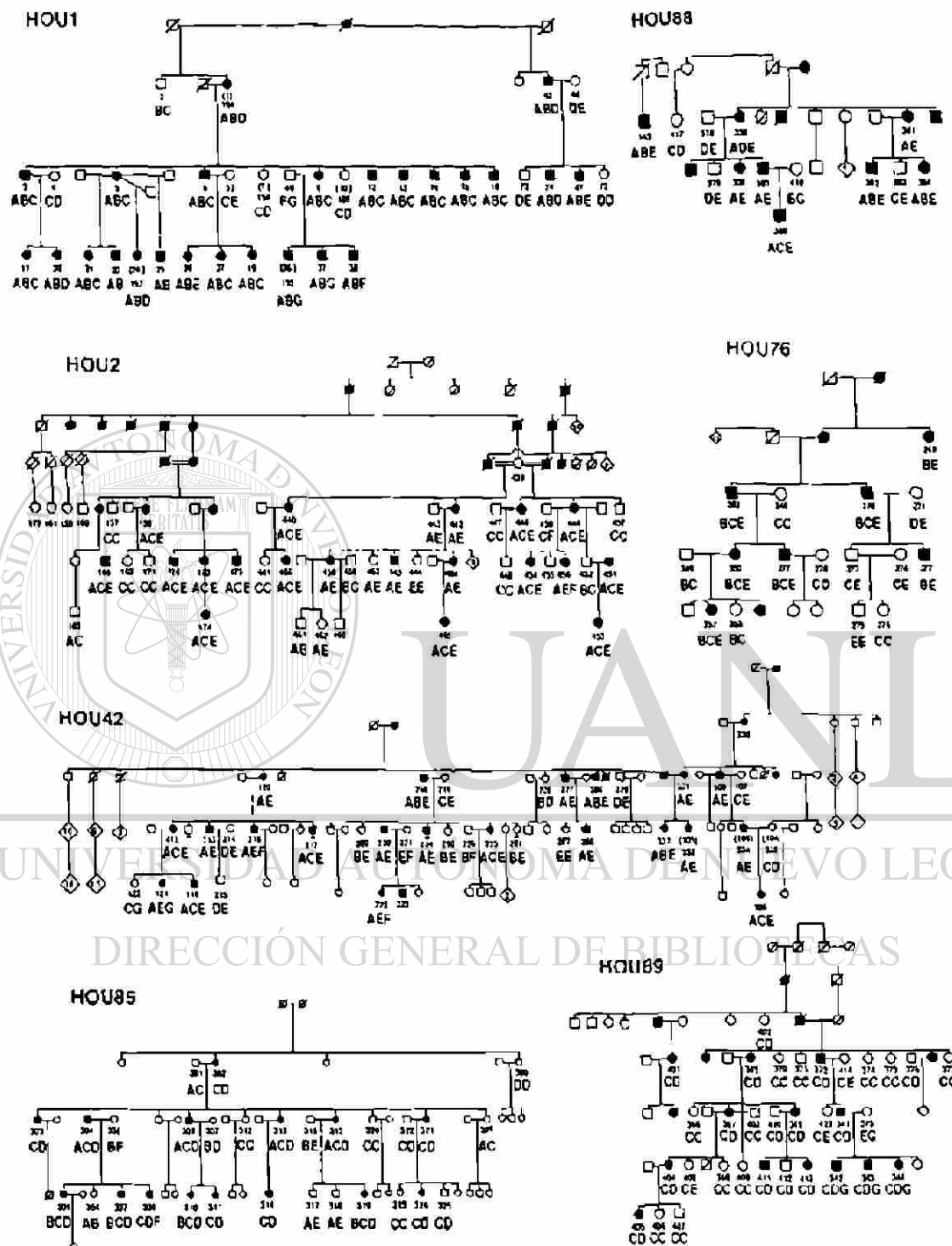


Figure 1. (GT), Genotypes at the D17S122 Locus for Kindreds Segregating Autosomal Dominant CMT1A

HOU1, HOU2 (Kilian and Koepler, 1979), HOU42, HOU85, HOU88, and HOU89 are of French-Canadian descent while HOU76 is of Ashkenazi Jewish descent. Standard pedigree symbols are used. Disease is indicated by the darkened symbols. The laboratory identification number and the (GT), genotype of each individual are indicated below the pedigree symbols. (GT), 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						θ	Z
	0.00	0.05	0.10	0.20	0.30	0.40		
Fc γ RII	$-\infty$	-16.17	-9.42	-5.66	-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	6.72	6.67	4.25	1.78	0.023	9.66
1516	15.89	14.63	13.24	10.16	5.75	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	5.47	2.65	0.013	15.84
MYH2	$-\infty$	1.10	2.91	3.56	2.81	1.45	0.180	3.58
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 (θ) between CMT1 and various genetic markers, and the peak LOD scores (Z) for nine loci are shown in Table 1. The immunoglobulin receptor Fc γ RII 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 Fc γ RII ($\theta = 0.5$, $Z = 0.0$, 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 Fc γ RII.

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 $\theta = 0$ 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, 4.28, 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 2-418 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 HOU86, with alleles A and B in HOU1, with alleles C and D in HOU85 and HOU89, and with alleles B and E in HOU76.



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

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

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

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

The five large French-Acadian pedigrees segregating CMT1A and the two small kindreds of French-Acadian (HOU88) and Ashkenazic Jewish (HOU76) descent were genotyped for RM11-GT. Genotype data from two nuclear CMT1A families within HOU88 and HOU76 are shown in Figure 2. These data demonstrate a striking observation:

six of eight CMT1A individuals show three (GT)_n alleles (e.g., individuals 88-340, 76-352), but all unaffected individuals are either homozygous or heterozygous for (GT)_n alleles. In certain matings, only two (GT)_n alleles were segregating and thus only two (GT)_n alleles could be detected in the affected child. However, careful examination of the autoradiograph often revealed that one of the two (GT)_n alleles was present in two copies (e.g., 88-339, 88-380 in Figure 2A). These data indicate that CMT1A patients of French-Acadian (Figure 2A) and Ashkenazic Jewish (Figure 2B) descent have three copies of the D17S122 locus, suggesting a duplication of this locus in CMT1A patients.

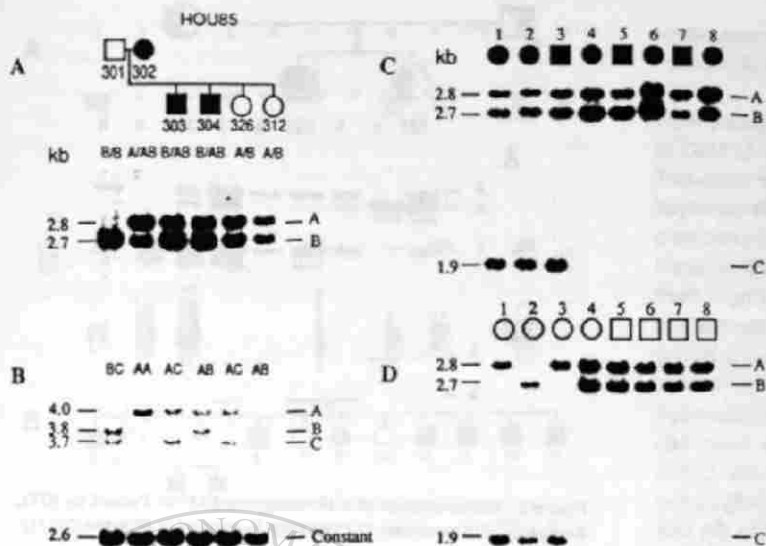
Genotypes for RM11-GT for all seven CMT1A pedigrees are shown in Figure 1 and demonstrate that three RM11-GT alleles are present only in affected individuals and are never observed in 53 unaffected offspring and 31 unaffected spouses. The transmission of this duplication is also highly specific. By considering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

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

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

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

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



lanes 1-3. This genotype clearly illustrates the duplication, but was observed in only 3 of 131 CMT1A patients. Lanes 4-6 show individuals who had two polymorphic alleles and in whom a duplication could be discerned by noting the difference in the relative intensity of one allele when compared to that of the other allele. This Southern blot was rehybridized with a control VNTR probe, YNH24 from chromosome 2 (Nakamura et al., 1987), and showed no difference in the intensity of the polymorphic alleles (data not shown). (D) Southern analysis of *Msp*I-digested genomic DNA from eight control individuals with the probe VAW409R3. Note the lack of dosage difference between alleles in all individuals.

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

To confirm this observation, 103 CMT1A patients from seven families (Figure 1) as well as 26 other unrelated patients were examined by Southern blot analysis with VAW409R3. Dosage of alleles was determined by visual examination and densitometry of autoradiographs or by quantitation of total radioactivity in each allele using a Betascope analyzer (Sullivan et al., 1987). Dosage was determined only in individuals who were heterozygous for the RFLP since the results were most reproducible and reliable in such cases. Seventy-six CMT1A patients were heterozygous for this RFLP and were conclusively demonstrated to have three copies of the D17S122 locus. In contrast, none of 63 controls (27 unaffected at-risk individuals with normal NCV and 36 controls with no family history of CMT) who were heterozygous for this marker showed dosage differences for this RFLP, suggesting that the genotype with dosage differences was specific to CMT1A patients ($\chi^2 = 48.72$; $P < 10^{-6}$). Similar dosage differences were observed with the marker VAW409R1 (data not shown).

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

(A) Southern analysis of *Msp*I-digested genomic DNA from a nuclear family (HOU85) with the probe VAW409R3 (D17S122). Southern analysis was conducted on 5 μ g of genomic DNA as described (Patel et al., 1990a). Squares and circles represent males and females, respectively. Note the difference in the relative intensity of alleles A and B in CMT patients (85-302, 85-303, 85-304) versus unaffected individuals (85-301, 85-312, 85-326). (B) Southern analysis with a probe from outside the duplication region. The Southern blot from (A) was rehybridized with the control probe 10-5, representing the myosin heavy chain locus in 17p13 (Schwartz et al., 1986; Nakamura et al., 1988). No difference in the intensity of the polymorphic alleles was noted.

