Am. J. Hum. Genet. 44:51–57, 1989

Fine Structure DNA Mapping Studies of the Chromosomal Region Harboring the Genetic Defect in Neurofibromatosis Type 1

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Summary

To better map the location of the von Recklinghausen neurofibromatosis (NF1) gene, we have characterized a somatic cell hybrid designated 7AE-11. This microcell-mediated, chromosome-transfer construct harbors a centromeric segment and a *neo*-marked segment from the distal long arm of human chromosome 17. We have identified 269 cosmid clones with human sequences from a 7AE-11 library and, using a panel of somatic cell hybrids with a total of six chromosome 17q breakpoints, have mapped 240 of these clones on chromosome 17q. The panel included a hybrid (NF13) carrying a der(22) chromosome that was isolated from an NF1 patient with a balanced translocation, t(17;22) (q11.2;q11.2). Fifty-three of the cosmids map into a region spanning the NF13 breakpoint, as defined by the two closest flanking breakpoints (17q11.2 and 17q11.2-q12). RFLP clones from a subset of these cosmids have been mapped by linkage analysis in normal reference families, to localize the NF1 gene more precisely and to enhance the potential for genetic diagnosis of this disorder. The cosmids in the NF1 region will be an important resource for testing DNA blots of large-fragment restriction-enzyme digests from NF1 patient cell lines, to detect rearrangements in patients' DNA and to identify the 17;22 NF1 translocation breakpoint.

Introduction

Genetic studies with DNA markers have localized the gene for von Recklinghausen neurofibromatosis (NF1) to chromosome 17 (Barker et al. 1987; Seizinger et al. 1987). Subsequent genetic studies have indicated that a centromeric marker, p3.6 (D17Z1), and the locus defined by DNA probe pHHH202 (D17S33) are very close to the gene (White et al. 1987) and that little evidence can be found for genetic heterogeneity in this disorder (linkage data summarized in Skolnick et al. 1987). Physical studies have indicated that the most closely linked marker, pHHH202, maps to the proximal long arm of chromosome 17 (vanTuinen et al. 1987). This observation is supported by a report of an NF1 patient carrying a chromosome 1;17 balanced translocation with its breakpoint at chromosome 17q11.2 (Schmidt et al. 1987).

We would like to take advantage of this mapping information to identify tightly linked markers that will permit the cloning of the gene responsible for NF1. One approach is to create somatic cell hybrids containing fragments of chromosome 17, by using the method of microcell-mediated chromosome transfer (Killary and Fournier 1984). Chromosomes from a human × rodent somatic cell hybrid carrying a genetically marked chromosome 17 are broken during the process of transfer to recipient cells. The resulting hybrid lines can be

Received July 15, 1988; revision received September 16, 1988.

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0002-9297/89/4401-0011$02.00

characterized with respect to the segments transferred, because a detailed genetic map for human chromosome 17 has been constructed (Nakamura et al. 1988). Southern blots of DNA from the microcell hybrid cell lines can be probed with elements of this map to determine the presence or absence of a particular chromosome 17 locus. This approach can be optimized if a panel of somatic cell hybrids containing translocation breakpoints that subdivide the region of interest is available. This allows the sorting of probes so that only those in the target region need to be analyzed further.

In this report, we characterize a microcell hybrid designated 7AE-11 and describe the mapping, with a somatic cell hybrid panel, of 240 cosmid clones from this cell line. The panel includes a cell line (NF13) carrying a der(22) chromosome isolated from a lymphoblastoid cell line derived from an NF1 patient (lab no. 11405) whose karyotype showed a constitutional 17;22 translocation. Cosmid clones that map into the breakpoints adjacent to that of NF13 can be used as probes to examine large-fragment restriction-enzyme digests of DNA from the lymphoblastoid cell line of patient 11405. This allows us to search regions a few hundred kilobases long for a restriction-enzyme fragment that has been altered by the translocation event. If a new, large fragment, present only in the translocation, can be detected with one of the cosmids from the target region, we can design experiments to clone the breakpoint region and, eventually, the NF1 gene itself.

