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Physical Mapping of a Translocation Breakpoint in Neurofibromatosis

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The gene for von Recklinghausen neurofibromatosis (NF1), one of the most common autosomal-dominant disorders of humans, was recently mapped to chromosome 17 by linkage analysis. The identification of two NF1 patients with balanced translocations that involved chromosome 17q11.2 suggests that the disease can arise by gross rearrangement of the *NF1* locus, and that the NF1 gene might be identified by cloning the region around these translocation breakpoints. To further define the region of these translocations, a series of chromosome 17 Not I–linking clones has been mapped to proximal 17q and studied by pulsed-field gel electrophoresis. One clone, 17L1 (D17S133), clearly identifies the breakpoint in an NF1 patient with a t(1;17) translocation. A 2.3-megabase pulsed-field map of this region was constructed and indicates that the NF1 breakpoint is only 10 to 240 kilobases away from 17L1. This finding prepares the way for the cloning of *NF1*.

VON RECKLINGHAUSEN NEUROFIBROMATOSIS (NF1) is an autosomal-dominant human genetic disease, characterized by café-au-lait spots, multiple neurofibromas that increase in size and number with age, hamartomas of the iris (Lisch nodules), learning disabilities, bone abnormalities, and an increased risk of malignancy (especially glioma and neurofibrosarcoma) (*1*). The incidence of the disease is about 1 in 4000. The specific manifestations and severity are remarkably variable, even within the same family, and the spontaneous mutation rate is high, with 30 to 50% of cases representing new mutations (*2*).

The NF1 gene (*NF1*) has been recently mapped to chromosome 17 by linkage analysis (*3*), and genetic analysis of 142 families by an International Consortium has indicated that *NF1* lies on proximal 17q (*4*). Markers on both sides of the gene have been identified that are within 5 centimorgans of *NF1* (*5*).

In support of this localization, two unrelated patients with NF1 and apparently balanced translocations involving chromosome 17 [t(1;17) and t(17;22)] have been identified (*6, 7*). In each instance, the chromosome 17 breakpoint is in band q11.2, precisely where *NF1* maps by linkage analysis. Somatic cell hybrids have been constructed that contain the translocation chromosomes from these patients (*7, 8*). Flanking genetic markers for *NF1* map on opposite sides of the translocation breakpoints (*7–10*), supporting the hypothesis that these translocations directly disrupt the gene. Other somatic cell hybrids created by microcell-mediated gene transfer have been used to further define the location of markers around the *NF1* locus (*9, 10*). The combination of linkage and physical mapping has now ruled out several chromosome 17 genes, including *ERBA1*, *ERBB2*, and *NGFR*, as candidates for direct involvement in NF1 (*9–11*).

Thus *NF1* is an appropriate target for cloning by reverse genetics (*12*). We have reported (*9*) initial results of physical mapping of this region by pulsed-field gel electrophoresis (PFGE). Although these results allowed us to physically connect some of the closely linked genetic markers, gaps were present in the map and none of these markers detected either NF1 translocation breakpoint. Therefore additional markers were required to visualize this region in more detail. The use of linking clones, which are genomic fragments containing rare restriction sites (*13*), has advantages in such an effort: such clones allow convenient construction of a physical map, and often mark the site of expressed genes. We generated a phage library of Not I–linking clones from flow-sorted chromosome 17 material (*14*) and localized these clones using a somatic cell hybrid panel (*9*). The clones that mapped to 17q11 were tested on PFGE blots (*15*) to see whether abnormal fragments were present in DNA from either NF1 patient with a translocation.

Of 16 linking clones studied, one (called 17L1) identified novel PFGE bands in DNA from the t(1;17) NF1 patient. Specifically, a 0.8-kb Not I–Xho I fragment of 17L1, denoted 17L1A, detected abnormal fragments with the enzymes Bss HII, Sac II, and Not I (Fig. 1A) and also with Mlu I. No abnormality was seen with Sfi I, Eag I, or Xho I (*16*).

