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ORIGINAL INVESTIGATION

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Identification and characterization of *NF1*-related loci on human chromosomes 22, 14 and 2

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Abstract Neurofibromatosis type 1 (NF1) is a frequent hereditary disorder. The disease is characterized by a very high mutation rate (up to 1/10000 gametes per generation). *NF1*-related loci in the human genome have been implicated in the high mutation rate by hypothesizing that these carry disease-causing mutations, which can be transferred to the functional *NF1* gene on chromosome arm 17q by interchromosomal gene conversion. To test this hypothesis, we want to identify and characterize the *NF1*-related loci in the human genome. In this study, we have localized an *NF1*-related locus in the most centromeric region of the long arm of chromosome 22. We demonstrate that this locus contains sequences homologous to cDNAs that include the GAP-related domain of the functional *NF1* gene. However, the GAP-related domain itself is not represented in this locus. In addition, cosmids specific to this locus reveal, by in situ hybridization, *NF1*-related loci in the pericentromeric region of chromosome arm 14q and in chromosomal band 2q21. These cosmids will enable us to determine whether identified disease-causing mutations are present at the chromosome 22-associated *NF1*-related locus.

Introduction

Neurofibromatosis type 1 (NF1) is a frequent, dominantly inherited disorder with an incidence of about 1 in 3000 births. The disease primarily affects the growth of neural crest-derived tissues. NF1 is characterized by a very high mutation rate (estimates range from 3.1×10^{-5} to 10^{-4} gametes per generation; Huson and Hughes 1994), resulting in about half of the cases being due to new mutations. The *NF1* gene in band q11.2 of chromosome 17 has been identified by positional cloning Cawthon et al. 1990; Viskochil et al. 1990; Wallace et al. 1990). It is a very large gene containing 59 exons, which are distributed over a genomic region of approximately 350 kb (Li et al. 1995). The mutation rate of *NF1*,, which is 100-fold higher than usual for a single gene, cannot solely be attributed to the large target size of the gene. To explain this high mutation rate, it has been hypothesized that *NF1*-related sequences (pseudogenes) in the human genome are reservoirs of mutations, which, during meiosis, can be crossed into the functional *NF1* gene on 17q by interchromosomal gene conversion (Marchuk et al. 1992). To test this hypothesis, we are in the process of identifying the *NF1*-related loci in the human genome and determining whether these harbor the mutations that are found in the functional *NF1* gene of patients. In addition, since interchromosomal gene conversion would require close proximity of the functional *NF1* locus and the *NF1*-related loci, we want to determine their spatial arrangement in the interphase and meiotic prophase nucleus by developing locus-specific probes and use these in in situ hybridization experiments.

Preliminary evidence suggests that *NF1*-related loci might be present on chromosomes 2, 12, 14, 15, 20, 21, and 22 (Legius et al. 1992; Marchuk et al. 1992; Cummings et al. 1993; Gasparini et al. 1993). However, they are ill-defined with respect to location, genomic organization, and extent of homology to the functional *NF1* gene.

We report here the identification, fine mapping, and partial characterization of an *NF1*-related locus on chromosome 22. In addition, we demonstrate that *NF1*-related loci, homologous to the one on chromosome arm 22q, are present in the pericentromeric region of chromosome arm 14q and in band q21 of chromosome 2.

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Materials and methods

Regional mapping

The human × rodent somatic cell hybrids, containing different regions of chromosome 22, have been characterized previously (Delattre et al. 1991; Demczuk et al. 1995). Cell line NF13 of this mapping panel contains the der(22) from a t(17;22) (q11.2;q11.2) translocation with the breakpoint in intron 31 of the functional *NF1* gene on 17q (Li et al. 1995). The pAC5 sequence (see Results) is on the der(17). PgMe25Nu and Wegroth-B3 are human × mouse hybrid cell lines with, respectively, chromosome 22 and chromosome 14 as the sole human contribution (De Klein et al. 1982; Geurts van Kessel et al. 1983). The two cell lines and the mouse-only cell line, Wehi-TG, were kindly provided by A. Geurts van Kessel (University of Nijmegen, Nijmegen, The Netherlands).

Screening of a chromosome 22 cosmid library

The chromosome 22 cosmid library, LL22NC03 (from Lawrence Livermore National Laboratory, Human Genome Center, Livermore, CA 94550, USA), was screened with probe pAC5, according to procedures described previously (Hulsebos et al. 1995).

