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ARTICLE

NF1 microdeletion breakpoints are clustered at flanking repetitive sequences

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Neurofibromatosis type 1 patients with a submicroscopic deletion spanning the *NF1* tumor suppressor gene are remarkable for an early age at onset of cutaneous neurofibromas, suggesting the deletion of an additional locus that potentiates neurofibromagenesis. Construction of a 3.5 Mb BAC/PAC/YAC contig at chromosome 17q11.2 and analysis of somatic cell hybrids from microdeletion patients showed that 14 of 17 cases had deletions of 1.5 Mb in length. The deletions encompassed the entire 350 kb *NF1* gene, three additional genes, one pseudogene and 16 expressed sequence tags (ESTs). In these cases, both proximal and distal breakpoints mapped at chromosomal regions of high identity, termed NF1REPs. These REPs, or clusters of paralogous loci, are 15–100 kb and harbor at least four ESTs and an expressed SH3GL pseudogene. The remaining three patients had at least one breakpoint outside an NF1REP element; one had a smaller deletion thereby narrowing the critical region harboring the putative locus that exacerbates neurofibroma development to 1 Mb. These data show that the likely mechanism of *NF1* microdeletion is homologous recombination between NF1REPs on sister chromatids. *NF1* microdeletion is the first REP-mediated rearrangement identified that results in loss of a tumor suppressor gene. Therefore, in addition to the germline rearrangements reported here, NF1REP-mediated somatic recombination could be an important mechanism for the loss of heterozygosity at *NF1* in tumors of NF1 patients.

INTRODUCTION

Haploinsufficiency for neurofibromin is the likely molecular basis of neurofibromatosis type 1 (NF1), a common autosomal disorder that predisposes to the development of benign and malignant tumors. Genetic, biochemical and proliferative studies of cells from NF1-associated tumors are consistent with a tumor suppressor function for neurofibromin. Tumor suppressor activity is due, at least in part, to a ras-GTPase activating protein (ras-GAP) domain which accelerates the conversion of activated GTP-ras to inactivated GDP-ras (1). Evidence in human and mouse shows that neurofibromin-deficient tumor cells have increased activated ras and dysregulated proliferative properties (2,3), which may be mediated by the ras-dependent mitogen-activated protein kinase signaling pathway (4). Both benign and malignant tumors show homozygous inactivation of *NF1* resulting in lack of functional neurofibromin. Although *NF1* inactivation in a tumor progenitor cell can occur by numerous mechanisms, the identification of defined intragenic *NF1* mutations in primary tumor tissue argues that lack of neurofibromin is central to their development (5–7).

Over 70% of germline mutations of the *NF1* gene are intragenic and predict a premature truncation of neurofibromin (8). These mutations are distributed throughout the coding region. They are generally unique for a given patient or family, and are not predictive for any of the diverse clinical manifestations that can develop in this multisystemic disorder. Nearly all NF1 patients develop café-au-lait macules, axillary and inguinal freckling, multiple neurofibromas, and Lisch nodules, which are hamartomas of the iris of the eye. Other significant, but less common, manifestations of the disorder include learning disabilities, optic glioma, bony abnormalities (sphenoid bone dysplasia, pseudoarthrosis, scoliosis), increased risk of specific malignancies, and others (9,10). NF1 has been considered to be primarily a disorder of cells derived from the neural crest, which is supported by recent evidence consistent with neurofibromas arising by clonal proliferation of a neurofibromin-deficient Schwann cell (11).

Previously, we identified five patients that carried a deletion of one entire *NF1* allele. These patients were remarkable for an early age (<10 years) at onset of dermal neurofibromas, an increased number or heavy burden of neurofibromas relative to their age, and certain atypical facial features (12,13). The asso-

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ciation of an *NF1* microdeletion with this phenotype was subsequently confirmed by us and other investigators (14–18). In addition, the identification of families segregating an *NF1* microdeletion demonstrated that the rearrangement was co-inherited with the remarkable facial and tumor features (17,19,20).

The molecular basis for precocious neurofibromagenesis in microdeletion patients is unknown. Previously, we estimated the microdeletions at 0.7–2 Mb, which, even accounting for the large 350 kb *NF1* gene, implies that many additional genes are deleted (13,14,19). Theoretically, early age at onset of neurofibromagenesis could be attributed to: (i) deletion of the *NF1* gene alone; (ii) co-deletion of *NF1* and one of the three genes of unknown function that are embedded in an *NF1* intron; (iii) co-deletion of *NF1* and a novel contiguous gene(s); or (iv) dysregulation of a gene at the deletion breakpoint. We consider it unlikely that neurofibromin haploinsufficiency alone could account for early onset of tumorigenesis. Over 70% of NF1 patients are heterozygous for a mutation that predicts premature truncation of neurofibromin, yet in a population-based study only ~14% of subjects developed dermal neurofibromas before 10 years of age (21,22). However, it is unknown whether neurofibroma development could be ameliorated in any of these patients due to possible residual activity from the mutant *NF1* allele. The role of a putative co-deleted locus has been difficult to assess because the number of deletion patients is small and information regarding number and age at onset of neurofibromas and deletion magnitude are not always evaluated or reported. Recently, however, we described 12 unrelated *NF1* microdeletion patients with early onset and/or high burden of neurofibromas with deletion breakpoints that clustered in the same centromeric and telomeric locus intervals (K. Maruyama, M. Weaver, K. Leppig, A.S. Aylsworth, M.O. Dorschner, R. Farber, J. Ortenberg, A. Rubenstein, L. Immken, C. Curry and K. Stephens, submitted for publication). Towards mapping and identifying a locus that potentiates neurofibromagenesis in NF1 patients, we constructed a 3.5 Mb physical map of the *NF1* region, precisely mapped the deletion, and examined deletion genotype with patient phenotype. We report that the breakpoints in the majority of patients are clustered at flanking genomic segments of paralogous sequence (sequence similarity due to duplication). These results have important implications regarding germline and somatic rearrangements involving *NF1*.