(C) Southern analysis of *Msp*I-digested genomic DNA from eight unrelated CMT1A patients with the probe VAW409R3 (D17S122). Note the presence of three polymorphic alleles in

Demonstration of Two (GT)_n Alleles in *Msp*I Fragments Showing Dosage Differences

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

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

Homozygosity for the Duplication Mutation in a Severely Affected Individual

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

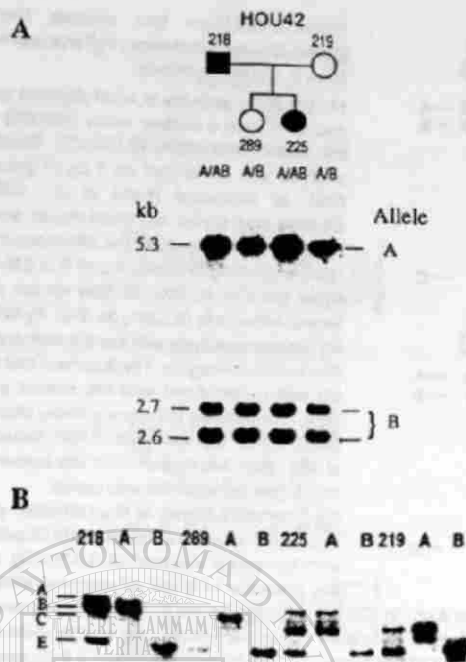


Figure 4. Demonstration of Two (GT)_n Alleles in Polymorphic MspI Fragments at the D17S122 Locus Showing Double Dosage by Allele Separation

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

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

For further confirmation, somatic cell hybrids retaining individual chromosome 17 homologs from patient 42-333, her affected mother (42-331), and her affected sister (42-

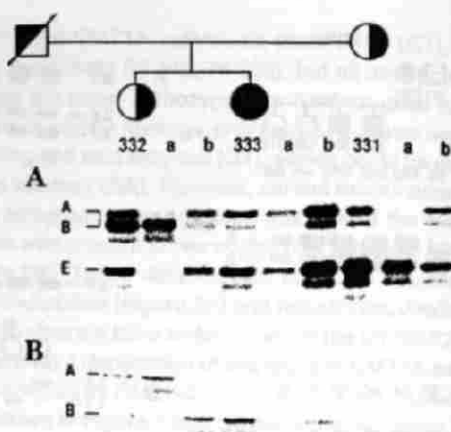


Figure 5. Demonstration of a Homozygous CMT1A Patient by (GT)_n Analysis of Chromosome 17 Homologs Separated in Somatic Cell Hybrids

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

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

PFGE Analysis Identifies a Novel SacII Fragment in CMT1A Patients

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

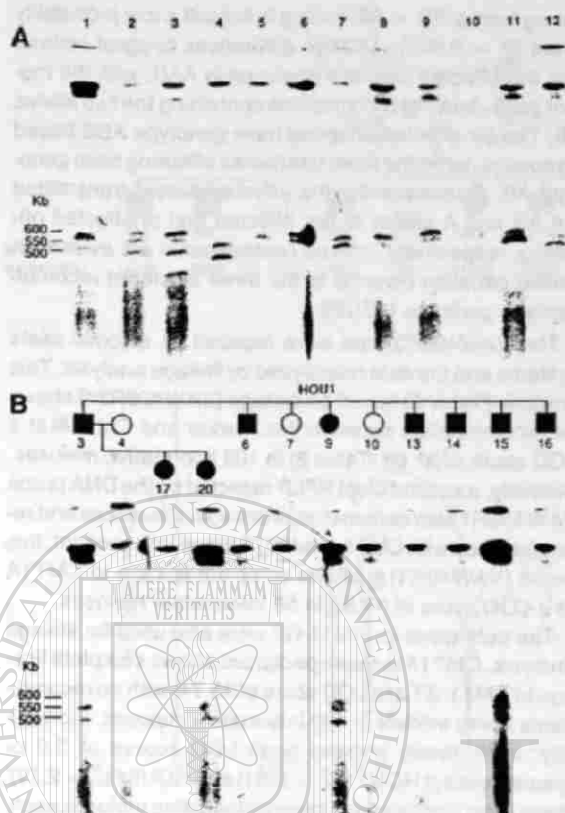


Figure 6. An Additional *Sac*II Allele Is Identified in CMT1A Individuals by PFGE

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

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

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

similar size in CMT1A patients of French-Acadian and Ashkenazic Jewish origin.

FISH Analysis Reveals a Duplication in Nuclei of CMT1A Patients

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

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

Consequences of the Duplication on Linkage Analysis for CMT1A Gene Localization

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

This important phenomenon is illustrated in Figure 8A

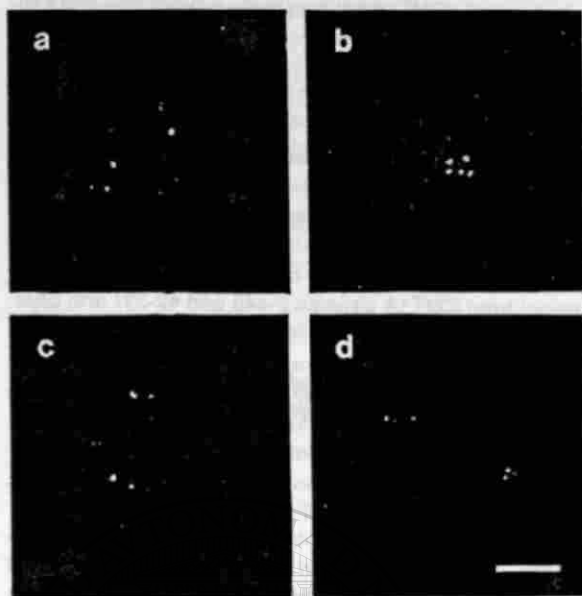


Figure 7. FISH Analysis of Interphase Nuclei from CMT1A and Normal Individuals with VAW409 and c1516

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

with the VAW409R3 MspI RFLP data from a nuclear family from HOU85. If dosage differences are ignored, the affected mother has genotype AB with the A chromosome carrying the CMT1A mutant gene; the unaffected father is BB. Since all nine offspring are AB but six are affected and three are unaffected, the unaffected individuals are recombinant. (Note that the segregation of nine AB off-

spring from a BB \times AB mating is in itself a low probability event [$P = 0.002$].) Dosage differences suggest instead that the affected mother's genotype is AAB, with the mutant gene-bearing chromosome containing the two alleles AB. The six affected offspring have genotype ABB based on dosage, while the three unaffected offspring have genotype AB. Consequently, the affected parent transmitted the AB and A alleles to her affected and unaffected offspring, respectively, and no recombinants are evident. A similar situation pertains to the three clustered recombinants in pedigree HOU89.

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

The genotypes for RM11-GT were also used for linkage analysis. CMT1A in these pedigrees shows complete linkage to RM11-GT at a LOD score of 36.74, with no recombinants being evident in 122 informative meioses. Individually, each family showed peak LOD scores of 3.0 or greater, except HOU76 ($\hat{Z} = 2.01$) and HOU88 ($\hat{Z} = 2.19$); these latter families have seven informative meioses each. Thus, taking dosage differences into account at VAW409R1, VAW409R3, and RM11-GT, locus D17S122 shows complete linkage to CMT1A.

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

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

Marker	Recombination Value						$\hat{\theta}$	\hat{Z}
	0.00	0.05	0.10	0.20	0.30	0.40		
409R3	— ∞	6.75	6.76	5.60	3.89	1.98	0.073	6.86
409R3d	31.08	28.26	25.35	19.23	12.67	5.70	0.000	31.08
409R1	— ∞	5.34	4.81	3.42	1.99	0.72	0.027	5.45
409R1d	17.56	15.86	14.21	10.55	6.86	3.10	0.000	17.56
RM11-GT	36.74	31.76	29.96	22.67	14.85	6.47	0.000	36.74

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

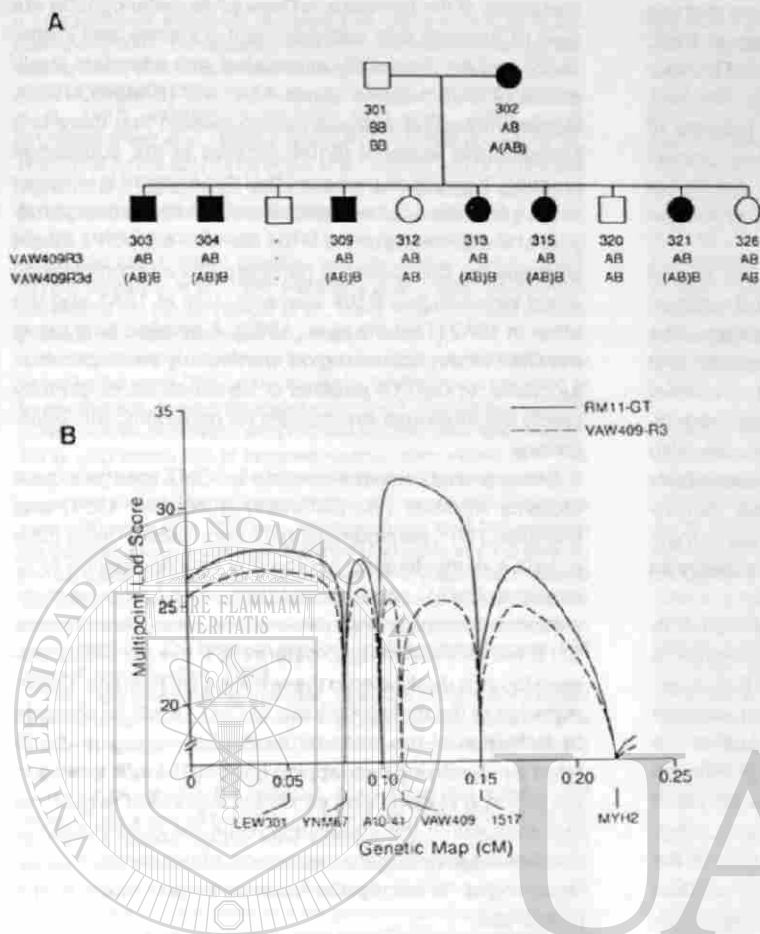


Figure 8. Consequences of Duplication Mutation on Linkage Analysis

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

(B) Multipoint linkage mapping of CMT1A on a genetic map of chromosome 17p. The locus positions of the markers are indicated on the horizontal axis. The height of the curves represents the relative likelihood of location (Lod score) at any specified point along the map. When dosage differences are ignored (VAW409R3), the most likely position of the CMT1A gene is proximal to LEW301; however, the RM11-GT locus data clearly place CMT1A at RM11-GT.

