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Genetic and physical map of the von Recklinghausen neurofibromatosis (*NF1*) region on chromosome 17

(genetic linkage/long-range physical mapping/hereditary disease)

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ABSTRACT **The von Recklinghausen neurofibromatosis 1 (*NF1*) locus has been previously assigned to the proximal long arm of chromosome 17, and two NF1 patients have been identified who have constitutional balanced translocations involving 17q11.2. We have constructed a cosmid library from a chromosome-mediated gene transfectant, KLT8, that contains approximately 10% of chromosome 17, including 17q11.2. Cosmids isolated from this library have been mapped across a panel of somatic cell hybrids, including the hybrids from the two patients, and have been localized to seven small regions of proximal 17q. We have 5 cosmids that map directly above the two *NF1* translocations, and 11 cosmids that map directly below. Of these, 2 cosmids in each region are linked to the disease locus and 3 of these cosmids show no recombination. One distal cosmid, 2B/B35, detects the two *NF1* translocations by pulsed-field gel analysis and has been used to produce a long-range restriction map that covers the translocations.**

Von Recklinghausen neurofibromatosis 1 (NF1) is one of the most common dominantly inherited disorders in man with an estimated frequency of 1/3000 and a high proportion of new mutations, estimated between 30 and 50% (1). Its expression is extremely variable, even within affected families, and ranges from minor skin manifestations (e.g., cafe-au-lait spots) to severely disabling and sometimes lethal neurological tumors. Linkage of this disorder to markers on proximal 17q has been demonstrated (2, 3) and linked flanking markers have been defined (4, 5). As with several other dominantly inherited tumor-forming syndromes (e.g., retinoblastoma or adenomatous polyposis coli), it seemed likely that NF1 might be the result of mutations in a tumor-suppressor gene. However, there are as yet no reports of loss of heterozygosity in NF1 for markers close to the *NF1* locus.

The finding of two individuals with NF1 carrying different constitutional balanced reciprocal translocations with breaks at 17q11.2 (6, 7) strongly suggested that the region of these translocations would contain the *NF1* gene. Probes that map close to the translocations have been used to make long-range maps of this region (8, 9). By using a chromosome-mediated gene transfectant (CMGT), KLT8, containing about 10% of chromosome 17, including the *NF1* region (10), we have isolated cosmids flanking the *NF1* translocations. Restriction fragment length polymorphisms (RFLPs) in these cosmids have allowed us to map them by genetic linkage. Our closest probe lies distal to the translocations and has no recombinants with *NF1*. It contains a CpG-rich island and has been used by analysis of pulsed-field gel electrophoresis (PFGE) results to define a long-range restriction map covering the translocations that differ from the published maps (27). In this paper we present the genetic linkage data on our flanking probes and our physical map of the translocation region.

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MATERIALS AND METHODS

Cell Lines. KLT8, PLT6B, and PLT8 are CMGTs containing fragments of chromosome 17 (10). PCTBA1.8 (11) is a chromosome 17-only hybrid and PJT2/A1 (12) is a hybrid containing the 15q+ t(15;17)(q21;q11.2-12) chromosome from an acute promyelocytic leukemia (APL) patient. TriD62 is a hybrid containing 17q (13). The two *NF1* parental lines t(1;17)(p34.3;q11.2) (6) and t(17;22)(q11.2;q11.2) (7) and two hybrids derived from them (7, 14) were kindly provided by David Ledbetter (Baylor College of Medicine Houston, TX). Conditions for growth of these cells have been described in the appropriate references.

NF1 Families. Linkage was done on a set of 18 British and South African families with NF1. Families ICR01-08, -11, -12, -15, and -17 through -20 have been reported (15); families ICR22, -24, and -25 are three further families of British origin. The diagnostic criteria for NF1 were those described (16, 17).

Lod scores, recorded in Table 1, were calculated using the program LINKAGE, version 4.7 (18).