RESULTS

Construction of a 3.5 Mb contig

Recently, we determined that both the centromeric and telomeric breakpoints in 14 of 15 NF1 patients with submicroscopic deletions were clustered in two distinct marker intervals. Quantitative PCR and the analysis of somatic cell hybrid lines carrying deleted chromosome 17 of each patient mapped the centromeric breakpoints between marker loci *D17S2120* and *UT172* and the telomeric breakpoints between *D17S1800* and *D17S1880* (K. Maruyama *et al.*, submitted for publication) (Fig. 1). Although these data were suggestive of breakpoint clustering, the length of each interval was unknown. In addition, the number and unique order of other markers within each of these initial breakpoint intervals was unknown. To refine the

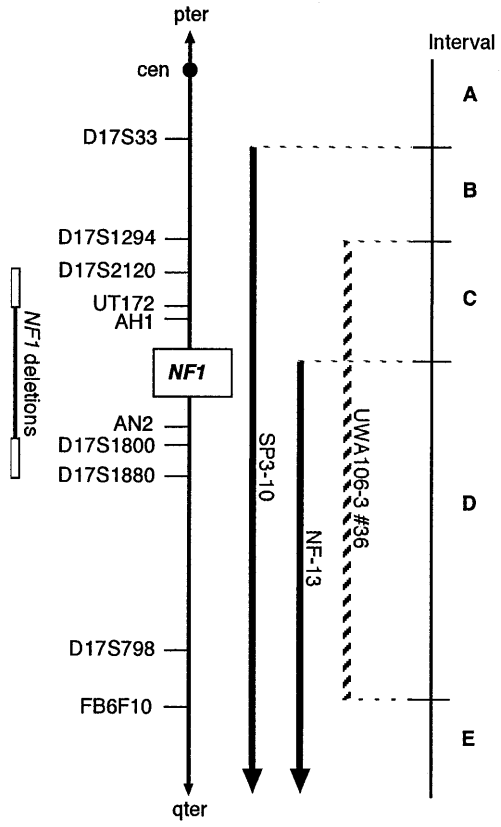

Figure 1. Hybrid mapping panel for the *NF1* region. The location of markers in the *NF1* region are depicted, along with the *NF1* deletion previously defined in patients, with open bars representing the breakpoint cluster regions (K. Maruyama *et al.*, submitted for publication). Loci were mapped to one of five intervals (A–E) on chromosome 17 by their presence or absence in human/rodent somatic cell hybrid lines. The hybrid line UWA106-3-#36 was constructed from *NF1* microdeletion patient UWA106-3 and carries a chromosome 17 deleted for the indicated segment (13). Line SP3-10 carries human chromosome segment 17q11.2 to qter (76) and NF13 carries a segment from *NF1* intron 27b to qter (77).

location of the deletion breakpoints, we sought to construct a physical map encompassing both breakpoint cluster regions. Initially, chromosome 17 loci reported to map at or near band q11.2 were gleaned from the literature and publicly available electronic databases and screened by PCR against a somatic cell hybrid mapping panel. This placed each locus into one of five possible chromosomal intervals (Fig. 1). Loci that mapped to intervals C and D were used to identify and construct a contig of novel and previously reported bacterial artificial chromosome (BAC) and P1-derived artificial chromosome (PAC) clones. Initial database searches identified five sequenced clones that served as a framework for contig construction. Two BACs, 468F23 and 41C23, were found to harbor *AH1* and *AN2*, respectively, which are end sequences of a previously described *NF1* yeast artificial chromosome (YAC) contig (23) (Fig. 2). A 297 kb sequence carrying a large portion of the *NF1* gene (GenBank accession no. AC004526), and clones 542B22 and 307A16, were identified from database searches. Together, the three clones 499I20, AC004526 and 41C23 comprise a 476 kb contiguous sequence spanning from intron 1 of the *NF1* gene to *D17S1800* (Fig. 2). The remainder of the contig was assembled by screening a BAC library with