A potential pitfall in this analysis can arise from variability in cutting of rare restriction sites. This can occur as a result of actual sequence polymorphism (*17*) or, more commonly, as a result of DNA methylation differences (*15*). Strong evidence that such effects cannot account for the data shown here include: (i) No novel PFGE fragments have been seen with 17L1A in Not I analysis of more than 60 normal chromosomes in several different tissues. (ii) These fragments cannot be accounted for by the effects of incomplete digestion; for example, the intentional partial Not I digest in Fig. 1A demonstrates that the t(1;17)-specific band at 550 kb (lane 9) is distinct from the 460- and 680-kb partial fragments in normal individuals (lanes 10 and 11). (iii) The t(1;17) DNA, which also includes a normal chromosome 17, always shows a normal band as well as the abnormal band, in approximately a 1:1 ratio. (iv) The abnor-

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mal bands segregate in the t(1;17) NF1 family (*6*) along with the translocation and NF1 (Fig. 1B). Lanes 3 to 5 represent Sac II analysis of peripheral blood leukocyte DNA from the mother (proband) and her two affected offspring. Similar results were obtained with Not I and Bss HII (*16*). (v) The abnormal bands are not a result of acquired rearrangement in the t(1;17) lymphoblastoid cell line; results were identical in the cell line (Fig. 1B, lane 2) and fresh blood leukocytes (Fig. 1B, lane 3) from the proband. (vi) A 2300-kb map has been constructed, based on single and double digests (Fig. 2). The normal and t(1;17) maps are identical to the left of 17L1A, but remarkably different on the right. Thus the combination of these observations provides unequivocal evidence that the chromosomal rearrangement is located just to the right of 17L1A, within the interval indicated by cross-hatching in Fig. 2 (*18*).

By means of somatic cell hybrids, 17L1 has been shown to be proximal to both the t(1;17) NF1 and t(17;22) NF1 breakpoints, since it is absent in the der(1) and der(22) hybrids derived from these patients (*9, 19*). This allows the map in Fig. 2 to be oriented with the centromere to the left. We have also studied DNA from the t(17;22) NF1 patient (*7*) by PFGE. No abnormalities were seen with 17L1A after complete digestion with Bss HII (Fig. 1A, lane 1), or with Not I, Eag I, or Mlu I (*16*). This indicates that the t(17;22) breakpoint lies distal to t(1;17). Preliminary data based on Not I partial and Nru I complete digests, however, indicate that the t(17;22) breakpoint lies in the 220-kb Not I–Nru I interval in Fig. 2. No abnormal Not I fragments have been identified with 17L1A in 22 cytogenetically normal unrelated NF1 patients (an example is shown in Fig. 1A, lane 8).

Thus we have identified a cloned DNA fragment that lies 10 to 240 kb from the t(1;17) NF1 breakpoint, effectively narrowing by an order of magnitude the previous localization of the gene. As this chromosomal rearrangement is likely to directly involve *NF1* itself, efforts are now under way to identify potential exons in this region. On the basis of the high mutation rate and the occurrence of translocations within or near the gene, the *NF1* locus may be quite large, similar to the Duchenne muscular dystrophy locus (*20*). In this regard, it is interesting that 17L1, like many HTF island linking clones (*21*), is strongly conserved across species (*16*) and therefore has to be considered itself as a candidate for part of *NF1*.