DNA Preparation. DNA was prepared for Southern blot analysis and the cosmid library by the standard phenol extraction procedure (19), with care taken to ensure that the DNA for the cosmid library remained very high molecular weight. DNA was prepared for PFGE analysis from cultured cells that were resuspended with an equal volume of 1.2% low-melting-point agarose to a concentration of 0.5, 0.75, or 1.0×10^6 cells per insert and processed according to the published protocol of Herrmann *et al.* (20).

Library Preparation. High molecular weight DNA prepared from the KLT8 hybrid was partially digested with *Mbo* I and dephosphorylated with calf intestine alkaline phosphatase to prevent religation of noncontiguous DNA fragments. Vector pCos8 (A. Craig, personal communication) was prepared by cleaving between the two *cos* sites with *Pvu* II, dephosphorylating the ends, and cutting at the *Bam*HI site, which created cohesive ends with *Mbo* I. The vector and insert DNA were then ligated with T4 DNA ligase and packaged in Gigapack Gold (Stratagene) as recommended by the supplier. The packaged mix was adsorbed onto ED8767 cells and plated out onto Hybond-N filters (Amersham) on agar containing L broth and kanamycin at 50 μg/ml. The resulting 5×10^5 colonies were screened with total human DNA, and 250 of the approximate 2000 positive colonies were picked for further characterization.

PFGE. All pulsed-field gels were electrophoresed on the LKB 2015 Pulsaphor system with a hexagonal electrode. Gels of 1% agarose in 0.25× TBE were electrophoresed at 170 V. (1× TBE = 90 mM Tris/64.6 mM boric acid/2.5 mM EDTA,

Abbreviations: NF1, von Recklinghausen neurofibromatosis 1; CMGT, chromosome-mediated gene transfectant; RFLP, restriction fragment length polymorphism; PFGE, pulse-field gel electrophoresis; APL, acute promyelocytic leukemia.

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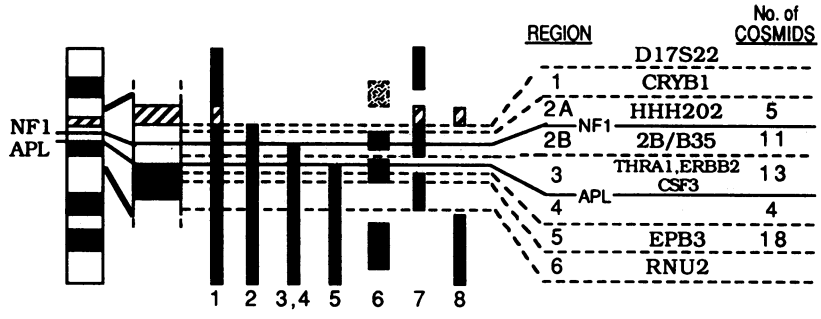

FIG. 1. Cosmids from a KLT8 library were mapped on a panel of chromosome 17 CMGTs and translocation hybrids. Chromosome 17 is shown with the *NF1* region enlarged. The following five human–mouse hybrids were used in the panel. Bars: 1, PCTBA1.8, chromosome 17-only (11); 2, TriD62, chromosome 17q-only, (17q11.2–qter) (13); 3, DCR-1, an *NF1* translocation hybrid, t(1;17)(p34.3;q11.2) (5); 4, NF13, an *NF1* translocation hybrid, t(17;22)(q11.2;q11.2) (14); 5, PJT2/A1, an APL hybrid 15q+, t(15;17)(q22;q11.2–12) (12). In the three translocation hybrids only one of the translocation products and no normal chromosome 17 was retained. The following three CMGTs were used in the panel. Bars: 6, KLT8, the CMGT from which the library was made; 7, PLT6B, contains markers that flank *NF1* but does not span the APL breakpoint; 8, PLT8 contains the area of the *TK1* locus. Designation of regions 1–6 defined by these hybrids and CMGTs with the markers that are known to map to these regions and the number of cosmids from the KLT8 library that have been isolated in them are shown. The D-numbers of the cosmids mapping to these regions are as follows: D17S136–140 in region 2A, D17S141–150 and -159 in region 2B, D17S151–158 and -160–164 in region 3, D17S166, -167, -174, and -175 in region 4, and D17S181–198 in region 5.

pH 8.3.) Periods of electrophoresis were 30–36 hr with a 100-sec pulse or 24–30 hr with a 45-sec pulse.