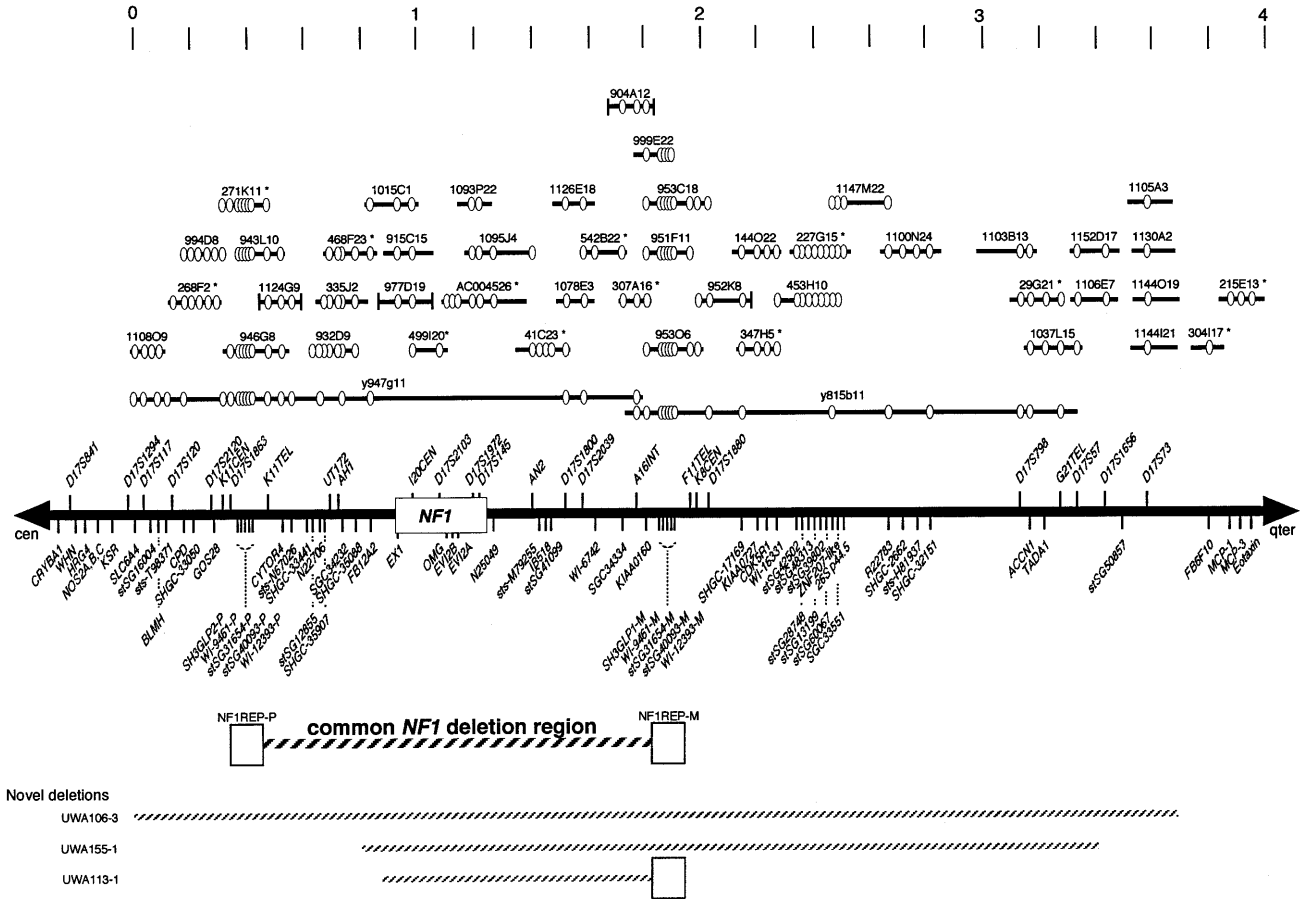

Figure 2. Physical contig of the *NF1* region. The thick black bar is a schematic of the chromosome 17q11.2 region with STS loci placed above the bar and genes and EST loci below. The BAC, PAC and YAC clones comprising the contig are shown above; open elipses are aligned with the loci on the chromosome schematic and indicate a positive hit in the clone; sequenced BAC/PAC clones are indicated with an asterisk. Vertical bars at the ends of BACs represent insert termini that were sequenced and submitted to GenBank, but not converted to amplimers. The scale in Mb is at the top of the figure. The size and extent of microdeletions of NF1 patients are shown below the chromosome. The common *NF1* deletion region was identified in 14 of 17 unrelated patients; whereas three patients had novel deletions as shown. The open boxes represent flanking repetitive sequences (NF1REP) where the majority of breakpoints mapped.

selected loci that mapped in intervals C and D (Figs 1 and 2) and by utilizing newly released chromosome 17 sequences from the Whitehead Institute for Biomedical Research/MIT Center for Genome Research (http://www-genome.wi.mit.edu/). The clones comprising the BACs are listed in Table 1.

The contig consisted of 39 BAC/PAC and two YAC clones (Fig. 2, Table 1). The new marker *A16INT* linked together the two YACs y947g11 and y815b11 (http://www-genome.wi.mit.edu/), creating a YAC contig of the region. In addition, loci prominent for their previous use in genetic mapping and loss of constitutional heterozygosity (LOH) analyses were mapped precisely. *UT172*, previously estimated to be 1.5 Mb centromeric of *NF1* (24), is only ~250 kb distant within BAC 468F23. *D17S117* and *D17S120* are located ~1 Mb centromeric of *NF1*; the latter marker actually lies within an intron of the carboxypeptidase D (*CPD*) gene. *D17S798* is located ~1.8 Mb telomeric of *NF1*.

Fine mapping of the *NF1* microdeletion breakpoints

Over 10 loci were placed precisely in each of the breakpoint cluster regions (Fig. 1), thereby facilitating fine mapping of the breakpoints of all 17 microdeletion patients. Fourteen microdeletion patients had proximal breakpoints in the locus interval of *SH3GLP2* to *CYTOR4* (*SHGC-37343*) and distal breakpoints in the interval between *SH3GLP1* and *D17S1880* (Fig. 2). The remaining three deletion cases had at least one novel breakpoint (Fig. 2). Patient UWA113-1 had a novel centromeric breakpoint between *FB12A2* and exon 1 of the *NF1* gene. Both breakpoints of patient UWA155-1 were novel and located between the intervals defined by *SHGC35088–FB12A2* and *D17S1656–stSG50857*. Patient UWA106-3, who had the largest deletion in our cohort (13), also had two unique breakpoints. The telomeric breakpoint mapped in the interval of *D17S73* to *FB6F10* and the centromeric breakpoint was mapped previously between *D17S1294* and *SCL6A4* during construction of a physical contig of the latter gene that encodes the serotonin transporter (25).

The contig provided more precise estimates of the physical lengths of both the region and the patient deletions. Because YACs 947g11 and 815b11, each estimated at 1.7 Mb (http://www-genome.wi.mit.edu/), are completely contained within the deletion of UWA106-3, this patient's deletion is approximated at 3.5 Mb. YAC 947g11 spans from *SLC6A4* to *A16INT*

Table 1. BAC/PAC clones from 17q11.2

Clone source	Clone name	GenBank accession no.	Size (kb)
hRPK	1108 O 9		
hRPK	268 F 2	AC006050	163
hRPK	994 D 8		
hRPK	946 G 8		
hRPK	271 K 11	AC005562	199
hRPK	943 L 10		
hRPK	1124 G 9		
hRPK	932 D 9		
hCIT	335 J 2		
hCIT	468 F 23	AC004666	120
hRPK	1015 C 1		
hRPK	915 C 15		
hRPK	997 D 19		
hCIT	499 I 20	AC004222	119
		AC004526[a]	297
hRPK	1093 P 22		
hRPK	1095 J 4		
hCIT	41 C 23	AC003101	208
hRPK	1078 I 13		
hRPK	1126 E 16		
hCIT	542 B 22	AC004523	131
hCIT	307 A 16	AC003041	78
hRPK	904 A 12		
hRPK	999 E 22		
hRPK	953 O 6		
hRPK	951 F 11		
hRPK	953 C 18		
hRPC	144 O 22		
hRPK	952 K 8		
hCIT	347 H 5	AC002119	109
hCIT	453 H 10		
hRPK	227 G 15	AC005899	184
hRPK	1147 M 22		
hRPK	1100 N 24		
hRPK	1103 B 13		
hRPC	29 G 21	AC003687	141
hRPK	1037 L 15		
hRPK	1106 E 7		
hRPK	1152 D 17		
hRPK	1014 I 16		
hRPK	1144 I 21		
hRPK	1130 A 2		
hRPK	1105 A 3		
hCIT	304 I 17	AC004147	139
hRPK	215 E 13	AC005549	147

hRPK, clones from RPCI-11 Human Male BAC library; hCIT, clones from CITB Caltech Human BAC library; hRPC, clones from RPCI Human PAC library.

[a]Contiguous sequence of two overlapping BAC clones.

and overlaps 815b11, which extends to just beyond *D17S798*. The known lengths of sequenced BACs and the average length of 185 kb for non-sequenced BACs derived from the RPC1-11 library were subtracted from the YAC lengths to give the estimated scale in Figure 2. The length of the common *NF1* microdeletion was estimated at 1.5 Mb.