Material and Methods

Microcell-mediated Chromosome Transfer

Cell line 7AE-11 is a primary human fibroblast × rat hepatoma somatic cell hybrid line that retains fragments of human chromosome 17. It was isolated in the following manner: human foreskin fibroblasts were transfected with the ZIP-*neo* SV(X)1 retrovirus and were selected to grow in 2 mg G418/ml (Lugo et al. 1987). The infected fibroblasts were used as donor cells in a microcell fusion (Killary and Fournier 1984) with the murine line LtK$^-$, aprt$^-$ (Wigler et al. 1979). The hybrid cells were isolated by selection in hypoxanthine/aminopterin/thymidine (HAT), G418, and 2,6-diaminopurine (DAP) media. This procedure selects for karyotypically simple hybrids that contain a human chromosome 17 and the ZIP-*neo*SV(X)1 vector. One such hybrid clone, L(17n)E, which contains a *neo*-marked human chromosome 17, was used as a donor to make secondary microcell hybrids with the rat hepatoma cell line PCTA-7A (Wynshaw-Boris et al. 1984). The hybrids resulting from this secondary fusion retain human chromosome 17—or microcell fusion–generated fragments thereof—in a rat hepatoma background. These cell lines were characterized by DNA marker analysis with 12 mapped sequences from chromosome 17. All cells were cultured in 1:1 Hams F12:Dulbecco's modified Eagle's medium with 10% FBS. The *neo*-containing hybrids were grown in medium containing 500 μg G418/ml.

Somatic Cell Hybrid Lines

The somatic cell hybrid lines used for subregional localization included microcell transfers 7AE-11, L(17n)E, and L(17n)C; the last is an independent isolate, from the procedure described above, that contains the long arm of human chromosome 17. The balance of the lines were human × mouse somatic cell hybrids described by vanTuinen et al. (1987): SP3, a hybrid containing a 15;17 translocation with its breakpoint at 17q11.2; P12.3B, a hybrid containing a 15;17 translocation from a patient with acute promyelocytic leukemia with a breakpoint in 17q11.2 or 12; and MH-41, a 17;19 hybrid with a breakpoint in 17q23. A new hybrid cell line, NF13, contains a der(22) chromosome from NF patient 11405; this patient showed a balanced translocation, t(17;22) (q11.2;q11.2). The clinical and cytogenetic data for patient 11405, as well as details of the construction of hybrid NF13, are presented by Ledbetter et al. (1989) elsewhere in this issue. Figure 1 summarizes the somatic cell hybrid panel in a graphic format.

Cosmid Library Construction and Screening

A cosmid library for hybrid 7AE-11 was constructed in a pWE15 cosmid vector (Stratagene, La Jolla, CA). 7AE-11 genomic DNA was isolated according to Bell et al.'s (1981) procedure, modified to reduce shearing of DNA. Standard methods were used to accomplish partial *Sau*3A digestion and sucrose-density gradient centrifugation of the genomic DNA. Ligations took place in the presence of 10% PEG8000; the ligation mixture was packaged with Gigapack™ (Stratagene). Colony transfers and probes with radiolabeled human DNA were carried out as described elsewhere (Cavenee et al. 1984; Nakamura et al. 1987).

Pulsed-Field Gel Electrophoresis

Pulsed-field gel electrophoresis was carried out with a Beckman Geneline 1000™ transverse alternating field electrophoresis (TAFE) device. DNA was isolated in agarose plugs as described elsewhere (Julier and White

