

ONLINE MUTATION REPORT

Automated comparative sequence analysis identifies mutations in 89% of NF1 patients and confirms a mutation cluster in exons 11–17 distinct from the GAP related domain

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Neurofibromatosis type 1 (NF1), formerly known as Von Recklinghausen Neurofibromatosis, is a common genetic disorder affecting approximately 1 in 3000–5000 people. It is a fully penetrant autosomal dominant disorder. Strict diagnostic criteria that include café au lait spots, neurofibromas, plexiform neurofibromas, freckling in the axillary or inguinal regions, Lisch nodules (iris haematomas), optic or chiasma glioma, pseudoarthrosis, and sphenoid dysplasia define NF1. Most disease features are present in more than 90% of patients at puberty.¹ Further manifestations are known to occur in this disorder, including macrocephaly, short stature, learning difficulties, scoliosis and certain malignancies.^{2–4} There is, however, great intra and interfamilial phenotypic variability. In addition a number of patients who have a clinical picture suspected to be NF1 do not fulfil the diagnostic criteria particularly in the younger age groups. As a consequence genetic testing would have a major impact on the diagnosis and management of these families.

The NF1 gene maps to chromosome 17q11.2 and is thought to be a tumour suppressor gene because loss of heterozygosity is associated with the occurrence of benign and malignant tumours in tissues derived from the neural crest^{5–7} as well as myeloid malignancies.⁸ It spans a region of about 350 kb of genomic DNA and contains 60 exons.^{9–11} It harbours at least three other embedded genes—EV12A, EV12B, and ONGP—transcribed from the opposite strand of NF1 intron 27b. The NF1 gene transcribes several mRNAs in the size range 11–13 kb expressed in neurones, oligodendrocytes and non-myelinating Schwann cells.¹² The most common transcript codes for a polypeptide of 2818 amino acids called neurofibromin.^{13–15} A 360 amino acid region of the predicted protein product shows homology with the GTPase activating (GAP) family of proteins in yeast and mammals.¹⁶ The GAP related domain (GRD) is the only known functional domain of the NF1 gene and spans exons 20–27a (bases 3497–4661). It has been found to interact with p21ras¹⁷ and GTP, and is thought to prevent uncontrolled cell proliferation by converting active RasGTP to inactive RasGDP. In keeping with this, RasGTP levels may be elevated in human NF1 peripheral nerve tumours in which neurofibromin is reduced or absent.¹⁸ This region of the gene has also been reported as representing a “hot spot” for mutations consistent with an important functional role.¹⁹ More recently, it has also been shown that learning deficiencies associated with NF1 may be caused by excessive Ras activity, which leads to impairments in long term potentiation caused by increased GABA mediated inhibition.²⁰ A putative second hot spot in a possible functional domain upstream of GRD, comprising exons 11–17, has been described.²¹ The region encodes a domain rich in cysteine and serine, with three cysteine pairs suggestive of ATP binding, as well as three potential cAMP dependent protein kinase recognition sites. Further evidence for cAMP

Key points

- Neurofibromatosis type 1 (NF1) is a common autosomal dominant genetic disorder caused by mutations in the NF1 gene. Mutation detection in NF1 has been a major challenge due to the large size of the gene and lack of mutational hotspots.
- We report here a study on 91 subjects fulfilling the National Institutes of Health (NIH) NF1 diagnostic criteria, in which we achieve a mutation detection rate of 89% using automated comparative sequence analysis (ACSA). This detection rate is the highest for a single technique and is therefore appropriate for routine clinical practice.
- Our data show that mutations are evenly distributed along the coding sequence of the NF1 gene and not clustered at the GAP-related domain (GRD). However, we confirm the presence of a second putative functional domain upstream of the GRD at exons 11–17.

signalling being perturbed in NF1 comes from mutations in *Drosophila* neurofibromin that have been shown to inhibit the cAMP and protein kinase signalling pathway.