Screening of a chromosome 22 yeast artificial chromosome library

Fragments of inserts from pAC5-positive cosmids were hybridized to filter copies of the high-density gridded array of the chromosome 22 yeast artificial chromosome (YAC) collection of the Sanger Centre, essentially as described by Collins et al. (1995).

Southern blot analysis

Exons of the functional *NF1* gene on chromosome 17q were amplified by PCR using DNA extracted from cell line GM10498B (NIGMS Human Genetic Mutant Cell Repository, Camden, NJ 08103, USA) as template. This human × mouse hybrid cell line contains chromosome 17 as the sole human contribution. PCR primers and conditions were according to Li et al. (1995). Inserts of plasmids or cosmids and PCR products were recovered from low melting temperature agarose and labelled with α-[^{32}P]dCTP by primer elongation (Feinberg and Vogelstein 1984). All other Southern blot procedures and conditions have been described previously (Hulsebos et al. 1991).

Fluorescence in situ hybridization analysis

Fluorescence in situ hybridization (FISH) and measured of the map position of the hybridizing signal on chromosome 2 were performed according to Hoovers et al. (1992), with minor modifications. The map position, expressed as the fractional length of the whole chromosome relative to pter (FLpter, 95% c.i.) of the hybridizing signal on chromosome 2 was 0.547 +/– 0.019. This corresponds to chromosomal bands 2q21.1–q21.3.

Results

Probe pAC5 is a 1.5-kb *Eco*RI fragment that contains most of the exon 27b sequence and approximately 1.4 kb of 3′ flanking intron sequence of the functional *NF1* gene on 17q. It has been reported before that this probe cross-hybridizes to *NF1*-related sequences on chromosomes 14 and 22 (Marchuk et al. 1992). On southern blots of *Eco*RI digests, probe pAC5 identifies cross-hybridizing fragments of 4.2 and 4.8 kb on human chromosome 14 (in hybrid cell line Wegroth-B3; Fig. 1, lane 2) and similarly sized fragments on human chromosome 22 (in hybrid cell line PgMe25Nu; Fig. 1, lane 4). To determine the location of the *NF1*-related sequence on chromosome 22, we hybridized pAC5 to a mapping panel that divides this chromosome into 30 segments (for a description of the panel see Demczuk et al. 1995). Cross-hybridizing sequences were detected in many cell lines of the panel (data not shown). However, as shown in Fig. 1, lane 5, the human-specific *Eco*RI fragments were still present in cell line NF13. This cell line contains the der(22) from a t(17;22) (q11.2;q11.2) translocation and no other human chromosmes (Ledbetter et al. 1989; E. H. Bijleveld and T. J. M. Hulsebos, unpublished results). Since the der(22) only contains the centromeric part of 22q, we conclude that the *NF1*-related sequence is located close to the centromere.

To obtain cosmids with sequences from the *NF1*-related locus on chromosome 22, we screened a chromosome 22 cosmid library (LL22NC03) with probe pAC5. About 25 pAC5-positive cosmids were found, of which four (29A9, 29D1, 112G5, and 127F6) were studied in