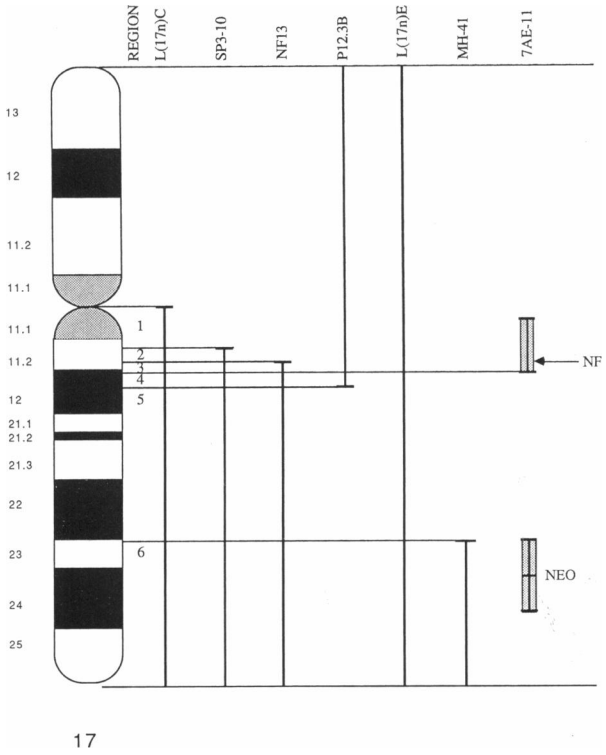

Figure 1 Karyogram of human chromosome 17, showing somatic cell hybrid translocation breakpoints. The regions defined by these breakpoints are numbered on the right of the chromosome. The approximate extents of the segments of chromosome 17 present in hybrid 7AE-11 are shaded.

1988). TAFE runs were carried out with 60–75-s switch times in 1% agarose gels, in 0.5 × TBE buffer at 250 V (constant voltage) for 20–22 h. For libraries from pulsed-field gel fragments, 30 μg of 7AE-11 DNA was loaded into a prep well and electrophoresed; the gel was then cut into 2-mm slices. DNA was electrophoretically isolated from each slice, extracted with phenol, precipitated with EtOH, and digested with *Bam*HI. A standard agarose gel was run with an aliquot of each fraction, and a DNA blot was prepared. For construction of the library, size fractions were selected by probing this filter with the probe of interest. The *Bam*HI fragments were cloned into Lambda Zap™ (Stratagene) and packaged as described above. Human clones were identified by probing with radiolabeled human genomic DNA.

Genotyping and Analysis of Data

Methods for the preparation of probe DNA, digestion with restriction enzymes, Southern blotting, radiolabeling of DNA, and hybridization have all been described elsewhere (Barker et al. 1984; Cavenee et al. 1984), except that we made the following modifications: DNA blots were made with alkaline transfer to charged nylon membrane (Gelman Biotrace RP™) according to the method of Reed and Mann (1985); radiolabeling of DNA probes was done by the random primer method (Feinberg and Vogelstein 1984); and our prehybridization and hybridization protocols substituted sheared, denatured human placental DNA for salmon sperm DNA to suppress hybridization of labeled human repetitive sequences when whole cosmids were used as probes.

DNA from 59 reference families, 40 of which constitute the CEPH (Human Polymorphism Study Center) collection of families (White et al. 1985; Dausset 1986) were used for linkage studies. DNA was extracted from blood or from lymphoblastoid cell lines by the method of Bell et al. (1981).

Genotypic data were entered into a computer data base, and the output listings were rechecked against the autoradiograms to avoid clerical errors. The computer program LINKAGE (Lathrop et al. 1984, 1985) was used for two-point and multipoint genetic linkage analysis. All data for closely linked markers (theta <.15) were reexamined by family to determine whether certain families demonstrated clusters of recombination events. Data from these families were rechecked to determine whether misidentification of parental or grandparental genotypes was inflating recombination estimates.

The probes pYNM67 (D17S29), pABL10-41 (D17S71), p3.6 (D17Z1), and pHHH202 (D17S33) have been described elsewhere (Barker et al. 1987; Nakamura et al. 1988). The *Eco*RI RFLP for pHAT-A1 (ERBA1) has been described elsewhere (Mathieu-Mahul et al. 1985), and we identified a new RFLP with *Msp*I by using a 3.0-kb *Xho*I fragment from this probe (table 1). All other probes were developed as part of the present study.