The failure to account for dosage differences at a two-allele RFLP in linkage analysis, when it exists, leads to misinterpretation of the parental origin of alleles, as shown in Figure 8A. These errors appear as multiple, clustered (within sibships) recombination events that reduce the LOD score and increase the recombination value between the disease and the marker. More importantly, when these errors are included, multipoint linkage analysis can seriously distort the positioning of the disease locus. This dramatic effect is shown in Figure 8B, where we present the multipoint LOD score for CMT1A versus a fixed map of the markers LEW301—YNM67—R5—A10-41—VAW409R3/RM11-GT—1517—MYH2. Figure 8B shows the multipoint LOD score curve for two analyses using CRI-MAP that are identical except that VAW409 was first coded as a two-allele RFLP without dosage (VAW409R3) and a second time using the RM11-GT polymorphism. Using the (GT)_n polymorphism, the multipoint LOD score is 31.4 and correctly places the CMT1A locus at VAW409. The 95% confidence limits on the CMT1A location define a 3 cM interval around the (GT)_n locus. On the other hand, ignoring the duplication produces a peak LOD score of 26.81 and incorrectly places CMT1A 1 cM proximal to LEW301. The 95% confidence limits on this location define a 6 cM interval around LEW301. Not only do these two confidence intervals fail to overlap, but the two LOD scores have an odds difference of 10^{4.9}! Furthermore, the correct location of CMT1 at VAW409 is 50 times less likely than the incor-

rect location when dosage at VAW409R3 is not scored. The misclassification leads to multiple recombination events with VAW409 and thus places the CMT1A locus toward LEW301, with which no recombinants were observed.

Discussion

We have demonstrated that CMT1A is associated with a DNA duplication using (GT)_n polymorphism and RFLP analysis, FISH analysis, and isolation of parental chromosomes in somatic cell hybrids. Three polymorphic markers at the D17S122 locus have displayed this duplication, namely, VAW409R3, VAW409R1, and RM11-GT, and in each case there is a perfect correlation between the duplication genotype and the CMT1A disease phenotype. PFGE suggests that the duplication includes a large genomic region. We have shown that failure to understand the molecular nature of the polymorphism leads to the mislocalization of CMT1A and reduced evidence for linkage. Preliminary data by RFLP analysis and dosage of polymorphic alleles indicate that two additional markers, VAW412R3 (D17S125) and EW401 (D17S67) (Wright et al., 1990), which are linked to VAW409, may also be duplicated, while other CMT1A-linked markers do not appear to show evidence for duplication.

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

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

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

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

The mechanism by which the duplication mutation arose is unknown. *De novo* mutations that include deletions and duplications have been observed in the proximal region of the short arm of chromosome 17 (Smith et al., 1986; Stratton et al., 1986; Magenis et al., 1986). It is possible that the same recombination mechanisms that result in microdeletion on one chromosome homolog can result in duplication on the reciprocal homolog. Recent studies on chromosomal duplications in *Escherichia coli* and humans have demonstrated that duplication junctions occur in regions containing repetitive extragenic palindromic (REP) sequences (Shyamala et al., 1990) as well as near *Alu* sequences (Kornreich et al., 1990; Devlin et al., 1990)

Mutations at the *Bar* locus in *Drosophila melanogaster* are gain of function and semidominant (Lindsley and Zimm, 1985) and are frequently associated with a tandem duplication of chromosome bands 16A1-A7 (Bridges, 1936). Recent molecular analysis has suggested that there is a transposable element (B104) located at the duplication junction. It has been proposed that this element is involved in the generation of the duplication. DNA sequencing analysis of the junctions of the B104 element support a model whereby the duplication is generated by a recombination event between two B104 elements, one in 16A1 and the other in 16A7 (Tsubota et al., 1989). A detailed analysis of the CMT1A duplicated region, particularly the duplication junctions, in CMT1A patients of varied ethnic origin may clarify the molecular mechanism for generating this duplication.

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

Experimental Procedures

Clinical Evaluation and Sampling of Families

All available at-risk members of pedigrees were subjected to a thorough clinical and electrophysiological examination. In pedigree HCU11, NCVs were initially determined only for clinically affected individuals. Further evaluation indicated that the clinically unaffected individuals 1-13, 1-27, and 1-36 had abnormal NCVs; therefore, the disease status of these individuals is different from that reported in the original pedigree (Patel et al., 1990a). In all other pedigrees at-risk individuals, whether clinically affected or unaffected, had motor NCVs determined. Diagnosis of CMT1 was established by slowed median and ulnar motor NCVs bilaterally (<40 m/s). A single normal motor NCV of the peroneal nerve excluded the diagnosis of CMT1 in patients 5 years or older. Blood was collected from each participating family member, under informed consent, and used to establish EBV-transformed lymphoblasts (Anderson and Gusella, 1984) and for isolating high molecular weight DNA (Miller et al., 1988). The variable numbers of tandem repeat locus YNH24 (D2S44) (Nakamura et al., 1987a, 1987b), in addition to the marker loci used in linkage analysis, were used to check parental origins for each individual in the seven pedigrees in Figure 1. Some parental exclusions were detected; these individuals were not incorporated in the linkage analysis.

Linkage Analysis

The chromosome 17p markers comprise 17 standard RFLPs and were detected using nine DNA probes and Southern analysis as previously described (Patel et al., 1990a, 1990b). The DNA probe FcyRII (chromosome 1q) was studied by Southern analysis to exclude linkage to chromosome 7. LOD score analysis used two point or multipoint methods (Morton, 1956; Ott, 1985; Lathrop and Lalouel, 1988) and the computer programs LINKAGE version 4.7 (Lathrop and Lalouel, 1988) and CRIMAP version 2.4 (Dennis-Keller et al., 1987). CMT1 was considered as

a fully penetrant autosomal dominant trait with a mutant gene frequency of 0.0001. The following markers, where alleles were codominant systems with frequencies as described in the literature, were used: LEW301 (D17S58) (Fain et al., 1987), pA10-41 (D17S71) (Barker et al., 1987); pYNM67-R5 (D17S29) (Ray et al., 1990); c1516 (D17S258) (Patel et al., 1990a); p1516-R4 (D17S258) (Franco et al., 1990); pS6-1-HB2 (D17S445) (Patel et al., 1990b); c1517 (D17S259) (Patel et al., 1990a) MspI allele lengths = 6.2/4.0/2.4 kb; c1541 (D17S260) (Patel et al., 1990a) BglII allele lengths = 3.4/2.0 + 1.4 kb; HindIII 14.0/13.0 kb, BamHI 11.0/7.6 kb (allele lengths for probes c1517 and c1541 reflect changes from the original report); p10-5 (MYH2) (Schwartz et al., 1986; Nakamura et al., 1988); VAW409R1, VAW409R3 (D17S122) (Wright et al., 1990); FcyRII, HFC3.0 (FCG2) (Hibbs et al., 1988; Grundy et al., 1989).

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

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

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

Detection of (GT)_n Polymorphic Markers and Genotype Determination

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

For PCR amplification either the GT strand (CAGAACCACAAAATG-TCTTGCATTC) or CA strand (GGCCAGACAGACCAGGCTCTGC) oligonucleotide primer flanking the (GT)_n repeat sequence was end-labeled at 37°C in a 15 μ l reaction volume containing 1.2 μ M primer, 100 μ Ci of [γ -³²P]ATP at 6000 Ci/mmol, 1 \times One Phor-All Plus buffer (Pharmacia), and 10 U of polynucleotide kinase (Pharmacia). The ki-

nase was inactivated at 65°C for 10 min and the primer used directly in the PCR reaction (0.4 μ l per reaction). PCR was performed using standard conditions in a 25 μ l reaction volume in a mixture containing 1 μ M each oligodeoxynucleotide primer, 250 μ M each dATP, dCTP, dGTP, and dTTP, 2.5 μ l of 10 \times PCR buffer (500 mM KCl, 120 mM Tris-HCl [pH 8.0], 1.5 mM MgCl₂, and 0.01% gelatin), 0.63 U of AmpliTaq (Cetus) DNA polymerase, and 0.4 μ l of end-labeled GT primer reaction mix. The amplification conditions were an initial denaturation at 94°C for 5 min followed by 30 cycles of 94°C denaturation for 1 min, 55°C annealing for 1 min, and 72°C extension for 2 min in an automated thermal cycler (Perkin-Elmer/Cetus). Reaction products (1.5 μ l) were mixed with 2 μ l of formamide stop solution (United States Biochemical Corporation) and electrophoresed in a 6% polyacrylamide DNA sequencing gel at 40 W for 3.5 hr. Gels were dried and autoradiographed for 2–12 hr by exposure to Kodak XAR-5 film at -70°C .

Southern Analysis and Dosage Determination

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

Allele Separation for PCR Analysis

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

Construction and Analysis of Somatic Cell Hybrids

Somatic cell hybrids were used to separate the maternal and paternal chromosomes 17 of individuals 42-331, 42-332, and 42-333. Hybrids were constructed as described by Zoghbi et al. (1989) using a23, a thymidine kinase-deficient Chinese hamster cell line (Westerveld et al., 1971) as the rodent parent. Briefly, two 100 mm plates were seeded with 10⁷ a23 cells per plate 16–20 hr before fusion. The cells were washed with Dulbecco's modified Eagle's medium (DMEM). To a 10 ml suspension of 5 \times 10⁷ lymphoblasts in Hanks' balanced salt solution (GIBCO), 250 μ l of a 1 mg/ml phytohemagglutinin (Sigma) solution was added. Five milliliters of this cell suspension was added to each plate of a23 cells, and the plates were incubated for 15 min at 37°C. The solution was aspirated, and 2 ml of 50% polyethylene glycol 1500 (Boehringer Mannheim Biochemicals) was spread over the surface of the plate. After 1 min the polyethylene glycol was aspirated, and the cells were washed three times with DMEM and incubated with 10 ml of DMEM for 30 min at 37°C. The medium was aspirated and the plates were incubated overnight with 10 ml of DMEM with 10% fetal calf serum (FCS). Hybrids were selected by growth in DMEM containing 10% FCS, 0.1 mM hypoxanthine, 0.001 mM aminopterin, and 0.01 mM thymidine. Hybrids were isolated with cloning rings 10–14 days later and transferred to 24-well microtiter plates.