Southern Blots. Gels were blotted onto Hybond-N in 20 × SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0) or Hybond-N+ in 0.4 M NaOH as recommended in the protocols provided by Amersham.

Labeling of Probes. Whole cosmids were used as probes for RFLPs, as recommended by Sealey *et al.* (21). Cosmid DNA was linearized by digesting with *Bam*HI, and 60 ng was used in the random-primer labeling method as described by Feinberg and Vogelstein (22). The labeled probes were separated from the unincorporated radionucleotides by using spun column chromatography (29). The probe was boiled for 10 min with 100 μg of human placental DNA (Sigma) in a total volume of 400 μl, placed on ice for 3 min, and incubated at 65°C for 3–5 hr. The probe was added to 10 ml of prewarmed hybridization fluid and added to the filters that had been prehybridized overnight with human placental DNA (50 μg/ml).

For pulsed-field gel analysis, nonrepetitive fragments were isolated from the cosmids. The probes were incubated for 2–3 hr as above, and prehybridization was done on new filters only. Although the probes were nonrepetitive, as judged by their lack of hybridization to total human DNA, it was found that the signal-to-noise ratio was improved by incubating the probes as above.

RESULTS

A series of somatic cell hybrids containing fragments of chromosome 17 was produced by chromosome-mediated gene transfer (10). One of these, KLT8, was used to construct a cosmid library, as it was known to contain about 10% of chromosome 17, from dot-blot analysis, and to contain the *NF1* region. Human clones from this library were mapped against a panel of hybrids containing fragments of chromosome 17 from constitutional rearrangements, leukemic rearrangements, hybrids with random breaks, and CMGTs. With this panel we were able to divide the proximal portion of 17q (17cen-q12) into seven regions. Fig. 1 shows a diagram of this panel with the seven regions and the number of cosmids clones mapped to each region. Previously mapped markers in this region of chromosome 17 are also shown (23). Our results place *CRYB1* in region 1 and *HHH202* distal in region 2A. Regions 2A and 2B flank the *NF1* translocations, with region 2B defined by 11 of our cosmids but no previously mapped genes. Regions 3 and 4 flank the t(15;17) translocation found in APL, with region 3 containing *THRA1*, *ERBB2*, and *CSF3*. Region 4 contains 4 of our cosmids but no previously assigned markers. Region 5 contains the erythrocyte surface protein band 3 EPB3 (24) and region 6 has *RNU2*. The two hybrids containing the *NF1* translocations (1;17 and 17;22) contain the *TK*-selected portion of chromosome 17; that is, chromosome 17 from the breakpoint at 17q11.2 to 17qter. These translocations are indicated by the same line in Fig. 1 (bar 3, 4), although they are not identical.

Five cosmids were isolated in region 2A, 11 cosmids were isolated in region 2B, and these cosmids were screened for RFLPs by using whole cosmids as probes. Polymorphisms were detected in 6 cosmids, and, in all cases, both allele frequencies were high and the probes were generally informative in the NF1 families. Fragments detecting the RFLPs were subcloned into pUC8 or pBluescript for further use. Five of these cosmids, 2 from region 2A and 3 from region 2B, were used in genetic linkage studies on 18 NF1 families, and these results are presented in Table 1. Both cosmids in region