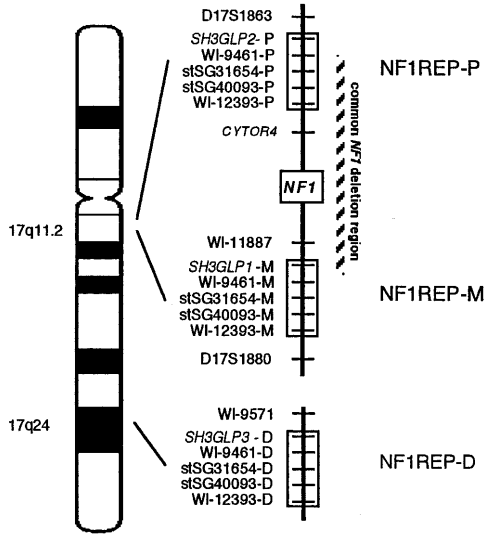

Figure 3. NF1REP domains on chromosome 17. The locations of the three NF1REP regions, designated -P, -M and -D (for proximal, medial and distal) are shown along with the five loci they are known to contain. In addition, the closest unique marker flanking each REP is indicated. The cross-hatched bar represents the 1.5 Mb region commonly deleted in *NF1* microdeletion patients.

NF1 microdeletion breakpoints cluster at repetitive sequences

Fine mapping of the region led to the discovery of two *SH3GL* expressed pseudogenes, *SH3GLP2* and *SH3GLP1*, that mapped near the breakpoints of the common *NF1* deletions (Fig. 2). Because low copy repeats are known to flank deletions/duplications responsible for some contiguous gene syndromes (26), a search for additional multicopy transcripts was initiated. A third expressed pseudogene, *SH3GLP3*, was reported to map distally at 17q24 (27). BLAST analyses of the *SH3GL* pseudogenes identified BACs 271K11 and 147L13, which carried *SH3GLP2* and *SH3GLP3*, respectively. The sequence-tagged site (STS)/expressed sequence tag (EST) content of the BAC clones was obtained (http://www-genome.wi.mit.edu/) and BLAST analyses identified their locations within each clone. This revealed that two ESTs, *WI-12393* and *WI-9461*, were present in both BACs and located near each respective *SH3GL* pseudogene. Systematic BLAST analyses of loci reportedly mapping near *NF1* in publicly available genome databases revealed that *stSG40093* and *stSG31654* were not only in BAC 271K11 centromeric to *NF1*, but were also harbored by BAC 147L13 at chromosome 17q24 (http://www.ncbi.nlm.nih.gov/genemap98). Together these analyses identified two clusters of five transcripts for which the order and relative distance between markers was conserved. These clusters of paralogous loci were designated as NF1REP, using the suffixes -P and -D to distinguish the proximal repeat at 17q11.2 from the distal repeat at 17q24 (Figs 2 and 3).

To determine whether the unsequenced region surrounding *SH3GLP1* comprised an additional NF1REP, PCR primers for

Table 2. Physical features of subjects with *NF1* microdeletions

Patient ID[a]	Cutaneous neurofibromas[b]		Ht[c] (%)	Macrocephaly (cm)	Facial features	Intelligence[d]	Hands/feet	Heart[e]	Other tumors	
	Age (years)	Number							Type, number	Age (years)
Patients with deletion breakpoints at NF1REP-P and NF1REP-M										
69-3	8	Many	5	– (54.5)	Hypertelorism	IQ 59	'Normal'		Plexiform neurofibroma, 2	10
119-1	22	TNTC	50	+	Hypertelorism	IQ 80s, 'dull normal'	NI	–		
123-3	5	Many	Normal	+	Hypertelorism, ptosis	LD	25–50%	ASD	Plexiform neurofibroma, 1 Neurofibrosarcoma, 1	5 9
128-3	7 29	Several TNTC	3	– (55)	Ptosis, downslanting palpebral fissures	Normal	10–25%	–		
147-3	6	Numerous	40	–	Hypertelorism, ptosis, coarse, downslanting palpebral fissures	Significant delays	NI	–		
156-1	31	TNTC	NI	NI	'Dysmorphic'	Mild MR with severe LD	NI	–	Optic glioma	
160-1	11 35	Several TNTC	<3	NI	'Noonan-like', coarse	LD, special education	NI	–	Plexiform neurofibroma, 1	7
166-1	39	TNTC	NI	NI	Telecanthus, downslanting palpebral fissures	'Dull'	'Large'	–		
166-2	7 19	Several TNTC	90	– (56.5)	Hypertelorism, downslanting palpebral fissures	Special education	>97%	–		
166-3	4	None	NI	– (49)	Downslanting palpebral fissures, 'unusual face'	'Normal', speech impediment	97%	–		
166-4	5 6	None 9	90	– (48.5)	Downslanting palpebral fissures	'Normal', speech problems	97%	–		
167-1	4 7	7 20	NI	NI	Ptosis, broad neck	Mild development delay	NI	ASD, PS		
169-1	18	Present	NI	+	Coarse, hypertelorism, downslanting palpebral fissures	'Dull normal', LD	'Large'	Dilated aortic valve replaced	f	
172-1	Childhood 35	None TNTC	NI	NI	'Dysmorphic'	Severe LD	'Large'	–	Optic gioma, 1 Plexiform neurofibroma, 2	
176-1	13	NI	10	–	Webbed neck, 'Noonan'	WISC-III, mild/ borderline MR	97%	–	Plexiform neurofibroma, 2	
183-1	12	10	25–50	NI	Hypertelorism, ptosis, broad nose	Borderline development delay	50%	PS	Plexiform neurofibrma, 1	1
184-1	5	TNTC	NI	NI	Telecanthus, coarse face	IQ 71 full-scale, 81 verbal, 66 performance	97%	–		
Patients with at least one unique *NF1* deletion breakpoint										
106-3	18	TNTC	Normal	+ (61.5)	Coarse	IQ 46	'Large'	–	Plexiform neurofibroma, 1 Spinal neurofibromas, TNTC	3 25
11311	8 15	TNTC TNTC	10	+ (59)	Coarse, hypertelorism	IQ 88 LD	>97%	–	–	
15511	27	TNTC	NI	+ (59.5)	Coarse, ptosis	Moderate MR	>97% hands, 75% feet	–	Spinal neurofibroma, 1 Neurofibrosarcoma, 1[g]	Sym 27 28

[a]The UWA- prefix for patient identifiers is not shown. All patients are unrelated, with the exception of four members of family UWA166.
[b]TNTC, too numerous to count.
[c]Height in percentile; NI, no information available.
[d]LD, learning disabilities; MR, mental retardation.
[e]ASD, atrial septal defect; PS, pulmonic stenosis.
[f]Patient's mother and sister with NF1, presumably due to the same *NF1* microdeletion, had retroperitoneal fibrosarcoma with metastases (age ~30) and cerebellar medulloblastoma (age 16), respectively.
[g]Spinal neurofibroma was symptomatic at age 27; patient inherited *NF1* from his father who died of malignant central nervous system tumor in fifth decade.