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

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

PFGE

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

FISH

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

Acknowledgments

We are grateful to the CMT patients and families for their continued cooperation, and the Muscular Dystrophy Association (MDA) clinics in New Orleans, Baton Rouge, and Lafayette, Louisiana, for clinical evaluation, treatment, and patient specimen collection. We thank Drs. R. Malamut and G. Parry for electrophysiological evaluation of CMT1A families and Dr. F. Axelrod (New York University Medical Center) for clinical evaluation and collection of HOU76. The excellent technical assistance of R. Wright, Zhang Heju, S. Davis, V. Holliday, and H. Massa, graphics assistance by R. Ross, and manuscript preparation by L. Hayway are gratefully acknowledged. We thank all investigators who provided probes. We gratefully appreciate the critical review of the manuscript by Drs. A. Beaudet, H. Bellen, J. Belmont, D. Ledbetter, D. Nelson, and H. Zoghbi. Work of B. J. T. at Lawrence Livermore National Laboratories was performed under US Department of Energy contract number W-7405-ENG-48 with support from US Public Health Service grant HG-00256-01. This research was also supported by an MDA Task Force on Genetics grant, a Texas Higher Education Advanced Technology Program grant, and NIH grants RO1 NS-27042 to J. R. L. and P. I. P. and HG 00344 to A. C., and by Baylor Mental Retardation Center grant HD 24064-02. R. M. is a recipient of a fellowship from the National Council of Science and Technology (CONACYT) of Mexico. V. G. is the recipient of an MDA postdoctoral fellowship. A. C. is a recipient of a Research Career Development Award from the NIH (HD 00774). J. R. L. acknowledges support from the Pew Scholars Program in Biomedical Sciences.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC Section 1734 solely to indicate this fact.

Received May 15, 1991; revised June 5, 1991.

References

- Anderson, M. A., and Gusella, J. R. (1984). The use of cyclosporin A in establishing Epstein-Barr virus-transformed human lymphoblastoid cell lines. *In Vitro* 20, 856–858.
- Barker, D., Wright, E., Nguyen, K., Cannon, L., Fain, P., Goldgar, D., Bishop, D. T., Carey, J., Baty, B., Kivlin, J., Willard, H., Wayne, J. S., Greig, G., Leinwand, L., Nakamura, Y., O'Connell, P., Leppert, M., Lalouel, J.-M., White, R., and Skolnick, M. (1987). Gene for von Recklinghausen neurofibromatosis is in the pericentromeric region of chromosome 17. *Science* 236, 1100–1102.
- Bedford, M. T., and van Helden, P. D. (1990). A method to analyze allele-specific methylation. *BioTechniques* 9, 744–748.
- Beeson, D., Jeremiah, S., West, L. F., Povey, S., and Newsom-Davis, J. (1990). Assignment of the human nicotinic acetylcholine receptor genes: the alpha and delta subunit genes to chromosome 2 and the beta subunit gene to chromosome 17. *Ann. Hum. Genet.* 54, 199–208.
- Bird, T. D., and Kraft, G. H. (1978). Charcot-Marie-Tooth disease: data for genetic counseling relating age to risk. *Clin. Genet.* 14, 43–49.
- Bird, T. D., Ott, J., and Giblett, E. R. (1982). Evidence for linkage of Charcot-Marie-Tooth neuropathy to the Duffy locus on chromosome 1. *Am. J. Hum. Genet.* 34, 388–394.
- Boulter, J., O'Shea-Greenfield, A., Duvoisin, R. M., Connolly, J. G., Wada, E., Jensen, A., Gardner, P. D., Ballivet, M., Deneris, E. S., McKinnon, D., Heinemann, S., and Patrick, J. (1990). $\alpha 3$, $\alpha 5$, and $\beta 4$: three members of the rat neuronal nicotinic acetylcholine receptor-related gene family form a gene cluster. *J. Biol. Chem.* 265, 4472–4482.
- Bridges, C. B. (1936). The Bar "gene" a duplication. *Science* 83, 210–211.
- Chance, P. F., Bird, T. D., O'Connell, P., Lipe, H., Lalouel, J.-M., and Leppert, M. (1990). Genetic linkage and heterogeneity in type 1 Charcot-Marie-Tooth disease (hereditary motor and sensory neuropathy type 1). *Am. J. Hum. Genet.* 47, 915–925.
- Charcot, J.-M., and Marie, P. (1886). Sur une forme particulière d'atrophie musculaire progressive souvent familiale debutant par les pieds et les jambes et atteignant plus tard les mains. *Rev. Méd.* 6, 97–138.
- Conneally, P. M., Edwards, J. H., Kidd, K. K., Lalouel, J.-M., Morton, N. E., Ott, J., and White, R. (1985). Report of the committee on methods of linkage analysis and reporting. *Cytogenet. Cell. Genet.* 40, 356–359.
- Dausset, J. (1986). Le Centre d'Etude du Polymorphisme Humain. *Presse Med.* 15, 1801.
- Devlin, R. H., Deeb, S., Brunzell, J., and Hayden, M. R. (1990). Partial gene duplication involving exon-Alu interchange results in lipoprotein lipase deficiency. *Am. J. Hum. Genet.* 46, 112–119.
- Donis-Keller, H., Green, P., Helms, C., Cartinhour, S., Weiffenbach, B., Stephens, K., Keith, T. P., Bowden, D. W., Smith, D. R., Lander, E. S., Botstein, D., Akots, G., Rediker, K. S., Gravius, T., Brown, V. A., Rising, M. B., Parker, C., Powers, J. A., Watt, D. E., Kauffman, E. R., Brickner, A., Phipps, P., Muller-Kahle, H., Fulton, T. R., Ng, S., Schumm, J. W., Broman, J. C., Knowlton, R. G., Barker, D. F., Crooks, S. M., Lincoln, S. E., Daly, M. J., and Abrahamson, J. (1987). A genetic linkage map of the human genome. *Cell* 51, 319–337.
- Fain, P. R., Barker, D. F., Goldgar, D. E., Wright, E., Nguyen, K., Carey, J., Johnson, J., Kivlin, J., Willard, H., Mathew, D., Ponder, B., and Skolnick, M. (1987). Genetic analysis of NF1: identification of close flanking markers on chromosome 17. *Genomics* 1, 340–345.
- Falconer, D. S. (1951). Two new mutants, "trembler" and "reeler," with neurological action in the house mouse (*Mus musculus* L.). *J. Genet.* 50, 192–201.