Table 1. Pairwise lod scores for *NF1* and KLT8 cosmids

Cosmid	Recombination fraction (θ)							$\hat{Z}$	$\hat{\theta}$
	0.00	0.05	0.10	0.15	0.20	0.30	0.40		
2B/A28	$-\infty$	2.05	2.42	2.38	2.17	1.45	0.56	2.43	0.12
2A/A34	4.53	4.03	3.53	3.02	2.50	1.45	0.49	4.52	0.00
2B/B35	4.25	3.89	3.52	3.11	2.69	1.75	0.72	4.25	0.00
2B/B412	0.89	0.79	0.70	0.60	0.51	0.33	0.17	0.88	0.00
2A/D218	5.34	4.90	4.43	3.92	3.38	2.20	0.90	5.33	0.00

$\hat{Z}$, maximum lod score; $\hat{\theta}$, recombination fraction at $\hat{Z}$. Cosmids detected polymorphisms with the following enzymes and allele frequencies: 2B/A28 with *Bam*HI, a1/a2 = 0.7/0.3; 2A/A34 with *Pst* I, 0.8/0.2; 2B/B35 with *Sph* I, 0.4/0.6; 2B/B412 with *Msp* I, 0.65/0.35; 2A/D218 with *Bgl* II, 0.35/0.65. lod scores were calculated using the program LINKAGE, version 4.7 (18).

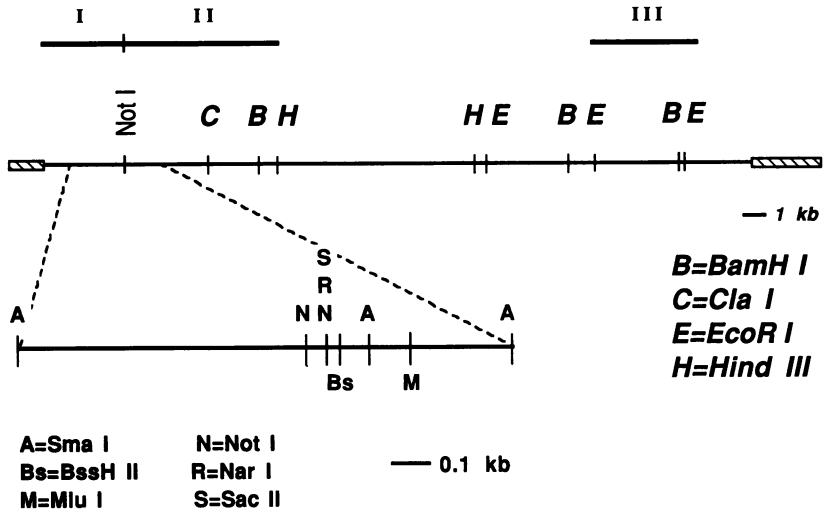

FIG. 2. Map of cosmid 2B/B35 (D17S145) showing the rare-cutting enzyme sites around the CpG-rich island. Two *Sac* II sites near the *Not* I sites are not shown. The three probes used to map the cosmid on the mapping panel and pulsed-field gels are shown above the cosmid.

2A, 2A/A34 and 2A/D218, are linked to *NF1* at 0% recombination with maximum lod scores of 4.5 and 5.3, respectively. One cosmid in region 2B, 2B/A28, is linked at 12% recombination, a considerable distance from the disease locus. Two other cosmids in region 2B show no recombination with *NF1*; 2B/B35 has a lod score of 4.3 and 2B/B412 has a low maximum lod score of 0.9.

Four of the cosmids with no recombinants were then used to determine whether any of them could detect an altered band size in the translocation chromosomes compared with the normal chromosome 17 on pulsed-field gels. One of the clones, 2B/B35, from region 2B, distal to both translocations, did indeed detect altered bands in both the translocation hybrids and the parental material with several rare-cutting enzymes, including *Nru* I, *Not* I, *Mlu* I, *Bss*HII, and *Sac* II. No such alterations are seen in blood samples or lymphoblastoid lines from 11 normal individuals with *Nru* I or *Not* I (data not shown), suggesting that the altered bands are not due to restriction site polymorphisms. A restriction map of this cosmid clone is shown in Fig. 2. 2B/B35 contains restriction sites for a series of rare-cutting enzymes, including *Not* I, *Sac* II, and *Bss*HII. Double digests of genomic DNA with *Hin*dIII plus each of these enzymes gave the expected bands with probes I and II (defined in Fig. 2) from cosmid 2B/B35, indicating that this is a undermethylated CpG-rich island in the genome (data not shown).