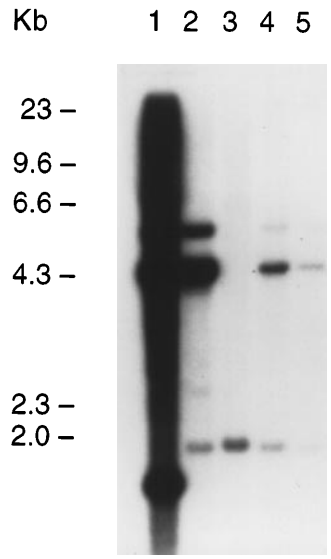

Fig. 1 Localization of the *NF1*-related locus on chromosome 22 and on chromosome 14. A Southern blot of *Eco*R1-digested human DNA and DNAs extracted from human × mouse cell lines was hybridized with probe pAC5. *Lane 1* human lymphocytes, *lane 2* cell line Wegroth-B3 with chromosome 14 as the sole human contribution, *lane 3* mouse-only cell line Wehi-TG, *lane 4* cell line PgMe25Nu with chromosome 22 as the sole human contribution, *lane 5* cell line NF13 with the der(22) from a t(17;22) (q11.2;q11.2) translocation as the sole human contribution. Probe pAC5 hybridizes to itself in total human DNA (1.5 kb fragment, lane 1) and cross-hybridizes to a mouse-specific fragment of 1.8 kb *(lanes 2–5)*, and to fragments of 4.2 and 4.8 kb on chromosomes 14, 22, and the der(22) in NF13 (*lanes 2, 4, and 5*, respectively). Additional cross-hybridizing fragments larger than 4.8 kb are present in total human DNA. However, these are not readily visible due to overexposure of the autoradiograph to reveal the cross-hybridizing fragments in the cell lines

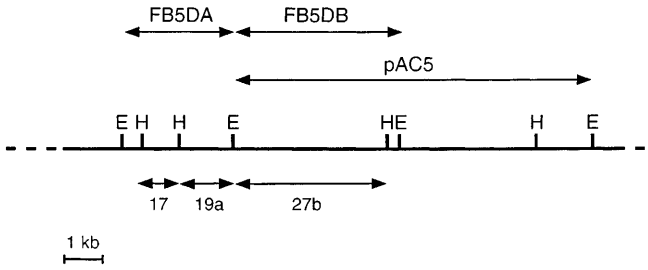

Fig. 2 Restriction map of the pAC5-positive region in cosmids from the *NF1*-related locus on chromosome 22. The partial restriction map was constructed by single- and double-digestion with *Eco*R1 *(E)* and *Hin*dIII *(H)* of the pAC5-positive cosmids and hybridization with probes pAC5, FB5DA, FB5DB, and with restriction fragments from the relevant region. The regions that contain sequences homologous to pAC5, to the GAP-related domain cDNA sequences FB5DA and FB5DB, and to exons 17, 19a, and 27b of the functional *NF1* gene are indicated

more detail. They all revealed, upon probing of *Eco*RI digests with pAC5, strongly hybridizing fragments of 4.2 and 4.8 kb, i.e., in their genomic configuration. The cosmids were analyzed for the presence of *NF1*-related sequences by hybridizing the *NF1* cDNA probes FF13, FB5DA, FB5DB, and P5 to *Eco*RI and *Hin*dIII digests. FF13 and P5 originate from the 5′ and 3′ part, respectively, of the cDNA of the functional *NF1* gene. FB5DA and FB5DB are from the middle part of the cDNA and include the 5′ and 3′ half, respectively, of the GAP-related domain (Marchuk et al. 1991). Only FB5DA- and FB5DB-homologous sequences were present in the cosmids. Their positions are indicated in the restriction map of the pAC5-positive region of the cosmids, which is shown in Fig. 2. To gain further insight into the presence of homologous exons in the cosmids, we hybridized PCR products from selected exons of the functional *NF1* gene to digests of the cosmids. PCR products of exons 15, 17, 19a, 21, 23a, 25, 27a, 27b, 29, and 31 were generated by using DNA from the chromosome 17-only human × mouse hybrid cell line GM10498B as template and published primer sequences and conditions (Li et al. 1995). As expected, the PCR product of exon 27b, of which most of the sequence is present in probe pAC5, displayed a positively hybridizing band on the Southern blots. Except for exons 17 and 19a, the PCR products of all other exons did not hybridize to the cosmid filters (data not shown). The positions of the sequences homologous to exons 17, 19a, and 27b of the functional *NF1* gene are indicated in Fig. 2.

To determine the chromosomal location of the *NF1*-related sequence on chromosome 22, we next performed fluorescence in situ hybridization (FISH) with the four cosmids. As expected, they all hybridized to the pericentromeric region of 22q (Fig. 3). Interestingly, these cosmids additionally hybridized to the pericentromeric region of chromosome arm 14q and to band q21 of chromosome 2. No hybridization signal was seen on chromosome arm 17q, which contains the functional *NF1* gene, or on any other chromosome.