- Franco, B., Rincón-Limas, D., Nakamura, Y., Patel, P. I., and Lupski, J. R. (1990). An *MspI* RFLP at the D17S250 locus. *Nucl. Acids Res.* 18, 7198.
- Green, M. C. (1989). *Genetic Variants and Strains of the Laboratory Mouse* (New York: Oxford University Press).
- Grundy, H. O., Peltz, G., Moore, K. W., Golbus, M. S., Jackson, L. G., and Lebo, R. V. (1989). The polymorphic *Fcy receptor* gene maps to human chromosome 1q. *Immunogenetics* 29, 331-339.
- Herrmann, B. G., Barlow, D. P., and Lehrach, H. (1987). A large inverted duplication allows homologous recombination between chromosomes heterozygous for the proximal t complex inversion. *Cell* 48, 813-825.
- Hibbs, M. L., Bonadonna, L., Scott, B. M., McKenzie, I. F. G., and Hogarth, P. M. (1988). Molecular cloning of a human immunoglobulin G Fc receptor. *Proc. Natl. Acad. Sci. USA* 85, 2240-2244.
- Killian, J. M., and Kloepper, H. W. (1979). Homozygous expression of a dominant gene for Charcot-Marie-Tooth neuropathy. *Ann. Neurol.* 5, 515-522.
- Konreich, R., Bishop, O. F., and Desnick, R. J. (1990). α -Galactosidase A gene rearrangements causing Fabry disease. Identification of short direct repeats at breakpoints in an *Alu*-rich gene. *J. Biol. Chem.* 265, 9319-9326.
- Lathrop, G. M., and Lalouel, J.-M. (1985). Efficient computations in multilocus linkage analysis. *Am. J. Hum. Genet.* 42, 498-505.
- Lawrence, J. B., Singer, R. H., and McNeil, J. A. (1989). Interphase and metaphase resolution of different distances within the human dystrophin gene. *Science* 249, 928-932.
- Lindsley, D., and Zimm, G. (1985). The genome of *Drosophila melanogaster*. Part 1: genes A-K. *Dros. Inf. Serv.* 62.
- Litt, M., and Luty, J. A. (1989). A hypervariable microsatellite revealed by in vitro amplification of a dinucleotide repeat within the cardiac muscle actin gene. *Am. J. Hum. Genet.* 44, 397-401.
- Lupski, J. R., Garcia, C. A., Parry, G. J., and Patel, P. I. (1991). Charcot-Marie-Tooth polynuropathy syndrome: clinical, electrophysiological, and genetic aspects. In *Current Neurology*, S. Appel, ed. (Chicago: Mosby-Yearbook), pp. 1-25.
- Magenis, R. E., Brown, M. G., Allen, L., and Reiss, J. (1986). De novo partial duplication of 17p [dup(17)(p12-p11.2)]. *Clinical Report Am. J. Med. Genet.* 24, 415-420.
- McAlpine, P. J., Feasby, T. E., Hahn, A. F., Komarnicki, L., James, S., Guy, C., Dixon, M., Qayyum, S., Wright, J., Coopland, G., Lewis, M., Kaita, H., Phillips, S., Wong, P., Koopman, W., Cox, D. W., and Yee, W. C. (1990). Localization of a locus for Charcot-Marie-Tooth neuropathy type IA (CMT1A) to chromosome 17. *Genomics* 7, 408-415.
- McKusick, V. A. (1990). *Mendelian Inheritance in Man* (Baltimore: The Johns Hopkins University Press).
- Middleton-Price, H. R., Harding, A. E., Monteiro, C., Berciano, J., and Malcolm, S. (1990). Linkage of hereditary motor and sensory neuropathy type I to the pericentromeric region of chromosome 17. *Am. J. Hum. Genet.* 46, 92-94.
- Miller, S. A., Dykes, D. D., and Polesky, H. F. (1986). A simple salting out procedure for extracting DNA from human nucleated cells. *Nucl. Acids Res.* 14, 1215.
- Morton, N. E. (1955). The detection and estimation of linkage between the genes for elliptocytosis and the Rh blood type. *Am. J. Hum. Genet.* 8, 80-96.
- Nakamura, Y., Leppert, M., O'Connell, P., Wolf, R., Holm, T., Culver, M., Martin, C., Fujimoto, E., Huff, M., Krumm, E., and White, R. (1987a). Variable number of tandem repeat (VNTR) markers for human gene mapping. *Science* 235, 1818-1822.
- Nakamura, Y., Gillilan, S., O'Connell, P., Leppert, M., Lathrop, G. M., Lalouel, J.-M., and White, R. (1987b). Isolation and mapping of a polymorphic DNA sequence pYNH24 on chromosome 2 (D2S44). *Nucl. Acids Res.* 15, 10073.
- Nakamura, Y., Lathrop, M., O'Connell, P., Leppert, M., Barker, D., Wright, E., Skolnick, M., Kondoleon, S., Litt, M., Lalouel, J.-M., and White, R. (1988). A mapped set of DNA markers for human chromosome 17. *Genomics* 2, 302-309.
- Ott, J. (1978). A simple scheme for the analysis of HLA linkages in pedigrees. *Ann. Hum. Genet.* 42, 256-257.
- Ott, J. (1985). *Analysis of Human Genetic Linkage* (Baltimore: The Johns Hopkins University Press).
- Patel, P. I., Franco, B., Garcia, C., Slaugenhaupt, S. A., Nakamura, Y., Ledbetter, D. H., Chakravarti, A., and Lupski, J. R. (1990a). Genetic mapping of autosomal dominant Charcot-Marie-Tooth disease in a large French-Canadian kindred: identification of new linked markers on chromosome 17. *Am. J. Hum. Genet.* 46, 801-809.
- Patel, P. I., Garcia, C., Montes de Oca-Luna, R., Malamut, R. J., Franco, B., Slaugenhaupt, S. A., Chakravarti, A., and Lupski, J. R. (1990b). Isolation of a marker linked to the Charcot-Marie-Tooth disease type 1A gene by differential *Alu*-PCR of human chromosome 17-retaining hybrids. *Am. J. Hum. Genet.* 47, 926-934.
- Reymaekers, P., Timmerman, V., De Jonghe, P., Swerts, L., Gheuens, J., Martin, J.-J., Muylle, L., De Winter, G., Vandenberghe, A., and Van Broeckhoven, C. (1989). Localization of the mutation in an extended family with Charcot-Marie-Tooth neuropathy (HMSN I). *Am. J. Hum. Genet.* 45, 953-958.
- Ray, R., Rincón-Limas, D., Wright, R. A., Davis, S. M., Lupski, J. R., and Patel, P. I. (1990). Three polymorphisms at the D17S29 locus. *Nucl. Acids Res.* 18, 4958.
- Sanger, F., Nicklen, S., and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74, 5463-5467.
- Schwartz, C. E., McNally, E., Leinwand, L., and Skolnick, M. H. (1986). A polymorphic human myosin heavy chain locus is linked to an anonymous single copy locus (D17S1) at 17p13. *Cytogenet. Cell. Genet.* 43, 117-120.
- Schwartz, D. C., and Cantor, C. R. (1984). Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis. *Cell* 37, 67-75.
- Shyamala, V., Schneider, E., and Ferro-Luzzi Ames, G. (1990). Tandem chromosomal duplications: role of REP sequences in the recombination event at the join-point. *EMBO J.* 9, 939-946.
- Skre, H. (1974). Genetic and clinical aspects of Charcot-Marie-Tooth's disease. *Clin. Genet.* 6, 98-118.
- Smith, A. C. M., McGowan, L., Robinson, J., Waldstein, G., Macfarlane, J., Zanona, J., Reiss, J., Lahr, M., Allen, L., and Magenis, R. E. (1986). Interstitial deletion of (17)(p11.2p11.2) in nine patients. *Am. J. Med. Genet.* 24, 393-414.
- Southern, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98, 503-517.
- Stratton, R. F., Dobyns, W. B., Greenberg, F., De Sans, J. B., Moore, C., Fidone, G., Runge, G. H., Feldman, P., Sekhri, G. S., Pauli, R., and Ledbetter, D. H. (1986). Interstitial deletion of (17)(p11.2p11.2): report of six additional patients with a new chromosome deletion syndrome. *Am. J. Med. Genet.* 24, 421-432.
- Sullivan, D. E., Auron, P. E., Quigley, G. J., Watkins, P. C., Stanchfield, J. E., and Boion, C. (1987). The nucleic acid blot analyzer. I: High speed imaging and quantitation of ³²P labeled blots. *BioTechniques* 5, 672-678.
- Timmerman, V., Reymaekers, P., De Jonghe, P., De Winter, G., Swerts, L., Jacobs, K., Gheuens, J., Martin, J.-J., Vandenberghe, A., and Van Broeckhoven, C. (1990). Assignment of the Charcot-Marie-Tooth neuropathy type I (CMT1a) gene to 17p11.2-p12. *Am. J. Hum. Genet.* 47, 680-685.
- Trask, B. J., Massa, H., Kenwick, S., and Gitachier, J. (1981). Mapping of human chromosome Xc28 by two-color fluorescence in situ hybridization of DNA sequences to interphase cell nuclei. *Am. J. Hum. Genet.* 48, 1-15.
- Tsubota, S. I., Rosenberg, D., Szostak, H., Rubin, D., and Schedl, P. (1989). The cloning of the *Sar* region and the B breakpoint in *Drosophila melanogaster*: evidence for a transposon-induced rearrangement. *Genetics* 122, 861-890.
- Vance, J. M., Nicholson, G. A., Yamaoka, L. H., Stajich, J., Stewart, C. S., Speer, M. C., Hung, W.-Y., Foses, A. D., Barker, D., and Pericak-Vance, M. A. (1989). Linkage of Charcot-Marie-Tooth neuropathy type 1a to chromosome 17. *Exp. Neurol.* 104, 166-169.

Vance, J. M., Barker, D., Yamagata, L. H., Stajich, J. M., Loprest, L., Hung, W.-Y., Fischbeck, K., Roses, A. D., and Pericak-Vance, M. A. (1991). Localization of Charcot-Marie-Tooth disease type 1a (CMT 1a) to chromosome 17p11.2. *Genomics* 9, 623-628.

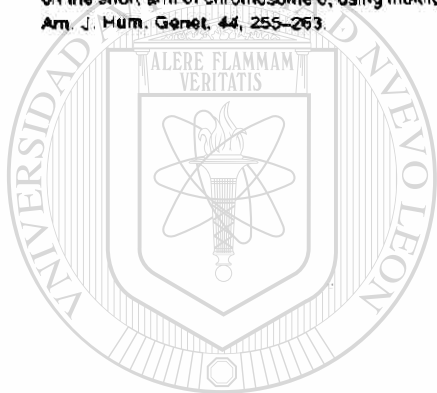
Weber, J. L. (1990). Human DNA polymorphisms based on length variation in simple-sequence tandem repeats. In *Genome Analysis, Vol. 1, Genetic and Physical Mapping*, K. E. Davies and S. M. Tilghman, eds. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press), pp. 157-181.

Weber, J. L., and May, P. E. (1989). Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. *Am. J. Hum. Genet.* 44, 388-396.

Westerveld, A., Visser, R. P. L. S., Khan, P. M., and Bootsma, D. (1971). Loss of human genetic markers in man-Chinese hamster somatic cell hybrids. *Nature New Biol.* 234, 20-24.

Wright, E. C., Golgar, D. E., Fain, P. R., Barker, D. F., and Skolnick, M.H. (1990). A genetic map of human chromosome 17p. *Genomics* 7, 103-109.

Zoghbi, H. Y., Sandkuijl, L. A., Orr, J., Daiger, S. P., Pollack, M., O'Brien, W. E., and Beaudet, A. L. (1989). Assignment of autosomal dominant spinocerebellar ataxia (SCA1) centromeric to the HLA region on the short arm of chromosome 6, using multilocus linkage analysis. *Am. J. Hum. Genet.* 44, 255-263.



UANL

UNIVERSIDAD AUTÓNOMA DE NUEVO LEÓN



DIRECCIÓN GENERAL DE BIBLIOTECAS

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

VITO GUZZETTA,*¹ BRUNELLA FRANCO,* BARBARA J. TRASK,† HEJU ZHANG,* ODILA SAUCEDO-CARDENAS,* ROBERTO MONTES DE OCA-LUNA,* FRANK GREENBERG,*‡ A. CRAIG CHINAULT,*§,|| JAMES R. LUPSKI,*§,† AND PRAGNA I. PATEL*§,2

*Institute for Molecular Genetics, §Human Genome Center, Departments of †Biochemistry and ‡Pediatrics, Baylor College of Medicine, Houston, Texas 77030; and †Biomedical Sciences Division, Lawrence Livermore National Laboratory, Livermore, California 94550

Received January 2, 1992; revised March 13, 1992

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

INTRODUCTION

Recently, much attention has been focused on the proximal region of the short arm of chromosome 17. Sys-

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

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

¹ Present address: Dipartimento Di Pediatria, III Cattedra, Via Pansini 5, Napoli, Italy.

² To whom correspondence should be addressed.

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

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

MATERIALS AND METHODS

Construction and characterization of somatic cell hybrids. Standard polyethylene glycol fusions were performed between lymphoblastoid cell lines from SMS patients (Greenberg *et al.*, 1991) and a thymidine kinase-deficient (TK⁻) hamster cell line, a23 (Westerveld *et al.*, 1971) as described by Lupski *et al.* (1991). Hybrids from patients 147 and 254 were constructed using a TK⁻ mouse cell line, CI-1D. For each fusion, 20-30 independent clones were isolated with cloning rings 10-14 days later and transferred to a 24-well microtiter plate. The clones were screened for the retention of the maternal or paternal homolog of human chromosome 17 by a polymerase chain reaction-based screening strategy. Cells representing each clone were obtained by trypsinization of a confluent well from the 24-well plate. One-fifth of the cells were washed with Hanks buffered saline and resuspended in 30 μ l of lysis buffer (10 mM Tris, pH 8.4, 50 mM KCl, 1.2 mM MgCl₂, 0.01% gelatin, 0.45% NP-40, 0.45% Tween 20), heated at 55°C for 1 h and at 95°C for 10 min, and centrifuged for 5 min to eliminate the debris. One microliter of lysate from each clone was used in a 100- μ l PCR reaction with STSs from two different loci mapping inside (D17S258) and outside (D17S124) the SMS critical region. It was possible to distinguish between clones containing the normal chromosome 17, which enabled amplification of both loci, and those retaining the deleted 17, which enabled the amplification of only one of the two STSs (D17S124). One clone identified as retaining the deleted chromosome 17 was selected from each fusion for more detailed analysis and for inclusion in the panel. The clone was expanded in a T75 flask, and genomic DNA was isolated as described by Miller *et al.* (1988).