WI-9461, *stSG31654*, *stSG40093* and *WI-12393* were used to amplify these loci from BACs overlapping the *SH3GLP1* locus. BAC clones 953C18, 951F11 and 95306 were positive for each of the transcripts; 999E22 was positive for all but the *WI-12393* locus. This medially positioned repeat cluster was designated NF1REP-M (Fig. 3). These results demonstrated that chromosome 17 carries at least three clusters of paralogous loci: *WI-9461*, *stSG31654*, *stSG40093* and *WI-12393*, each in association with a specific *SH3GL* pseudogene (Fig. 3). The absence of *WI-12393* from BAC 999E22 and preliminary sequence analysis (M. Dorschner, unpublished data) strongly suggests that NF1REP-P and -M are direct repeats of 15–100 kb in length. The repetitive sequences may extend further beyond *WI-12393*. The breakpoints of the patients carrying the common *NF1* microdeletion lie within, or adjacent to, NF1REP regions. The centromeric breakpoints were between *SH3GLP2* and *CYTOR4*, whereas the telomeric breakpoints occurred between *SH3GLP1* and *K8CEN*. Finer mapping of the breakpoints will require the development of REP-specific primers or Southern blot analyses that identify junction fragments. The size and orientation of NF1REP-D is unknown.

Several lines of evidence confirmed that, despite carrying sequences with a high degree of identity, BACs spanning NF1REP-P and -M were localized unambiguously. First, the primers for amplification of *SH3GLP1* and *SH3GLP2* were locus specific, exploiting base differences in the 5′ region of the transcripts (27). BACs 943L10 and 946G8 were the only clones that possessed *SH3GLP2*, whereas clones 953C18, 951F11, 999E22 and 95306 carried only *SH3GLP1*. In addition, these BACs harbored the expected unique loci based on our deletion analysis. BAC 943L10 was positive for *D17S1863* and *CYTOR4*, whereas BACs spanning the medial REP contained *KIAA0160* or *D17S1880*.

Mouse orthologs for all of the genes located in interval B (Fig. 1), *SLC6A4*, *CPD*, *CDK5R1* and the chemokine cluster, have been mapped to the same region of mouse chromosome 11 that carries the *NF1* ortholog. It appears that synteny has been conserved between human and mouse for the region from *CRYBA1* to at least the chemokine cluster.

NF1 deletion genotype/phenotype and parental origin of deletion

The physical features of the 13 unrelated *NF1* microdeletion patients and the four members of family UWA166 are summarized in Table 2. There were no obvious differences detected between the features present in those individuals with the common *NF1* deletion and the three with deletions of different lengths. No single feature was present or absent consistently within either group. The location of the putative gene that potentiates neurofibromagenesis was narrowed to an interval of 1 Mb between *FB12A2* and *SH3GLP1*, as defined by the deletion of patient UWA113-1 (Fig. 2). This critical region is known to harbor four genes, two pseudogenes, and seven ESTs (Fig. 2, Table 3).

A preference for *de novo* microdeletion of the maternally derived chromosome was observed. Among the eight cases with documented *de novo* microdeletions, six were derived from the maternal homolog (UWA patients 113-1, 119-1, 147-3, 167-1, 183-1, 184-1) and two from the paternal homolog (UWA106-3, UWA123-3) (13,19, data not shown). Three families inherited *NF1* microdeletions. Family UWA166 includes the affected mother UWA166-1 and her three affected children UWA166-2, -3 and -4 (19), patient UWA169-1 inherited NF1 from his affected mother (19,28), and UWA155-1 inherited NF1 from his affected father.

DISCUSSION

NF1REP elements

Three NF1REPs were mapped to chromosome 17. NF1REP-P and -M flank the *NF1* locus at 17q11.2 and are separated by ~1.5 Mb of DNA. The third, NF1REP-D, is located at 17q24. Each REP is composed of at least five transcripts/ESTs including an *SH3GL* pseudogene, *WI-9461*, *stSG31654*, *stSG40093* and *WI-12393* (Fig. 3). In a search for proteins containing SH3 (src homology region 3) domains, three functional genes were identified from a fetal brain cDNA library, *SH3GL1*, *SH3GL2* and *SH3GL3*. These genes map at chromosomes 19p13.3, 9p22 and 15q24, respectively (27), and function in signal transduction, cytoskeleton and aggregation of huntingtin (29–31). In addition, three expressed *SH3GL* pseudogenes were identified that mapped by FISH to chromosome 17 (Fig. 3) (27). It will be important to characterize the expression of the other paralogous loci, *WI-9461*, *stSG31654*, *stSG40093* and *WI-12393*, at each of the NF1REPs. Although these loci were originally isolated as ESTs expressed in multiple tissues (Unigene: www.ncbi.nlm.nih.gov/UniGene/index.html), it is unclear whether each paralogous locus in NF1REP-P, -M and -D is expressed and whether they represent pseudogenes, functional loci or residual gene fragments.