Results

Characterization of Cell Line 7AE-11

A panel of mapped chromosome 17 DNA markers (Nakamura et al. 1988) was used to test microcell transfer lines for segments of chromosome 17. 7AE-11 was selected for further study because analysis of DNA blots suggested that it contained only on the order of 10–20 million bp of human DNA, including chromosome 17 alpha-satellite sequences and the NF1-linked DNA marker locus, HHH202. 7AE-11 was negative for all other chromosome 17 markers, save for G418 resistance

Table 1

RFLPs at NF1 Locus

Locus No.	Probe	Region	Enzyme	Alleles (kb)	Heterozygosity
8.....	pYNM67	17p	*Taq*I	3.8/3.2	.41
			*Rsa*I	3.0/1.3	.18
			*Rsa*I	1.8/0.8	.31
9.....	pABL10-41	17p	*Msp*I	2.4/1.9	.50
			*Pvu*II	2.7/2.6	.29
1.....	p3.6	1	*Eco*RI	2.0/1.1,0.9	.45
2.....	c11-2B	2	*Msp*I (INS/DEL)	2.5/1.5	.40
3.....	pHHH202	2	*Rsa*I	2.1/1.8	.49
4.....	pTH17.19	2	*Bgl*II	16.0/12.0	.52
5.....	p11-3C4.2	2	*Msp*I	1.5/0.8	.30
10.....	p11-2C11.1	3	*Taq*I	6.0/4.0,2.0	.45
	p11-2C11.5	3	*Taq*I	2.0/1.8	.40
	p11-2C11.7	3	*Bam*HI	4.5/4.4	.40
11.....	p11-2F9.2	3	*Taq*I	4.4/3.2	.50
			*Pvu*II	3.1/2.8	.50[a]
			*Hin*dIII	4.5/4.2	.50[a]
	p11-2F9.8	3	*Bgl*II	7.5/6.0	.30
			*Taq*I	12.0/6.5	.25
6.....	pHAT-A1	4	*Msp*I	2.3/2.0	.37
			*Eco*RI	18.0/8.5	.09[a]

[a] Not run.

and the locus defined by pTHH59 (D17S4), both of which map distal to 17q23. These results indicate that 7AE-11 has at least two segments of chromosome 17: one near the centromere and another distal to 17q23. The presence of the HHH202 locus suggested that the centromeric chromosome 17 fragment was likely to contain the NF1 gene as well.

Cosmid Screening

A cosmid library was constructed from hybrid 7AE-11. Human DNA was used to screen four genome equivalents of the 7AE-11 library. Of 290 first-round positives, 267 passed a second round of screening. DNA prepared from these cosmids was characterized on Southern blots of the somatic cell hybrid panel (see fig. 1). Whole cosmids were used as probes in the presence of human DNA competitor. Those cosmids mapping distal to the MH-41 breakpoint (fig. 1) were excluded from further analysis, as they represented clones from the *neo*-marked segment of chromosome 17 (region 6).

Locus HHH202, which is distal to the SP3 breakpoint (vanTuinen et al. 1987), was found to be proximal to the NF13 breakpoint. Although this result suggested that NF1 might not be present in 7AE-11, 34 cosmids from the 7AE-11 library mapped distal to the NF13 breakpoint yet proximal to that of P12.3B. Therefore, 7AE-11 extends distally to the NF13 breakpoint and must contain it. We were able to infer a 7AE-11 chromosome 17q11.2-12 breakpoint proximal to that in cell line P12.3B, by using the following logic: The genes for *erb*A1 and *erb*B2 have been mapped distal to the NF13 breakpoint but proximal to the P12.3B breakpoint. Because these genes are not present in cell line 7AE-11 yet cosmids from 7AE-11 span the NF13 breakpoint, we have placed its breakpoint between those of NF13 and P12.3B. This conclusion means that the two oncogenes are unlikely to represent candidate loci for the NF1 gene.

These results are summarized in graphic form in figure 2; 30% of the cosmids mapped to the NF1 region (fig. 1, regions 1–3), and 63% mapped to the *neo* insertion region (region 6). About 7% of the cosmids could not be mapped because results on the somatic cell panel were unacceptable.

DNA Probes for the NF1 Region

Two methods were used to isolate and develop RFLP probes from cell line 7AE-11: (1) standard screening of