Results of PFGE analysis with three enzymes are shown in Fig. 3. Probes I and II, as indicated in Fig. 2, cover the proximal and distal sides of the *Not* I sites in the CpG-rich island. Probe III is a *Bam*HI fragment on the distal side. As seen in Fig. 3*a*, probe III detects a 600-kilobase (kb) *Nru* I band in normal human DNA (lanes 1 and 2) prepared from lymphoblastoid cell lines. This is the same 600-kb band reported by Fountain *et al.* (8) to be detected by the linking

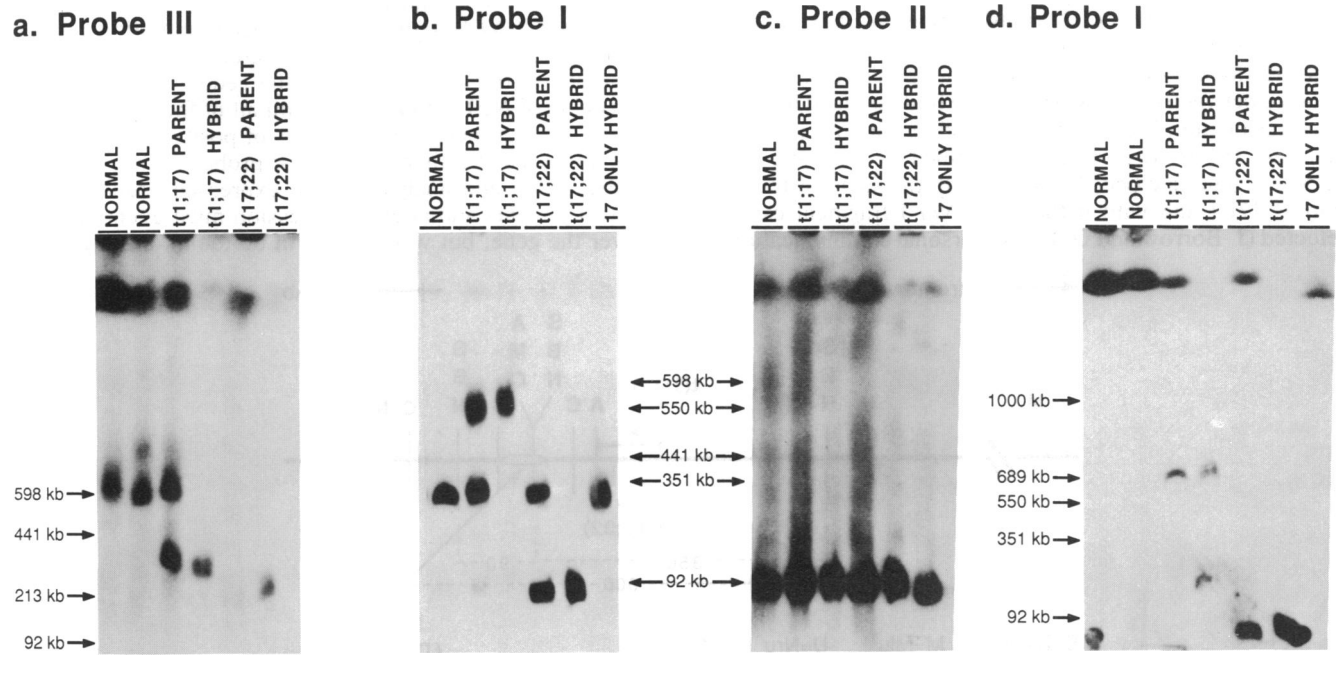

FIG. 3. 2B/B35 detects the *NF1* translocation breakpoints on pulsed-field gels with several enzymes. All gels were electrophoresed with a hexagonal electrode at 170 V. (*a*) *Nru* I digest, 36 hr, 90-sec pulse time. The t(17;22) parental line does not show clean bands on *Nru* I digests, presumably due to variations in methylation patterns. (*b* and *c*) *Not* I digests, 30 hr, 45-sec pulse time. (*d*) *Mlu* I digest, 32 hr, 90-sec pulse time. In samples of DNA from normal individuals, the *Mlu* I band detected by probe I is in the limiting mobility of the gel, >1100 kb.