Four previously described hybrids were also used in this study: hybrid DHA4, which is a clonal derivative of hybrid DH110-D1 from a SMS patient (Patel *et al.*, 1990a) and retains only a del(17)(p11.2) chromosome, hybrid HO-11, which retains 17p13.100 \rightarrow 17qter (van Tuinen *et al.*, 1988), hybrid JW-4, which retains 17p13.105 \rightarrow 17qter (van Tuinen *et al.*, 1987), and hybrid 88H3, which retains 17pter \rightarrow p11.2 (van Tuinen *et al.*, 1987).

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

TABLE 1A
Sequence-Tagged Sites for 17pter \rightarrow 17p12 Loci

Marker	Locus	Primer	Sequence (5'-3')	Size (bp)	Repeats	Heterozygosity	YACs
c1541	D17S260	IMG 3696	AATGGCTCCAAAAGGAGATATTG	\approx 147	(TGC) _n TTGG(GT) _n AT(GT) _n	50%	Not screened
cDNA of β -NACHR	CHRN1	IMG 3697	CTCCGCAACATGCTTTCCTC	450	(GT) _n	88%	No positives
		IMG 3829*	GCCTTGGCAACCCCGGGCATC	No positives			
		IMG 3830	CCATAGTGCAAATTCGCCAGTGG	No positives			
c10F4	CHRN1	IMG 4126*	CTCGAGCCCGCCGATTCAGAA	\approx 165	(GT) _n	88%	B164E1, B48B9, H164D11, B189A10, B236B9
		IMG 4128	AACCTTACTACAGGATTCACCC	No positives			
		IMG 3126	GCACCTCTTTAGTTTCAGAGCCA	No positives			
pVAW411R2	D17S124	IMG 3128	CAAAGGCTACAGCTCAGCCTACAG	350	(AT) _n N(GT) _n (AT) _n	14%	B240E1, A27A11, A56D6, A30D10
pEW405	D17S121	IMG 8311	GGCAACAGCTTTAATGATTTCTAGTGTG	260	(AT) _n N(GT) _n (AT) _n	14%	Not screened
pEW401	D17S81	IMG 8312	CTTCTTTGGCTGTATTTTCTTTTCAG	\approx 194			
		IMG 8289	CTTTACATAGGCAGAAATAGGAATC				
pVAW412R3	D17S126	IMG 8270*	GGAGATCGCTTGAACCTGGGAGG	200	(AT) _n N(GT) _n (AT) _n	14%	No positives
		IMG 3925	GTTCTTAAAGCTCCCTTGCCTCCG				
		IMG 3926	TTTCATCAGATATTTTACAGAGACC	200			

* Size of the product variable when it contains a repeat.

TABLE 1B

Sequence-Tagged Sites for 17p12 → 17cen LocI

Marker	Locus	Primer	Sequence (5'-3')	Size (bp)	Repeats	Heterozygosity	YACs
pVGI1	D17S122	IMG 4046	GGGATTTTGAAGCAAGCTTTAGG	250			CRU1, C24G1, A22U7, A217H6
pRM11-GT	D17S122	IMG 4846	GCATCTTTGGAAAGTCTTCTCTGC	≥169	(TA) _n (CA) _n (TA) _n	74%	A217H6
pS6.1-HB2	D17S446	IMG 3342*	CAGAACCACAAATGCTTTCATTC	231			R127H4, A60D11, H227H2, A276H2
OS2-GA3	D17S71	IMG 3124	GGCAGACAGACAGGCTTCTGC	166	(GAA) _n	63%	Not screened
pA10-41	D17S71	IMG 3184	TATGAGACAGCAAGTCTCTCTG	183			A185E7, A166R9
pFG2	D17S447	IMG 3517	GGTTCAGAGTGGAGGCTCTCTGC	231			Not screened
pYNM67-R5	D17S29	IMG 5313	GAITACGGAAITGAGCCATGCTC	400			A25H9
pYNM67-R5	D17S29	IMG 4473	ATTGGCTGGTACTGCTTTTCAC	222	(GTT) _n		Not screened
c1516	D17S259	IMG 1907	CAAGCCAAATCTGCTCCATG	209			R157H4, R157H5, A260R4
pFG1	D17S446	IMG 5314	GCCTCCAGCTGGGCAAGAGGTTG	660			A294E7
pEW301	D17S68	IMG 2813	TCTTCACTCCATAGCTATGAGCC	252			A164B2, R172C7, A276H1, R39B13, R11C4
		IMG 2917	CCAGTCTCAGAGATTTAATAATC				
		IMG 2918 ^a	GTACTCTCAGCTATTCGGAGGCTG				
		IMG 2915	TCTAACCCCAAGCAAGCCACTGC				
		IMG 2916	GAGACCTGGGATGATAATGATGG				
		IMG 4094	TTTCAACTCAAGCTGGGCTGTTC				
		IMG 4086	TTCTTGTGTAATGAATGGACAAC				
		IMG 3263	GTCTTCTCCCAACTAATAATAC				
		IMG 3264	GCCTTTCAACCATACTCATCACC				

* Primers for pRM11-GT from Lupski et al. (1991).

^a Primer has some homology to the *Alu* repeat element.

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

Polymerase chain reaction. The PCR was performed using standard conditions in a 50- μ l reaction volume in a mixture containing 1 μ M of each oligodeoxynucleotide primer with the exceptions noted below: 250 μ M of each dNTP, 5 μ l of 10× PCR buffer (500 mM KCl, 120 mM Tris-HCl, pH 8.0, 1.5 mM MgCl₂, and 0.01% gelatin), and 126 units of Amplitaq (Cetus) DNA polymerase. The amplification conditions were an initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 55°C or as specified below, and extension for 2 min at 72°C in a first-generation automated thermal cycler (Perkin-Elmer/Cetus). For STSs developed from c1516 and RM11-GT, an annealing temperature of 60 and 65°C, respectively, was used. When a primer had homology with the *Alu* repeat, it was used at 0.1 μ M. The PCR products were visualized by electrophoresing 10 μ l of the reaction in a 1.3% agarose gel in 1× TBE buffer.

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

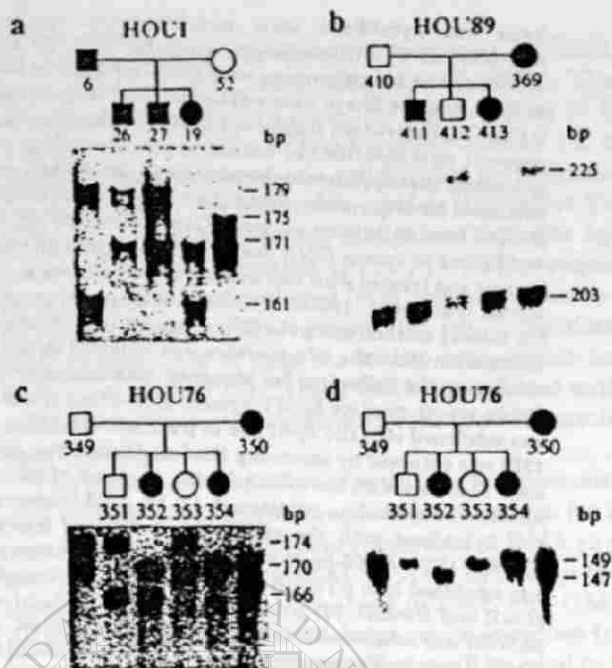


FIG. 1. Mendelian inheritance of simple repeat polymorphisms in 17p. Approximately 100 ng of genomic DNA from members of nuclear families segregating CMT1A was subjected to amplification with primers for the indicated loci and analyzed by electrophoresis in 6% polyacrylamide sequencing gels, as described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

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

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

To establish relative probe order along the chromosome, two probes, one biotinylated and one digoxigenin-labeled, were mixed and hybridized simultaneously to metaphase chromosomes. The biotinylated probe was subsequently labeled orange-red by incubation in avidin-Texas Red, biotinylated goat anti-avidin antibody, and avidin-Texas Red. The digoxigenin-labeled probe was labeled green with

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

RESULTS

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

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

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

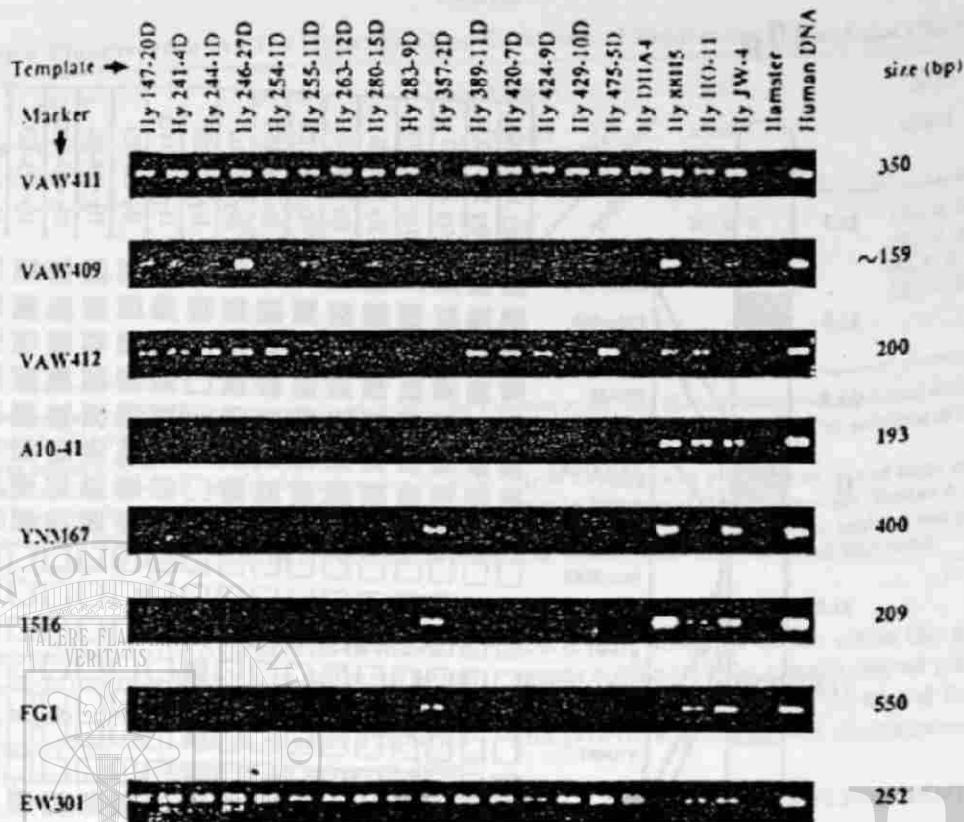


FIG. 2. PCR amplification products from representative loci in 17p obtained from somatic cell hybrids. 150 ng of genomic DNA from each hybrid was subjected to PCR amplification with primers representing each of the loci, as described under Materials and Methods. Ten microliters of the PCR reaction was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