NF1REP-mediated recombination

We propose that a high degree of homology between NF1REP-P and -M facilitates homologous recombination during meiosis or mitosis resulting in the deletion of intervening sequences. Consistent with this hypothesis, pseudogenes *SH3GLP1* and *-2* are 97.8% identical, whereas *SH3GLP3* shares only 90% identity with either of these (27). Further analysis of the identity between NF1REP-P and -M will require completing the sequence of the NF1REP-M domain; partial sequence analysis shows >98% identity (M. Dorschner, unpublished data). Recombination between the direct repeats NF1REP-P and -M could give rise to *NF1* microdeletions by either unequal recombination between sister chromatids or intrachromosomal recombination via a fold-back loop and excision. Distinguishing between these mechanisms will require further analyses to determine whether NF1REP-mediated recombination is associated with a meiotic crossover event. The apparent preference for *de novo NF1* microdeletion of the maternally derived chromosomes may provide a clue. Other REP-mediated rearrangements show a sex-dependent mechanism with maternally derived deletions resulting from excision of an intrachromatidal loop (32). Although in other cases, microdeletions mediated by flanking REP domains appear to arise by both mechanisms (33–35). If unequal meiotic recombination between sister chromatids underlies *NF1* microdeletion, it would predict the formation of a reciprocal duplication derivative. Whether a 1.5 Mb *NF1* duplication product would be stable is unknown; it may quickly undergo recombination and

Table 3. Genes and ESTs involved in *NF1* microdeletions

Gene/EST	Description	GenBank accession no.	Unigene cluster
SLC6A4	Serotonin transporter	L05568	553
stSG16004		R96721	155925
BLMH	Bleomycin hydrolase	X92106	78943
sts-T98371		T98371	15036
CPD	Carboxypeptidase D	U65090	5057
SHGC-33050		G29398	
GOS28	Golgi SNAP receptor complex member 1	AF073926	8868
SH3GLP2-P	SH3-domain GRB2-like 1 pseudogene	X99660	
WI-9461-P		G07297	183294
stSG31654-P		AA910341	191479
stSG40093-P		AI378068	124418
WI-12393-P	Moderately similar to KIAA0563 protein	G20446	14232
CYTOR4	Cytokine related receptor protein 4	G30528	119410
sts-N67026	Weakly similar to AD7C-NTP	N67026	206654
SHGC-33441		T70563	
stSG12855		H56424	221463
N22706		N22706	43234
SHGC-35907		D19648	94891
SHGC-34232	Weakly similar to IP4/PIP3 binding protein	G28215	28802
SHGC-35088		H79008	
FB12A2		T02847	
NF1	Neurofibromin	M89914	93207
OMG	Oligodendrocyte myelin glycoprotein	M63623	194772
EVI2A	Ecotropic viral integration site	M55267	41846
EVI2B	Ecotropic viral integration site	M60830	5509
AK3-p1	Adenylate kinase pseudogene	X60674	
N25049	Expressed only in olfactory epithelium	N25049	183219
sts-M79255		M79255	
IB518	Weakly similar to KIAA0665 protein	T03582	3454
stSG-41099		H29300	7985
WI-6742		R44280	8179
SHGC-34334		D19683	30670
KIAA0160		D63881	170329
SH3GLP1-M	SH3-domain GRB2-like 1 pseudogene	X99658	
WI-9461-M		G07297	183294
stSG31654-M		AA910341	191479
stSG40093-M		AI378068	124418
WI-12393-M	Moderately similar to KIAA0563 protein	G20446	14232
SHGC-17169		G19390	192761
KIAA0727	Similar to unconventional myosins	AB018270	39871
CDK5R1	CDK5 regulatory subunit 1/p35	X80343	2869
WI-16331		G20991	120762
stSG42502		H86705	40488
stSG28748		AA860832	
stSG48313		AA279265	97128
stSG13199		H75373	
stSG39802		AA084612	125286
stSG60067		AI017068	131740
ZNF207-like	Zinc finger transcription factor	AF046001	62112
SGC33551	*Homo sapiens* clone 23685 mRNA sequence	AF052093	9800
p44.5/ PMSD11	Macropain 26S proteosome subunit	AB003102	90744
R22783		R22783	
SHGC-2662	Weak similarity to ubiquitin-like protein 8	D11824	109701
sts-H81937	Moderate similarity to serine/threonine kinase	H81937	37528
SHGC-32151	Weak similarity to RAS gene family	AA199845	14202
ACCN1	Sodium channel (hBNaCl), degenerin	U57352	6517
TADA1	Maid-like gene	N37022	3447
stSG50857		AA400117	125747

Genes and ESTs in bold are within the 1.5 Mb commonly deleted NF1 region.

Table 4. Contiguous gene rearrangements involving low copy repeats

Disorder	Location	Rearrangement		REP size (kb)	Transcripts in REP	Reference
		Type	Size (kb)			
NF1	17q11.2	Deletion	1500	15–100	*SH3GLP, WI-9461, stSG31654, stSG40093, WI-12393*	This paper
VCFS/DGS CES	22q11	Deletion Duplication	2000/1500	200	*GGT, GGT-rel, BCRL, V7-rel, POM121-like*	45,48
Williams' syndrome	7q11.23	Deletion	2000	>30	*GTF2I, IB1445*	47
HNPP CMT1A	17p11.2	Deletion Duplication	1500	24	*COX10*	43
Smith–Magenis	17p11.2	Deletion	5000	200	*TRE, KER, SRP, CLP*	44
PWS/AS	15q11–13	Deletion	4000	50–200	*SGC32610, SHGC15126, SHGC17218, A006B10, MN7, A008B26, HERC2*	46,49
NPHP1 (nephronopthisis type 1)	2q12–13	Deletion	250	100	*D2S1735, D2S2087*	53

'revert' to a deletion (36). Non-mosaic trisomy 17 has not been reported in a live born, and partial 17p or 17q trisomy is rare and even mosaic cases are uncommon (37), suggesting that many such rearrangements are lethal.

Patterns of REP domains and chromosomal rearrangements

About 10 years ago it became clear that intragenic, or relatively small, deletions, duplications and inversions of the human genome could be mediated by homologous recombination between tandem genes, or other nearby repetitive sequences. Such rearrangements have been well described for the steroid sulfatase, α-globin, Factor VIII, LDL receptor and other genes (reviewed in refs 36,38). Recently, however, the breakpoints of large contiguous gene deletions and duplications from 1–5 Mb in length were mapped to flanking repetitive sequences of high identity. Such low copy repetitive elements have been designated as REPs, duplicons or paralogous regions (39,40). There is compelling evidence that homologous recombination between REPs is the molecular basis for a number of disorders (Table 4) (reviewed in refs 26,38). The precedence was established for the neuropathies Charcot–Marie–Tooth type 1A (CMT1A) and hereditary neuropathy with liability to pressure palsies (HNPP). Unequal recombination between flanking REPs during meiosis I results in duplication (CMT1A) or deletion (HNPP) of a 1.5 Mb segment of chromosome 17p11.2 (41,42). The two 24 kb CMT1A-REPs have 98.7% identity with an internal 557 bp recombination hotspot where 21 of 23 breakpoints occurred (43).