Finally, a very accurate map position of the *NF1*-related locus on 22q could be established by screening our chromosome 22 YAC library with probes E2.8 and E4.4. Probe E2.8 is the 2.8-kb *Eco*RI fragment that contains FB5DA-homologous sequences (see Fig. 2). Probe E4.4 is a 4.4-kb *Eco*RI fragment that originates from a region that is outside the restriction map shown in Fig. 2. Both probes

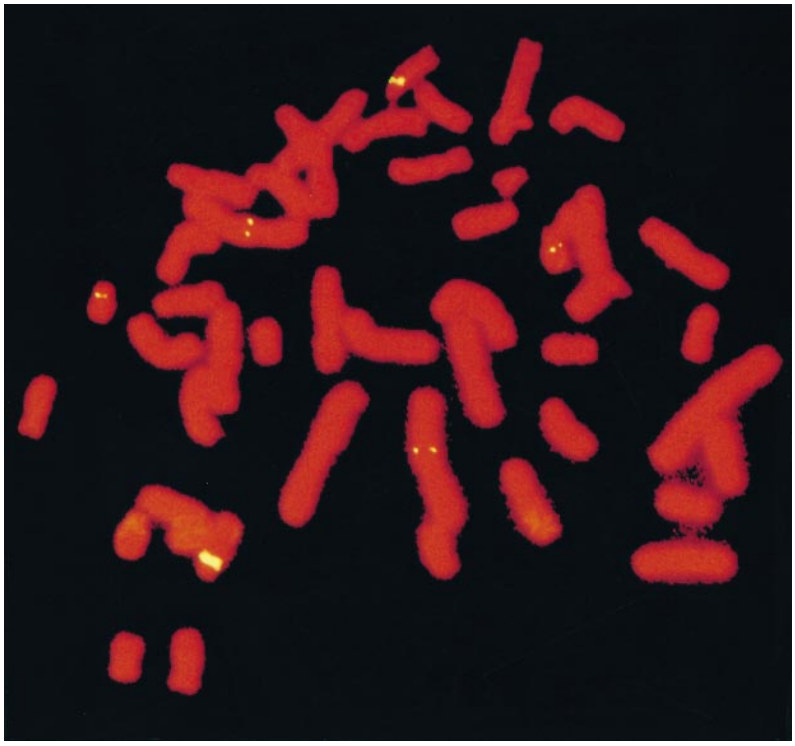

Fig. 3 Fluorescence in situ hybridization (FISH) of cosmid 29A9 from the *NF1*-related locus on chromosome 22 to normal human prometaphase chromosomes. The biotinylated cosmid was hybridized to prometaphase spreads and the hybridized signals were visualized, essentially as described by Hoovers et al. (1992). Chromosomes were counterstained with propidium iodide. The other three cosmids from the *NF1*-related locus on chromosome 22 gave identical FISH results

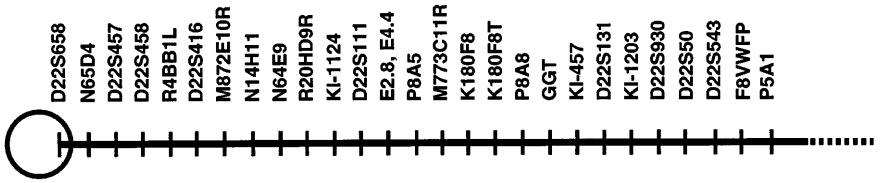

Fig. 4 Location of probes E2.8 and E4.4, isolated from cosmids specific to the *NF1*-related locus on chromosome 22, on the yeast artificial chromosome contig map of this chromosome. Order of markers in this most centromeric part of chromosome arm 22q was taken from Collins et al. (1995). The position of the centromere is indicated by the *circle* at the left

identify the same YACs in the library. These YACs (Y10C4, R20HD9, Y3C8, and M773C11) are all contained within our recently established YAC contig map of chromosome 22 (see Collins et al. 1995). We infer from the YAC mapping data that E2.8 and E4.4 are located between markers D22S111 and P8A5 (Fig. 4). Thus, the *NF1*-related locus maps very close to the centromere on the long arm of chromosome 22.