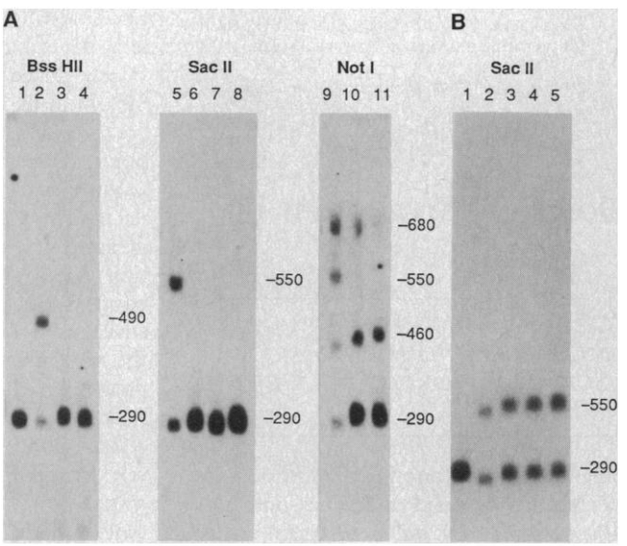

Fig. 1. Pulsed-field gel analysis with probe 17L1A. (**A**) PFGE analysis of patient DNA and normal controls. The DNA source in each lane is: 1, t(17;22) NF1; 2, 5, and 9, t(1;17) NF1; 3, 6, and 10, normal lymphoblastoid DNA; 4, 7, and 11, normal skin fibroblast DNA; 8, lymphoblastoid DNA from a cytogenetically normal NF1 patient. The Not I analysis is an intentional partial digest. Sizes are in kilobases. (**B**) Analysis of the t(1;17) NF1 family with Sac II. Source of DNA is: 1, normal lymphoblastoid DNA; 2, lymphoblastoid DNA from the t(1;17) NF1 proband; 3, peripheral blood DNA from the same individual; 4 and 5, peripheral blood from her son and daughter, who both have t(1;17) NF1. Note that the 550-kb band segregates with NF1. The slightly faster mobility of fragments in lane 2 is attributable to the presence of less DNA in this sample, which affects mobility in field-inversion gels (*22*). DNAs were prepared in gel blocks and analyzed as described (*9*). Briefly, half blocks (5 μg of DNA) were digested to completion with 8 to 30 U of Bss HII or Sac II, or partially digested with 5 U of Not I. Digested DNAs were separated by electrophoresis through 1% agarose field-inversion gels (*22*) in 0.5× TBE (tris, boric acid, and EDTA) (*23*). Forward to reverse pulse times were maintained at a constant ratio of 3:1. Gels were stained with ethidium bromide, ultraviolet light–nicked for 2.5 min on a 254-nm light box, and transferred to Hybond. Hybridizations were performed in 1*M* NaCl, 10% dextran sulfate, 1% SDS and 1× Denhardt's (*23*). An 800-bp Not I–Xho I fragment of the linking clone 17L1 (*14*), designated 17L1A, was labeled by the random primer method (*24*) and used as a probe in these studies. Filters were washed to a final stringency of 0.1× saline sodium citrate, 0.1% SDS at 65°C.

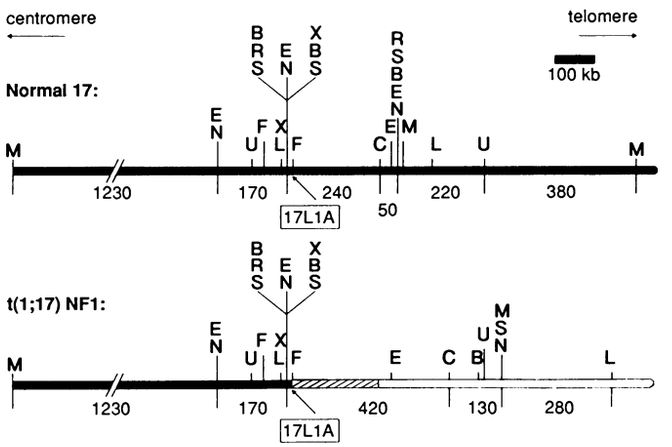

Fig. 2. Pulsed-field map of the t(1;17) NF1 breakpoint region on chromosome 17. Chromosome 17 is depicted in black, chromosome 1 is shown in white, and the cross hatched region represents the area where the t(1;17) breakpoint resides (*18*). The position of the 17L1A probe used in Fig. 1 is indicated. The centromere is to the left of the region portrayed here and the t(17;22) NF1 breakpoint is to the right. Single- and double-enzyme digests were performed on lymphoblastoid and fibroblast DNAs in order to derive this map. Enzyme sites are designated as follows: B, Bss HII; C, Cla I; E, Eag I; F, Sfi I; L, Sal I; M, Mlu I; N, Not I; R, Nar I; S, Sac II; U, Nru I; and X, Xho I. The order of sites in the HTF island that 17L1 contains are from left to right: Bss HII, Nar I, Sac II, Not I (Eag I), Sac II, Bss HII, and Xho I. Fragment lengths are indicated in kilobases beneath the chromosomes. For most enzymes only the sites closest to 17L1A are indicated.

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18. The limits of the position of the t(1;17) translocation breakpoint (cross-hatching in Fig. 2) were defined as follows: (i) the normal Sfi I site 10 kb telomeric to 17L1A is present on the translocation chromosome; (ii) in a Cla I + Not I double digest, the normal Cla I site 240 kb telomeric to 17L1A is replaced by a novel site 420 kb telomeric to 17L1A on the t(1;17) chromosome; (iii) despite restriction analysis with multiple other enzymes, no informative sites are present between Sfi I and Cla I to more accurately define this breakpoint. The Eag I and Nru I sites in chromosome 1 DNA shown in Fig. 2 apparently occur coincidentally in approximately the same location as their counterparts on the normal chromosome 17.
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Two NF1 Translocations Map Within a 600-Kilobase Segment of 17q11.2

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Balanced translocations, each involving chromosome 17q11.2, have been described in two patients with von Recklinghausen neurofibromatosis (NF1). To better localize the end points of these translocation events, and the NF1 gene (*NF1*) itself, human cosmids were isolated and mapped in the immediate vicinity of *NF1*. One cosmid probe, c11-1F10, demonstrated that both translocation breakpoints, and presumably *NF1*, are contained within a 600-kilobase Nru I fragment.