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

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

Five markers were deleted in most of the patients (D17S446, D17S258, D17S29, D17S71, and D17S445)

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

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

17p

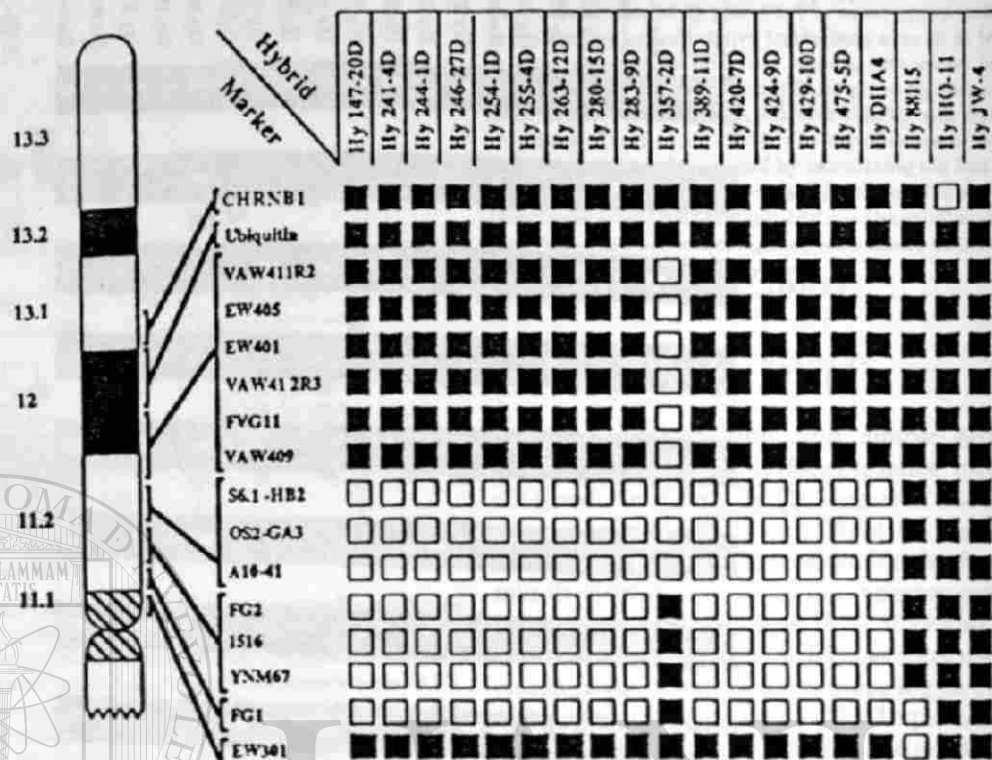


FIG. 3. Deletion status of 17p markers in the hybrids determined by PCR. An ideogram illustrating the short arm of chromosome 17, deletion intervals, and the relative position of individual markers are shown on the left. An open box indicates that the marker is deleted, while a solid box indicates that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

DISCUSSION

Much attention has been focused on efforts to characterize the human genome at two levels: the gross physical and genetic map of chromosomes and their DNA sequence (McKusick and Ruddle, 1987). The molecular dissection of specific chromosomal segments and the acquisition of region-specific polymorphic sequences are significant steps toward these goals. We describe the development of a somatic cell hybrid panel from SMS patients that should be very useful for the ordering of probes within the 17p11.2-17p12 intervals and provide valuable information on the molecular basis of the syndrome. The density of well-characterized markers available in this region did not allow us to differentiate the deletions in these patients except in patient 92-357. The extent of the region encompassed by the deletion in patient 92-357 has an interesting implication. This patient carries a deletion spanning the markers LEW401 (D17S61), VAW409 (D17S122), and VAW412 (D17S125), which span a genetic distance of ≈ 10 cM (Wright *et al.*, 1990) and have been demonstrated to map within a 1.5-Mb region duplicated in patients affected with CMT1A (Lupski *et al.*, 1991; our unpublished observations). This suggests that the proximal junction of the CMT1A-associated duplication may closely flank the distal breakpoint in del(17)(p11.2) patients. Patient 92-

357, who is deleted for all three markers, does not show any clinical signs of CMT1A, although further electrophysiological evaluation is required. This suggests that monosomy for the gene(s) in the CMT1A-associated duplication interval does not lead to clinical signs of CMT1A. The deletion in this patient extends more telomeric to include the VAW411R2 (D17S124) locus, which is not duplicated in CMT1A patients.

The gene for the β subunit of the muscle acetylcholine receptor had been previously mapped to the 17p11.2-17p12 region (Beeson *et al.*, 1989). Our studies clearly show that this gene maps to 17p13. This is based on PCR analysis of the hybrid panel using two different primer sets specific for this gene and FISH. Thus, the chromosomal location of this gene, which is much more distal than the markers mapping within the CMT1A-associated duplication, makes it an unlikely candidate for this disease.

FISH mapping confirmed the hybrid panel results. Probes from three of the seven intervals defined by the hybrid panel were assigned to cytogenetic band locations. In addition, FISH mapping placed cYNM67 proximal to c1516, c15F4 (NACHRB) in 17p13, distal VAW409, and placed two additional markers, c1541 (c17-68 (CHRNE) distal to c15F4 (CHRN1) in 17p

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

TABLE 2
Two-Color Fluorescence *In Situ* Hybridization Ordering of Probes on Metaphase Chromosomes

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

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

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

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

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

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

In conclusion, the use of somatic cell hybrids and PCR as well as fluorescence *in situ* hybridization has proved to be a rapid and reliable method for mapping probes

and for the study of SMS, while the development of reagents such as STSs, simple repeat polymorphisms, and region-specific YACs will be useful for further physical and genetic studies of this chromosomal segment.

ACKNOWLEDGMENTS

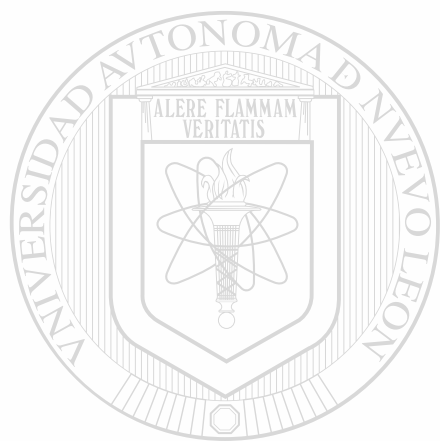
We thank Dr. David Ledbetter for providing hybrids DHA4, HO-11, JW-4, and 88H5. We thank Dr. Jim Patrick for providing the cDNAs for the β and ϵ subunits of the acetylcholine receptor gene. This research was supported by a Muscular Dystrophy Association Task Force on Genetics Grant, an Advanced Technology Program Grant from the state of Texas, and NIH Grant R01 NS-27042 to J.R.L. and P.I.P. Support was also obtained from a Baylor Mental Retardation Research Center Grant MD 24062-02 and a Human Genome Center Grant (HG 00210-01). Work of B.J.T. was performed under the auspices of the U.S. Department of Energy at LLNL under Contract W-7405-ENG-48 with support from USPHS Grant HG-00256-01. J.R.L. acknowledges support from the Pew Scholars Program in Biomedical Sciences. V.G. was a recipient of a Muscular Dystrophy Association postdoctoral fellowship. The hybrid Hy357-2D will be available from the NIGMS Mutant Cell Repository (Camden, NJ).

REFERENCES

- Barker, D., Wright, E., Nguyen, K., Cannon, L., Fain, P., Goldgar, D., Bishop, D. T., Carey, J., Baty, B., Kivlin, J., Willard, H., Waye, J. S., Greig, G., Leinwand, L., Nakamura, Y., O'Connell, P., Leppert, M., LaJouel, J.-M., White, R., and Skolnick, M. (1987). Gene for von Recklinghausen neurofibromatosis is in the pericentromeric region of chromosome 17. *Science* 236: 1100-1102.
- Beeson, D., Brydon, M., and Newsom-Davis, J. (1989). Nucleotide sequence of human muscle acetylcholine receptor beta-subunit. *Nucleic Acids Res.* 17: 4391.
- Bird, A. (1987). CpG islands as gene markers in the vertebrate nucleus. *Trends Genet.* 3: 342-347.
- Brownstein, B. H., Silverman, G. A., Little, R. D., Burke, D. T., Korsmeyer, S. J., Schlessinger, D., and Olson, M. V. (1989). Isolation of single-copy human genes from a library of yeast artificial chromosome clones. *Science* 244: 1348-1351.
- Burke, D. T., Carle, G. F., and Olson, M. V. (1987). Cloning of large segments of exogenous DNA into yeast by means of artificial chromosome vectors. *Science* 236: 806-812.
- Carlock, L. R., Skarecky, D., Dana, S. L., and Wasnuth, J. J. (1985).