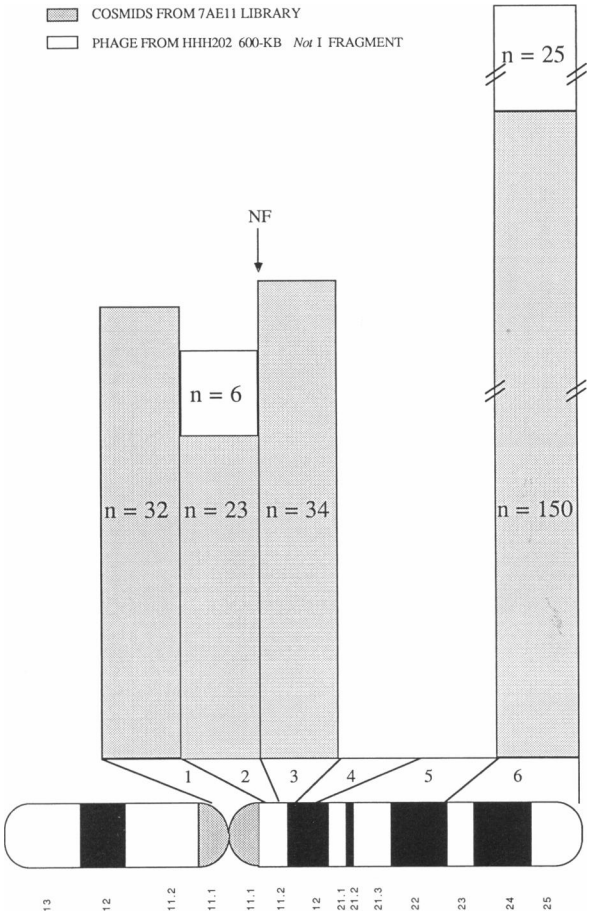

Figure 2 Histogram showing the distribution of clones from the 7AE-11 cell line on chromosome 17. The breakpoints shown are the same as those in fig. 1.

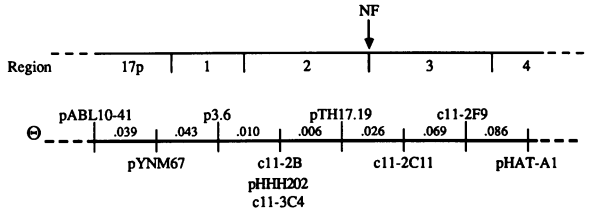

Figure 3 Genetic linkage map for the NF1 region. Recombination estimates (θ) and physical assignments are derived from the data in table 2. The location of the NF1 locus was defined by hybrid cell line NF13.

the cosmids that mapped into the regions adjacent to the NF13 breakpoint and (2) isolation of clones from the HHH202 600-kb *Not*I fragment.

Whole cosmids were used to probe Southern blots of DNA, from six individuals, cut with a panel of 10 restriction enzymes. Four cosmids from the 7AE-11 screen, c11-2B (D17S81), c11-3C4 (D17S84), c11-2C11 (D17S83), and c11-2F9 (D17S85) showed RFLPs (see table 1); subclones or whole cosmids were used as probes.

Because genetic studies have indicated that marker locus HHH202 is tightly linked to the NF1 gene, and because HHH202 resides on a relatively large (600-kb) *Not*I fragment, we decided to generate new RFLP probes from that fragment, by using the 7AE-11 cell line to enrich for the NF1 region. To build the *Not*I fragment library, ~25 ng of 7AE-11 DNA from the 600-kb-size class detected by pHHH202 was digested with *Bam*HI and ligated to a 1:1 ratio of the Lambda Zap™ phage vector ends. Of the 50,000 phage packaged, 31 hybridized to radiolabeled human DNA and were mapped by means of the somatic cell hybrid panel (see fig. 2). One clone of the six mapping to the same region as pHHH202, a clone designated pTH17.19 (D17S82), revealed a *Bgl*II RFLP. pTH17.19 is very tightly linked to the HHH202 locus (see fig. 3) and detects the same *Not*I, *Mlu*I, and *Bss*HI restriction-enzyme fragments as pHHH202 (data not shown).

Linkage Analysis

Genotypic data from markers derived from the same recombinant DNA clone (loci 8–11) were haplotyped prior to linkage analysis. Linkage analysis was performed with the LINKAGE programs (Lathrop et al. 1985). Loci were divided into initial subgroups, on the basis of two-locus lod scores and physical mapping against a hybrid cell panel. Flanking markers from a previously determined map of chromosome 17 (Nakamura et al. 1988) or from physical localizations (table 2A) were used to establish a trial map order. Recombination estimates under the trial order were used to test alternate gene orders within subgroups. Because c11-2B, pHHH202, and p11-3C4 (loci 2, 3, and 5) showed no recombination among each other, their order could not be resolved (data not shown). Likelihoods for different permutations and orientations within subgroups were tested to determine support for order within subgroups; these results are summarized in table 2B. Multipoint linkage analysis was carried out to establish estimates of recombination under the best-supported order. These results are shown in figure 3.