Although the characterization of REPs flanking large contiguous gene rearrangements is in its infancy, variability in REP length, number, complexity and orientation is apparent. REP length varies considerably (Table 4) and may correlate directly with the size of the intervening deletion/duplication. This suggests that recombination between distant REPs may require longer tracts of identity for efficient pairing (26). Although a single CMT1A-REP lies on each side of the CMT1A/HNPP rearrangement, the number of REPs and the apparent preference for recombination between specific REPs can vary considerably. Three Smith-Magenis syndrome (SMS)-REPs are found in the 17p11.2 region, yet nearly all SMS deletions are due to crossover events between the proximal and distal REPs (44). The identification of eight REPs at 22q11 suggests that recombination between specific REP pairs may account for different rearrangements underlying multiple congenital anomaly disorders that map to this chromosomal region, such as velocardiofacial syndrome/DiGeorge syndrome (VCFS/DGS) and cat eye syndrome (CES) (45). REP domains may be very complex repeats that include multiple subrepeats, which can be in tandem or interspersed, inverted or direct in orientation (46–48). REPs may even be dispersed among chromosomes; FISH experiments suggest that copies of the Prader-Willi syndrome/Angelman syndrome (PWS/AS) REP may be at 15q24 and 16p11 (49). The apparent preference for REP domains to occur near the centromere of chromosomes (Table 4) (26) is consistent with reports of a strong bias for these regions to acquire paralogous segments. This phenomenon is referred to as pericentromeric plasticity and presumably accounts for the varied *NF1*-related fragments that are scattered among the centromeric regions of seven different autosomes (50; reviewed in ref. 39). Homologous recombination events and the resulting chromosomal rearrangements are also dependent on the orientation of the repetitive sequences involved. For example, recombination between direct CMT1A-REPs results in deletion and duplication via unequal crossing-over between chromatids (reviewed in ref. 51), whereas recombination between indirect duplicons can result in either deletions or inversions (52–54).

Unique pathological aspects of NF1REP-mediated recombination

Several aspects of *NF1* microdeletions are unique among REP-mediated contiguous gene rearrangements in the human genome. For other disorders, REP-mediated rearrangements commonly account for a large fraction of analyzed cases. For example, >98% of CMT1A cases are caused by a duplication that results in partial trisomy of the 17p11.2 region that includes the *PMP22* locus, whereas <2% are due to missense mutations in the *PMP22* gene itself (55). In AS, large maternal deletions account for 70% of cases, uniparental disomy and imprinting mutations for an additional 5%, and inactivating mutations in *UBE3A* for another 5% (56). In marked contrast, only 2–13% of NF1 cases result from *NF1* microdeletions (16,18,57,58), whereas >70% result from intragenic mutations that predict premature truncation of neurofibromin (8). Under-

Table 5. New BAC-end derived loci

Locus	Forward primer (5′→3′)	Reverse primer (5′→3′)	Size (bp)	GenBank accession no.
F2-CEN	GCTGGAAGCCACATTTGTCTG	GCACACAAATTCTCTTGGGA	77	AC006050
F2-TEL	TCCCCTTGCAGCATTGCTAT	CAGACACTTCTCCCCTCTACCCT	150	AC006050
K11-CEN	ACACTGCTGCTCTTCACCATTG	CCACCCATGAGCAAGTTCG	150	AC005562
K11-TEL	AGGTGTGAGCCACTGTGCACT	GGCTCCCCTAGGAAGCTCC	200	AC005562
I20-CEN	CGAACTCCTGACCTCGTGATC	ACCTGGTGTCTAGAGCTGATG	788	AC004222
F11-TEL	TGAGACTGATTGTAGCAGAAGTC	ACCTGTGGCTGTTGAACACTTG	325	AF170177
K8-CEN	GCTCCATGTTCCATGCTATGAG	TCTTCTCCACTCATTCCTTTGTC	363	AF170179
G21-TEL	TTAGTTAGAGCCCACCCCTCC	CCATAGGTGTGCTGGCCAC	155	AC003687
A16-INT	GGCCTCCAACTTGGTAGTCTG	GTCTGCAAATGAGCTGACAAGCT	320	AC004523

standing why microdeletion is not the prevalent mutational mechanism may reveal important parameters that affect the efficiency of REP-mediated rearrangements. Perhaps the size and sequence identity of NF1REP-P and -M are comparatively less than those of other genomic disorders, thereby reducing the probability of *NF1–REP* pairing. Or, polymorphism in the number and orientation of, or identity between, NF1REPs may result in a haplotype that is recombination-prone. A precedent for an inversion polymorphism mediated by flanking repetitive repeats has been established (54).

Our data suggest that *NF1* microdeletion may also predispose patients to the development of malignant tumors. This hypothesis is supported by our observation that 2 of the 17 (11%) unrelated microdeletion patients had a neurofibrosarcoma (Table 2, UWA124-3 and UWA155-1). This clearly is greater than the expected occurrence of 1.4–3.5% in NF1 patients (21,59), more so given the young age of the microdeletion patients. In addition, first degree affected relatives of two microdeletion patients died of malignancies (Table 2, UWA155-1 and UWA169-1). Further studies are needed to confirm this hypothesis and to determine whether this effect is mediated by the same putative gene that causes early onset of benign neurofibromas. Two lines of evidence suggest that the increased burden of cutaneous neurofibromas in deletion patients would be an unlikely cause of an apparent increased frequency of malignancy. First, cutaneous neurofibromas do not undergo malignant transformation; in cases where neurofibrosarcomas are associated with a neurofibroma it is either a plexiform neurofibroma or a neurofibroma involving a large nerve or nerve plexus (60). Second, the malignancies of the affected first degree relatives of our patients were central nervous system and fibrosarcoma, not neurofibrosarcoma (Table 2).

NF1REP-P and -M-mediated deletion in early embryogenesis may be an underlying mechanism of somatic mosaicism of *NF1*. It has been proposed that somatic mosaicism may be common among NF1 patients and could explain, for example, cases of a mildly affected parent with a severely affected child (61,62). Patients with somatic mosaicism for an *NF1* deletion have been described (16,57,63–65). Because breakpoints were not mapped in these cases, it is not known whether these deletions involved the entire *NF1* gene and/or contiguous genes. The frequency of somatic mosaicism for an *NF1* deletion was estimated at 1.5% (16,57). However, this may be underestimated significantly due to the low detection rate of the methods employed.