LOCALIZATION OF THE NEUROfibromatosis type 1 (NF1) gene (*NF1*) to chromosome 17 by linkage analysis (*1, 2*) was followed by description of a marker, pHHH202, that showed no recombination in the Utah panel of NF1 families (*3*). Close linkage of pHHH202 to NF1 was confirmed by an international consortium (*4*). Subsequent studies (*5, 6*) physically localized pHHH202 to band 17q11.2, the same band that is involved in the two known rearrangements associated with NF1: t(1;17) (p34.3;q11.2) (*7*) and t(17;22) (q11.2;q11.2) (*8*).

To locate *NF1* more precisely, we isolated a large number of cosmid clones as a source of additional polymorphic markers and probes. Because intact chromosome 17 is too large (150,000,000 bp) for development of probes specifically targeted to the NF1 region, we used pHHH202 and other elements of our chromosome 17 map (*9*) to characterize a large number of human × rat microcell hybrids (*10*) that had been created by fusion of a somatic cell hybrid containing a *neo*-marked human chromosome 17 and a rat cell line (*11*). To avoid confusion caused by undetected deletions or rearrangements, we chose to search for new probes in cosmid libraries prepared from the two microcell hybrids that best represented the NF1 region, 7AE-11 (*6*) and FTHB(17)L4 (*12*).

Human cosmids identified by screening the microcell hybrid libraries with labeled human DNA were physically mapped by means of a panel that included somatic cell hybrids (*5*), the two chromosome 17 microcell hybrids 7AE-11 and FTHB(17)L4, and two cell lines with *NF1* translocation breakpoints (Fig. 1A). One of the translocation cell lines, NF13 (*6, 8*), contains the derivative 22 [der(22)] chromosome from a sporadic NF1 patient who carries a balanced translocation between chromosomes 17 and 22, t(17;22)(q11.2;q11.2). The other hybrid, designated DCR1, was isolated from a fusion of lymphoblasts from an NF1 patient with a balanced translocation between chromosomes 1 and 17, t(1;17)(p34.3;q11.2) (*7*) and contains the der(1) chromosome from this patient (*13*). The results of the physical mapping are summarized in Fig. 1B.

We determined the relation of clones in the NF1 region to *NF1* by a combination of genetic and physical mapping. A high-resolution genetic map for the NF1 region (*6*) indicated that two probes, pTH17.19 and c11-2C11, flank *NF1* about 2 centimorgans apart. Given the density of cosmid probes within the target regions, we reasoned that it should be possible to detect abnormal pulsed-field fragments in the two NF1 patients with balanced translocations. DNA samples from normal individuals, patients with sporadic NF1, the two patients with balanced translocations, and somatic cell hybrids were prepared in agarose blocks, digested with a number of rarely cutting enzymes, and subjected to pulsed-field gel electrophoresis (PFGE) (*14*).

One region 3 clone, c11-1F10, hybridized to a 600-kb Nru I fragment. When tested on PFGE blots (*15*) prepared with DNA from the patients with translocations, this clone showed new, translocation-specific Nru I fragments. The t(17;22) cell line showed the normal 600-kb Nru I fragment plus a fragment of 390 kb. The t(1;17) cell line gave the normal 600-kb Nru I fragment plus a fragment of 450 kb.

A potential caveat is that these observations may reflect heterogeneous Nru I digestion of the translocation cell lines, caused by differences in methylation between the normal and translocation chromosomes. In fact, many lymphoblastoid cell lines, including the t(17;22) balanced translocation line, require a large excess (100×) of Nru I to reach complete digestion (Fig. 2). However, we believe these new c11-1F10–hybridizing Nru I fragments are associated with the translocation events because (i) both translocations revealed aberrant bands, each a different size, as would be expected for two independent translocation events; (ii) Nru I digests of 40 additional lymphoblastoid cell lines from normal people and from NF1 patients yielded only the 600-kb normal Nru I band; (iii) the two derivative fragments are smaller than the normal fragment, a finding inconsistent with a digestion artifact; (iv) the two derivative fragments are

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