Discussion

This series of experiments has indicated that the microcell hybrid 7AE-11 is an effective tool for iden-

Table 2

Physical and Genetic Mapping of Loci in the NF1 Region

A. Physical Mapping of Probes against the Somatic Cell Hybrid Panel

Locus No.	Probe	Cell Line							Region
		Human	L17nE	L17nC	SP-3	NF13	12.3B	7AE11	
9	pABL10-41	+	+	−	−	−	+	−	17p
8	pYNM67	+	+	−	−	−	+	−	17p
1	p3.6	+	+	+	−	−	+	+	1
2	c11-2B	+	+	+	+	−	+	+	2
3	pHHH202	+	+	+	+	−	+	+	2
4	pTH17.19	+	+	+	+	−	+	+	2
5	c11-3C4	+	+	+	+	−	+	+	2
10	c11-2C11	+	+	+	+	+	+	+	3
11	c11-2F9	+	+	+	+	+	+	+	3
6	pHAT-A1	+	+	+	+	+	+	−	4

B. Most Likely Linear Orders Derived from Linkage Analysis[a]

Order	Likelihood	Odds Against
9-8-1-(2,3,5)-4-10-6	−1,702.8	1
9-8-1-4-(2,3,5)-10-6	−1,687.8	1, 823
9-8-1-3-10-11-6	−1,279.9	1
9-8-1-3-11-10-6	−1,269.9	149.2

[a] Locus numbers correspond to those defined in tables 1 and 2A.

tifying NF1 gene–linked cosmid clones. Physical and genetic mapping studies have indicated that clones from this library are tightly linked to the NF1 gene and flank it on both sides. The almost complete lack of crossovers within the cluster of markers (p3.6, c11-3C4, c11-2B, pHHH202, and pTH17.19) between the centromere and the NF13 breakpoint suggests that recombination may be suppressed in this region. The placement of pTH17.19 distal to pHHH202 has been confirmed by pulsed-field analysis, reported by Fountain et al. (1989) elsewhere in this issue, that placed this clone ~120 kb closer to the NF1 locus than is pHHH202. Note that the use of the NF13 breakpoint allows us to position the NF1 gene on the normal map; two loci, TH17.19 and 11-2C11, show only 2.6% recombination across this breakpoint. There is a good chance that these are the most tightly linked flanking markers for the NF1 gene.

The data described above suggest that only two segments of chromosome 17 are present in hybrid 7AE-11: a proximal 17q segment including the NF1 gene and a *neo*-marked region of distal 17q. The number of cosmids needed to blanket the NF1 gene region is uncertain, as our data do not address the issue of the relative size of the two segments of chromosome 17 in 7AE-11. This is because the NF1 region segment is not selected by G418, and it may not be present in all 7AE-11 cells.

There is a possibility that the NF1 region of 7AE-11 may exist as more than two segments; if so, the NF1 gene may not be present in this line. Should that be the case, we will have to turn to other microcell constructs that have the same characteristics as 7AE-11 but carry a different set of chromosome 17 breakpoints. What is clear is that if a sufficient density of human clones covering the regions adjacent to the NF13 can be obtained, TAFE analysis with a number of large restriction-enzyme fragments will almost certainly detect one of the two known NF1 breakpoints: t(17;22) or t(1;17). Once this goal is realized, procedures such as cloning from pulsed-field gel fragments or standard cosmid walking will allow the breakpoint to be cloned and the NF1 gene to be identified.

Acknowledgments

The authors wish to thank Dr. D. Mathieu-Mahul for probe pHAT-A1 and Dr. H. F. Willard for probe p3.6; John Cowan, Leslie Jerominski, and Lesa Nelson for technical assistance; and Ruth Foltz for editing the manuscript and preparing the

figures. R. White and J.-M. Lalouel are Investigators at the Howard Hughes Medical Institute.

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