This is the first report of a REP-mediated rearrangement resulting in the loss of a tumor suppressor gene. Therefore, in addition to the germline rearrangements reported here, NF1REP-mediated somatic recombination could be an important mechanism for the LOH at *NF1* in tumors of NF1 patients (5,7,66,67). This hypothesis is consistent with our recent analysis of LOH at *NF1* in primary leukemic cells of children affected with NF1 that developed malignant myeloid disorders (K. Stephens, M. Weaver, K. Leppig, K. Maruyama, E.D. Davis, R. Espinosa III, M.H. Freedman, P. Emanuel, L. Side, M.M. LeBeau and K. Shannon, unpublished data). LOH in 2 of 20 tumors arose by an interstitial deletion of a 1–2 Mb segment comparable with the germline deletions described here. Additional informative polymorphisms are needed to determine whether the deletion breakpoints are at NF1REP-P and -M. Other examples of clustered neoplasia-related rearrangements could also result from a REP-mediated recombination mechanism. For example, the interstitial 20q deletion in polycythemia vera and myeloid malignancies (54) and the i(17q)-associated hematologic malignancies (68).

The precocious neurofibromagenesis and severe tumor burden of patients with *NF1* microdeletions is consistent with our hypothesis that deletion of a gene or regulatory sequence, in conjunction with neurofibromin haploinsufficiency, potentiates development of neurofibromas. All of the deletion patients showed either childhood onset and/or large numbers of cutaneous neurofibromas (Fig. 2, Table 2), with the exception of UWA166-3 who is only 4 years old. Patient UWA113-1 has the smallest deletion of ~1 Mb, thereby establishing a critical interval between *FB12A2* and *SH3GLP1* as the location of the putative tumor-promoting gene (Fig. 2). These data excluded the strong candidate gene kinase suppressor of ras (*KSR*) (69). Currently, the critical region is known to harbor four genes, *NF1*, *OMG*, *EVI2A* and *EVI2B*, two pseudogenes and seven ESTs (Fig. 2, Table 3). The products of these genes are not strong candidates for potentiating neurofibromagenesis. *OMG*, *EVI2A* and *EVI2B* are genes of unknown function located entirely within intron 27b of the *NF1* gene, but they are transcribed from the opposite direction. *OMG* encodes a glycoprotein, OMgp, which is expressed only in the central nervous system in neurons and oligodendrocytes, and is displayed in central nervous system myelin (70,71). Although growth suppression of NIH3T3 fibroblasts overexpressing OMgp suggests that it plays a role in cell proliferation (72), its lack of expression in the peripheral nervous system makes it a poor candidate. *EVI2A* and *-B* genes are more widely expressed and

predict a putative transmembrane protein of unknown function (73); it is not known whether they are expressed in Schwann cells, which appear to be the progenitor cells of neurofibromas (11). *EVI2A* and *-B* are human orthologs of mouse loci where retroviral integration causes myeloid leukemia. Further investigation, however, revealed that it was inactivation of *NF1*, not the *EVI2* genes, that caused the leukemia (74). The identification of patients deleted for *OMG*, *EVI2A*, *EVI2B* or a segment of *NF1* along with flanking sequences would be a direct test of a role for these genes in the early onset of neurofibromas. Assuming exclusion of *NF1* and the embedded genes, the critical region is reduced to ~700 kb in length. The seven ESTs that we mapped to this region, and the sequence-ready contig, will provide the basis for identifying and characterizing the putative tumor-modifying gene.

MATERIALS AND METHODS

Subjects

Patients described previously include UWA106-3 (12,13); UWA69-3, UWA119-1, UWA123-3 and UWA128-3 (13); UWA166-2 and UWA169-1 (19); UWA147-3, UWA156-1, UWA160-1, UWA167-1, UWA172-1 and UWA176–1 (K. Maruyama *et al*., submitted for publication). Table 2 includes clinical findings from these reports and more recent clinical evaluations. This study was approved by the Institutional Review Boards of the University of Washington and Children's Hospital and Regional Medical Center (Seattle, WA). Immortalized cell lines and human/rodent somatic cell hybrid lines carrying a single human chromosome 17 were constructed as described previously (13).

BAC library screening

Marker loci were amplified in the presence of [^{32}P]dCTP as described previously (http://www.sanger.ac.uk). A cocktail of probes, 1×10^6–10^7 c.p.m./ml hybridization solution each, was used to screen the RPCI-11 human BAC library, segment 4 (BACPAC Resources, Buffalo, NY; http://bacpac.med.buffalo.edu) by hybridization. Membranes were prehybridized in 25 ml of hybridization buffer (75) at 65°C for 1 h, hybridized overnight, and washed four to six times at increasing stringency, with a final wash of 0.2× SSC/0.1% SDS for 45 min. Following autoradiography for 1–2 days at –70°C with intensifying screens, positive clone addresses were determined and obtained from BACPAC resources. BAC DNA was isolated from 3 ml overnight cultures using the Qiagen Spin miniprep plasmid kit (Qiagen, Chatsworth, CA) according to the manufacturer's directions.

STSs, ESTs and generation of new markers

Loci were amplified either as described in the database entry or using a program with an initial denaturation of 94°C for 2 min, followed by 35 cycles of 94°C for 15 s, 59°C for 15 s, and 72°C for 60 s, and a final extension of 8 min. Primers for the amplification of *D17S117* and *D17S120* were designed from partial sequence analysis of plasmid clones. *D17S117* was amplified with primers 5′-AGGATGGACTAGGATTCTTAGTG-3′ and 5′-GCTGTCAATCACCAAAGTCGAG-3′ for *D17S117*. *D17S210* were amplified with primers 5′-CTCGAAGGTAGGATAGTGACAG-3′ and 5′-GATAGTTTGAGCTCAGGAATGTG-3′.

New markers were developed from the ends of BAC clone inserts. DNA was extracted from 300 ml of overnight culture from selected BAC clones using the Qiagen MIDI prep plasmid kit. BAC end termini were sequenced using 0.8–1.0 μg of purified BAC DNA, T7 or SP6 primers, and BigDye terminator chemistry (Applied Biosystems, Foster City, CA). Nucleotide sequences were analyzed with Sequencher 3.0 (Gene Codes, Ann Arbor, MI) and primers were designed (Table 5).

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