Discussion

NF1-related loci on chromosomes 22, 14, and 2

Our regional mapping, in situ hybridization, and YAC screening data demonstrate that an *NF1*-related locus is present in the region very close to the centromere on 22q. The cosmids that we used in the in situ hybridization experiments all contained sequences homologous to probes (FB5DA and FB5DB) that include the GAP-related domain in the middle part of the functional *NF1* gene cDNA (Marchuk et al. 1991; see Fig. 2). However, our preliminary mapping data, using functional *NF1* exon-specific probes, indicate that only sequences homologous to exons 17, 19a, and 27b (and possibly the flanking exons 16, 18, 19b, 20, and 28, which were not tested) are present in the *NF1*-related locus on chromosome 22 (Fig. 2). Remarkably, sequences homologous to exons 21, 23-1, 25, and 27a (and possibly exons 22, 23-2, 24, 26 in between, which were not tested) were absent. This suggests that the GAP-related domain itself, defined as the region that has homology to bovine P120GAP, which extends from exon 21 to exon 27a (Li et al. 1995), is not represented in this locus. Probes from the 5′ and 3′ part of the functional *NF1* gene cDNA (FF13 and P5) failed to hybridize to the cosmids, indicating that their homologous sequences, if present at the *NF1*-related locus, were not included in these cosmids. Additional hybridizing signals were detected in the pericentromeric region of 14q and in 2q21 (Fig. 3). It was already apparent from the presence of similarly sized pAC5-positive fragments in the 14-only and 22-only cell lines (Fig. 1) and from earlier studies (Marchuk et al. 1992) that the *NF1*-related loci on chromosomes 14 and 22 closely resemble each other. The in situ hybridization signal on 2q21 is rather remarkable. This chromosomal band is supposed to contain the remnant of the centromere of ape chromosome 13, which became inactive after telomeric fusion with ape chromosome 12 to form human chromosome 2 (IJdo et al. 1991). It cannot be excluded at the moment that the hybridization signal at 2q21 is merely caused by the ancient centromeric sequences present at this position. However, our preliminary mapping studies indicate that weakly hybridizing pAC5-positive fragments, similar in size to the ones at the *NF1*-related loci on chromosomes 14 and 22, are present at this locus (E. H. Bijleveld and T. J. M. Hulsebos, in preparation). Other *NF1*-related loci, presumed to be present on chromosomes 12, 15, 20, and 21 (Legius et al. 1992; Marchuk et al. 1992; Cummings et al. 1993; Gasparini et al. 1993) did not hybridize with the cosmids. This suggests that these loci do not have the sequences contained within the cosmids from the 22q-specific *NF1*-related locus. Alternatively, the sequences at these loci differ too much from those in the 22q-specific *NF1*-related locus to be detected by in situ hybridization.

Despite the presence in the cosmids of sequences homologous to cDNAs that include the GAP-related domain, these cosmids did not reveal an in situ hybridization signal with the functional *NF1* gene on 17q. The cosmids were originally selected for the presence of pAC5-homologous sequences. This probe originates from the exon 27b region in the functional *NF1* gene. Exon 27b is flanked at the 5′ end by an intron of probably more than 4 kb and at the 3′ end by an intron of 45–50 kb (Li et al. 1995). Moreover, as discussed above, only a very limited number of exonic sequences of the functional *NF1* gene are represented in the *NF1*-related locus on chromosome 22. Taken together, these data indicate that most of the intronic sequences contained within the cosmids differ considerably from those in the functional *NF1* gene.

Interchromosomal gene conversion

It has been proposed for several human genes that they can acquire mutations present in a related gene or pseudogene by gene conversion (Gorski and Mach 1986; Harada et al. 1987; Higashi et al. 1988; Matsuno et al. 1992). In these cases, the presumed conversion event occurs intrachromosomally, i.e., between donor and target gene on the same chromosome. Recently, strong evidence has been given for the transfer of mutations by interchromosomal gene conversion, namely between the von Willebrand factor pseudogene *(F8VWFP)* on chromosome 22 and the functional von Willebrand factor gene *(F8VWF)* on chromosome 12 (Eikenboom et al. 1994). Interestingly, the von Willebrand factor pseudogene is located in the same centromeric region of 22q as the *NF1*-related gene (Fig. 4).

In case of NF1, interchromosomal gene conversion during meiosis would require close contact between the *NF1*-related genes and the functional *NF1* gene. The participation of extrachromosomal intermediates in such an exchange process cannot, however, be excluded. With the availability of cosmids that specifically hybridize to the *NF1*-related sequences on chromosomes 22, 14 and 2, the concept of close contact can be tested now. Experiments have been started, using these cosmids and cosmids specific to the functional *NF1* gene, to determine the spatial distribution of the *NF1* gene and of the *NF1*-related genes in the interphase and meiotic prophase nucleus by confocal laser scanning microscopy.

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