- Deletion mapping of human chromosome 5 using chromosome-specific DNA probes. *Am. J. Hum. Genet.* 37: 839-852.
- Casanova, J.-L., Pannetier, C., Jaulin, C., and Kourilsky, P. (1990). Optimal conditions for directly sequencing double-stranded PCR products with Sequenase. *Nucleic Acids Res.* 18: 4028.
- Charcot, J. M., and Marie, P. (1886). Sur une forme particuliere d'atrophie musculaire progressive souvent familiale debutant par les pieds et les jambes et atteignant plus tard les mains. *Rev. Med.* 6: 97-138.
- Elder, F. F. B., Nichols, M. M., Hood, O. J., and Harrison, W. R. (1985). Unbalanced translocation (15;17)(q13;p13.3) with apparent Prader-Willi syndrome but without Miller-Dieker syndrome. *Am. J. Med. Genet.* 20: 519-524.
- Elvin, P., Slynn, G., Black, D., Graham, A., Butler, R., Riley, J., Anand, R., and Markham, A. F. (1990). Isolation of cDNA clones using yeast artificial chromosome probes. *Nucleic Acids Res.* 18: 3913-3917.
- Fain, P. R., Ledbetter, D. H., and Solomon, E. (1991). Second international workshop on human chromosome 17 mapping. *Cytogenet. Cell Genet.* 57: 63-77.
- Franco, B., Rincon-Limas, D., Nakamura, Y., Patel, P. I., and Lupski, J. R. (1990). An *MspI* RFLP at the D17S238 locus in 17p11.2. *Nucleic Acids Res.* 18: 7196.
- Greenberg, F., Guzzetta, V., Montes de Oca-Luna, R., Magenis, E., Smith, A. C. M., Richter, S. F., Kondo, L., Dobyns, W. B., Patel, P. I., and Lupski, J. R. (1991). Molecular analysis of the Smith-Magenis syndrome: A possible contiguous-gene syndrome associated with del(17)(p11.2). *Am. J. Hum. Genet.* 49: 1207-1218.
- Guzzetta, V., Montes de Oca-Luna, R., Lupski, J. R., and Patel, P. I. (1991). Isolation of region-specific and polymorphic markers from chromosome 17 by restricted *Alu*-polymerase chain reaction. *Genomics* 9: 31-36.
- Kwiatkowski, T. J., Jr., Zogbi, H. Y., Ledbetter, S. A., Ellison, K. A., and Chinault, A. C. (1990). Rapid identification of yeast artificial chromosome clones by matrix probing and crude lysate PCR. *Nucleic Acids Res.* 18: 7191-7192.
- Ledbetter, S. A., Nelson, D. L., Warren, S. T., and Ledbetter, D. H. (1990). Rapid isolation of DNA probes within specific chromosome regions by interspersed repetitive sequence (IRS) PCR. *Genomics* 6: 475-481.
- Lupski, J. R., Montes de Oca-Luna, R., Slaugenhaupt, S., Pericak, L., Guzzetta, V., Trask, B. J., Saucedo-Cardenas, O., Barker, D. F., Killian, J. M., Garcia, C. A., Chakravarti, A., and Patel, P. I. (1991). DNA duplication associated with Charcot-Marie-Tooth disease type 1A. *Cell* 66: 219-232.
- McKusick, V. A., and Ruddle, F. H. (1987). Toward a complete map of the human genome (editorial). *Genomics* 1: 103-106.
- Mead, D. A., Szczesna-Skorupa, E., and Kemper, B. (1986). Single-stranded DNA 'blue' T7 promoter plasmids: A versatile tandem promoter system for cloning and protein engineering. *Protein Eng.* 1: 67-76.
- Middleton-Price, H. R., Harding, A. E., Montiero, C., Berciano, J., and Malcolm, S. (1990). Linkage of hereditary motor and sensory neuropathy type I to the pericentromeric region of chromosome 17. *Am. J. Hum. Genet.* 46: 92-94.
- Miller, S. A., Dykes, D. D., and Polesky, H. F. (1988). A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res.* 16: 1215.
- Nelson, D. L., Ledbetter, S. A., Corbo, L., Victoria, M. F., Ramirez-Solis, R., Webster, T. D., Ledbetter, D. H., and Caskey, C. T. (1989). *Alu* polymerase chain reaction: A method for rapid isolation of human-specific sequences from complex DNA sources. *Proc. Natl. Acad. Sci. USA* 86: 6686-6690.
- Nelson, D. L., Ballabio, A., Victoria, M. F., Pieretti, M., Bies, R. D., Gibbs, R. A., Maley, J. A., Chinault, A. C., Webster, T. D., and Caskey, C. T. (1991). *Alu*-primed polymerase chain reaction for regional assignment of 110 yeast artificial chromosome clones from the human X chromosome: Identification of clones associated with a disease locus. *Proc. Natl. Acad. Sci. USA* 88: 6157-6161.
- Oberle, J., Camerino, G., Klopfer, C., Moisan, J. P., Grzeschik, K. J., Hellkuhl, B., Hors-Cayle, M. C., Van-Cong, N., Weil, D., and Mandel, J. L. (1986). Characterization of a set of X-linked sequences of a panel of somatic cell hybrids useful for the regional mapping the human X chromosome. *Hum. Genet.* 72: 43-49.
- Patel, P. I., Franco, B., Garcia, C., Slaugenhaupt, S. A., Nakamura, Y., Ledbetter, D. H., Chakravarti, A., and Lupski, J. R. (1990a). Genetic mapping of autosomal dominant Charcot-Marie-Tooth disease in a large French-Canadian kindred: Identification of new link markers on chromosome 17. *Am. J. Hum. Genet.* 46: 801-809.
- Patel, P. I., Garcia, C., Montes de Oca-Luna, R., Malamut, R., Franco, B., Slaugenhaupt, S., Chakravarti, A., and Lupski, J. (1990b). Isolation of a marker linked to the Charcot-Marie-Tooth disease type 1A gene by differential *Alu*-PCR of human chromosome 17-retaining hybrids. *Am. J. Hum. Genet.* 47: 926-934.
- Raeymaekers, P., Timmerman, V., de Jonghe, P., Swerts, L., Ghevel, J., Martin, J. J., Muyllie, L., de Winter, G., Vandenberghe, A., and van Broeckhoven, C. (1989). Localization of the mutation in extended family with Charcot-Marie-Tooth neuropathy (Hx11). *Am. J. Hum. Genet.* 45: 953-958.
- Raeymaekers, P., Timmerman, V., Nelis, E., de Jonghe, P., Hoogdijk, J. E., Bass, F., Barker, D. F., Martin, J. J., de Visser, Bolhuis, P. A., van Broeckhoven, C., and the HMSN collaborative research group. (1991). Duplication in chromosome 17p11.2 Charcot-Marie-Tooth neuropathy type 1A (CMT 1A). *Neuromuscular Disorders* 1: 93-97.
- Ray, R., Rincon-Limas, D., Wright, R., Davis, S., Lupski, J. R., and Patel, P. I. (1990). Three polymorphisms at the D17S29 locus. *Nucleic Acids Res.* 18: 4958.
- Riley, J., Butler, R., Ogilvie, D., Finniear, R., Jenner, D., Powell, Anand, R., Smith, J. C., and Markham, A. F. (1990). A novel method for the isolation of terminal sequences from yeast artificial chromosome (YAC) clones. *Nucleic Acids Res.* 18: 2887.
- Schmickel, R. D. (1986). Contiguous gene syndromes: A component recognizable syndromes. *J. Pediatr.* 109: 231-241.
- Schweizer, D. (1976). Reverse fluorescent chromosome banding with chromomycin and DAPI. *Chromosoma* 58: 307-324.
- Skre, H. (1974). Genetic and clinical aspects of Charcot-Marie-Tooth's disease. *Clin. Genet.* 6: 98-118.
- Smith, A. C. M., McGavran, L., Robinson, J., Waldstein, G., Meriflane, J., Zonona, J., Reiss, J., Lahr, M., Allen, L., and Magenis (1988). Interstitial deletion of (17)(p11.2p11.2) in nine patients. *Am. J. Med. Genet.* 24: 393-414.
- Southern, E. M. (1975). Detection of specific sequences among fragments separated by gel electrophoresis. *J. Mol. Biol.* 98: 503-511.
- Stratton, R. F., Dobyns, W. B., Greenberg, F., Desana, J. B., Moore, C., Fidone, G., Runge, G. H., Feldman, P., Sekhon, G. S., Paik, R. M., and Ledbetter, D. H. (1986). Interstitial deletion (17)(p11.2p11.2): Report of six additional patients with a new chromosome deletion syndrome. *Am. J. Med. Genet.* 24: 421-432.
- Tooth, H. (1886). "Peroneal Type of Progressive Muscular Atrophy." H. K. Lewis, London.
- Trask, B. J. (1992). DNA sequence localization in metaphase and terphase cells by fluorescence *in situ* hybridization. In "Function Organization of the Nucleus—A Laboratory Guide" (B. A. Hekalo and S. C. R. Elgin, Eds.), Methods in Cell Biology, Vol. 35: 3-35, Academic Press, San Diego.
- Trask, B. J., Massa, H. F., Kenwick, S., and Gitschier, J. (1989). Mapping of human chromosome Xq28 by two-color fluorescent *in situ* hybridization of DNA sequences to interphase cell nuclei. *Am. J. Hum. Genet.* 48: 1-15.
- Vance, J. M., Nicholson, G. A., Yamaoka, L. H., Stajich, J., Stem, C. S., Speer, M. C., Hung, W.-Y., Roses, A. D., Barker, D., and Pericak-Vance, M. A. (1989). Linkage of Charcot-Marie-Tooth neuropathy type 1a to chromosome 17. *Exp. Neurol.* 104: 186-189.

- vance, J. M., Barker, D., Yamaoka, L. H., Stajich, J. M., Loprest, L., Hung, W.-Y., Fischbeck, K., Roses, A. D., and Pericak-Vance, M. A. (1991). Localization of Charcot-Marie-Tooth disease type 1a (CMT 1a) to chromosome 17p11.2. *Genomics* 9: 623-628.
- van Tuinen, P., Rich, D. C., Summers, K. M., and Ledbetter, D. H. (1987). Regional mapping panel for human chromosome 17: Application to neurofibromatosis type 1. *Genomics* 1: 374-381.
- van Tuinen, P., Dobyns, W. B., Rich, D. C., Summers, K. M., Robinson, T. J., Nakamura, Y., and Ledbetter, D. H. (1988). Molecular detection of microscopic and submicroscopic deletions associated with Miller-Dieker syndrome. *Am. J. Hum. Genet.* 43: 587-596.
- Verma, R. S., and Babu, A. (1989). "Human Chromosomes: Manual of Basic Techniques," Pergamon, New York.
- Wallace, M. R., Marchuk, D. A., Andersen, L. B., Letcher, R., Odeh, H. M., Saulino, A. M., Fountain, J. W., Brereton, A., Nicholson, J., Mitchell, A. L., Brownstein, B. H., and Collins, F. S. (1990). Type 1 neurofibromatosis gene: Identification of a large transcript disrupted in three NF1 patients. *Science* 249: 181-186.
- Weber, J. L. (1990). Human DNA polymorphisms based on length variations in simple-sequence tandem repeats. In *Genome Analysis Volume 1: Genetic and Physical Mapping*, pp. 159-181, Cold Spring Harbor Laboratory Press.
- Westerveld, A., Visser, R. P. L. S., Khan, P. M., and Bootsma, D. (1971). Loss of human genetic markers in man-Chinese hamster somatic cell hybrids. *Nature New Biol.* 234: 20-24.
- Wright, E. C., Goldgar, D. E., Fain, P. R., Barker, D. F., and Skolnick, M. H. (1990). A genetic map of human chromosome 17p. *Genomics* 7: 103-109.
- Yunis, J. J. (1976). High resolution of human chromosomes. *Science* 191: 1268-1270.



UANL

UNIVERSIDAD AUTÓNOMA DE NUEVO LEÓN

®

DIRECCIÓN GENERAL DE BIBLIOTECAS