clone 17L1 on the proximal side of the translocations and by O'Connell *et al.* (9) by c11-1F10 on the distal side of the translocations. The 600-kb band is also seen in the parental lymphoid line from the patient carrying the 1;17 translocation and in addition an altered 350-kb band is seen (lane 3). In the hybrid containing only the 1;17 translocation and no normal chromosome 17, only the altered band is seen (lane 4). The 17;22 parental line has consistently shown only faint, rather than clear, bands when *Nru* I digests are used; these bands appear to be at 600 kb and 300 kb. The hybrid containing only the 17;22 translocation clearly shows the 300-kb altered band (lane 6).

Fig. 3 *b* and *c* shows the same *Not* I filter probed with fragments I and II, from each side of the CpG-rich island. In Fig. 3*b*, by using fragment I, a 350-kb band is seen in the normal DNA (lane 1). The same size band is seen in a chromosome 17-only hybrid (lane 6). The t(1;17) parental line again shows the normal band as well as an altered band of 550 kb (lane 2). Only the altered band is seen in the t(1;17) hybrid (lane 3). Similarly, the t(17;22) parental line shows the normal band as well as an altered band at 92 kb (lane 4) and the hybrid shows only the altered 92-kb band (lane 5). These results demonstrate that there is a *Not* I site 350 kb proximal to the 2B/B35 island and that the two translocation breakpoints lie between. Fig. 3*c* shows the same blot probed with fragment II. All digests show a single 92-kb band, indicating that this is the distance to the next *Not* I site, distal to 2B/B35. Similar blots of *Bss*HII and *Sac* II digests probed with these same fragments show that these sites are the same distance from 2B/B35 on the normal chromosome 17, suggesting that these sites are clustered in CpG-rich islands (data not shown).

Fig. 3*d* shows the results of an *Mlu* I digest probed with fragment I. The band detected in normal DNA and in chromosome 17-only hybrid DNA (lanes 1, 2, and 7) is in the limiting mobility of the gel, at >1100 kb. The parental and hybrid *NF1* lines again show an altered band. For t(1;17), this is at 690 kb (lanes 3 and 4) and for the t(17;22) at 80 kb (lanes 5 and 6).

A composite map of the region is shown in Fig. 4 with the 2B/B35 clone 75–150 kb distal to the 1;17 translocation and 75–90 kb distal to the 17;22 translocation. We have not yet been able to find a rare-cutting enzyme that cuts between the translocation breakpoints and so cannot precisely define the distance between them. As shown, we find no island between the translocation breakpoints and define the next proximal island as 350 kb away from that defined by B35. We have isolated a linking clone, LCN6F2, with the same restriction map as 17L1 and with it the same *Not* I fragment can be detected (J. Borrow and D. Black, personal communication).

DISCUSSION

The genetic linkage data on *NF1* indicate that the gene responsible for this disease lies in the 17q11.2 region. There has been no evidence for heterogeneity in the location of this disease. To isolate probes within the *NF1* region so as to eventually identify the *NF1* gene, we have taken the approach of using libraries from hybrids with small fragments of chromosome 17. Our genetic linkage data on probes distal and proximal to the disease locus indicate that this approach is extremely efficient. Regions 2A and 2B, defined by CMGTs, are sufficiently small that four probes isolated at random are definitely linked, three with no recombination. The finding of no recombinants with a probe within 90 kb of one of the translocations confirms that these translocations must be within a small genetic distance from the gene, although the physical distance could still be quite large. To our knowledge, the probes in region 2B are the first reported probes distal to *NF1* with no recombinants and along with probes in region 2A should be extremely useful as informative markers in the genetic diagnosis of NF1 in affected families.

O'Connell *et al.* (25) using a similar approach produced a cosmid library from a microcell hybrid, 7AE-11, and were also able to isolate a distal clone, c11-1F10, that detected the translocations (9). It is clear that CMGTs, microcell fusion hybrids, or x-irradiation hybrids (26) are extremely powerful reagents for producing libraries for the isolation of clones from small regions of a chromosome. Caution must always be taken in their use for this purpose, however, as they often contain small interstitial deletions. This can be seen clearly in KLT8, which has at least four regions of chromosome 17 that have only become apparent as finer scale mapping has been done. The fact that we find *CRYB1* proximal to *HHH202* is in contradiction to the order found using the microcell fusion hybrids (27). This could be due to another small deletion in KLT8 or alternatively to a deletion or rearrangement in the microcell hybrid. Genetic studies do not clarify this inconsistency as neither *CRYB1* nor *HHH202* shows recombination with *NF1*.

Because of the high proportion of new mutations, it is possible that *NF1* exists in a wide range of mutational types, some of them large enough to be detected by PFGE. One approach to the identification of the gene is, therefore, the screening of NF1 patient DNA with all of the probes in this region. As yet there are no reports of positive results using this approach. Further isolation of probes surrounding the translocation and analysis of the expressed sequences for rearrangements and altered expression must eventually uncover the gene, but whether it will be found directly at the

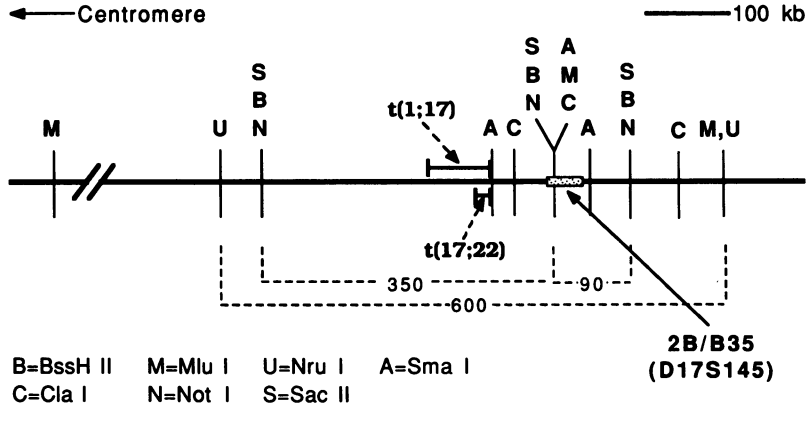

FIG. 4. Long-range map of the *NF1* region derived from PFGE analysis, using the three probes from 2B/B35. Not all sites in the cosmid are shown; see Fig. 2 for a more detailed map. The two *NF1* translocations have not been precisely localized: The t(1;17) breakpoint is 75–150 kb from the *Not* I site in 2B/B35 and the t(17;22) breakpoint is 75–90 kb from the same site.

translocations remains unknown. The nature of the gene is of great interest because its mutations result in a wide range of abnormalities of growth and development as well as in tumor formation. The extreme variability of expression of the mutations both within and between families suggests that different *NF1* mutations may have different phenotypic effects and also that there are strong genetic or environmental-modifying effects. With respect to tumor formation, if lack of allele loss persists even with the use of further probes in the region, the mutations would appear to be dominantly acting—a situation that, to date, would be unusual among the inherited cancer syndromes, the only other possible example being multiple endocrine neoplasia type 2 (28).

Note Added in Proof. Since this work was accepted for publication the cloning of the *NF1* gene has been published and the *NF1* gene is indeed in the region of the translocations (30–32).

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