Mutation detection in NF1 has been made difficult by the large size of the gene, the existence of a number of homologous pseudogene sequences spread throughout the genome, and the lack of defined mutational hotspots. To overcome these problems, a variety of techniques have been employed for screening the NF1 gene. Most studies have been based on single strand conformation polymorphism, heteroduplex analysis, temperature gradient gel electrophoresis and denaturing gradient gel electrophoresis. In the largest study to date, involving 500 patients, Fahsold et al²¹ used a protein truncation test, temperature gradient gel electrophoresis, and direct genomic sequencing to examine all of the individual exons, finding sequence variants in 301 patients. Within these variants 278 mutations were considered pathogenic. In two more papers published recently, the methodologies were used sequentially to raise mutation detection rates. In the study of Ars et al²² on 80 patients, using cDNA single strand conformation polymorphism and heteroduplex analysis, a detection rate of 70–80% of mutations was achieved. Messiaen et al²³ used a protein truncation test, fluorescence in situ hybridisation, southern blot and cytogenetic analysis

Abbreviations: ACSA, automated comparative sequence analysis; CSA, comparative sequence analysis; GRD, GAP-related domain; NF1, neurofibromatosis type 1; NIH, National Institutes of Health

to study 67 patients, and reported a detection rate of 95%, including a high frequency of unusual splicing defects. The sensitivity of each individual technique is hard to establish, as mutation analysis reports have either concentrated on groups of exons, included small numbers in their studies, or used a combination of techniques. In reviews of known NF1 mutations,¹ several mutation types are described, but no correlation with phenotype was documented. Most of the fully characterised NF1 mutations are either nonsense or frameshift mutations, which presumably lead to premature truncation of neurofibromin. Large deletions of the NF1 gene are thought to account for less than 10% of cases. A relationship between whole gene deletions and a more severe NF1 phenotype has been reported.¹

Although the application of these different techniques allows a clinically useful mutation detection rate, the extremely time consuming nature of the work makes them impractical for routine clinical work. An alternative strategy based on automated sequencing techniques would clearly be a more sensitive and informative method for mutation testing. However these techniques are not widely used as a first screen since analysis of the results can be both cumbersome and time consuming, and with current methodologies raw sequence data often require extensive manipulation before analysis. We have previously described a novel analytical approach, comparative sequence analysis (CSA), to overcome this problem.²⁴ This technique allows automation of data analysis, with accurate analyses of 96 lanes, and is therefore achievable in 1–2 minutes. Complete processing of four 96 well plates is therefore now feasible for a high throughput team in a single day. It is particularly suited to the scanning of large genes, with large numbers of different mutations, which are expected to be in heterozygous form. Here we apply this technique to the problem of NF1 gene mutations.

MATERIALS AND METHODS

Subjects

One hundred and sixty seven subjects were screened in three batches designated D02, D04, and D05. These included 131 unrelated patients suspected of having NF1, 24 relatives of the probands, and 12 normal controls. All subjects were assessed using the criteria of the National Institutes of Health (NIH)²⁵ to determine NF1 status. Ninety one unrelated subjects fulfilled the NIH criteria for NF1 and were categorised as definitely affected. Thirteen unrelated subjects fell marginally outside the NIH criteria and were categorised as possibly affected. The remaining 27 unrelated patients were classified as unaffected.

Polymerase chain reaction

Genomic DNA was extracted from peripheral blood using the Nucleon BACC2 kit and resuspended in TE buffer to a concentration of approximately 500 µg/ml. The entire reading frame of the NF1 gene was PCR amplified exon by exon, in a total of 58 amplicons. In general primers were designed so that each amplicon comprised an exon flanked by at least 50 base pairs of intron on either side. One primer in each pair was tailed with an M13 primer sequence (M13F, TGTAACACGACGGCCAGT or M13R, CAGGAAACA GCTAT GACC) to provide a binding site for M13 universal sequencing primer. Care was taken to ensure any repetitive sequences flanking the exon lay at the opposite end of the amplicon with respect to the M13 sequencing primer sequence. In general the PCR temperature profile was: initial denaturation at 95°C for 2 min, followed by 30 cycles with annealing for 30 s, elongation at 72°C for 1 min, and denaturation at 95°C for 30 s. Primer sequences, annealing temperatures and buffers used are given in Table 1.

Sequencing

Polymerase chain reaction products were purified by adding 1 U shrimp alkaline phosphatase (Amersham) and 10 U exonuclease I (NEB) to each reaction and incubating at 37°C for 30 min followed by 95°C for 10 min to denature the enzymes. Purified PCR products were then sequenced in a single orientation dependant on the location of the M13 tail on the PCR primers. Reaction mixtures comprised 2 µl of PCR product, 0.7 µl BigDye V sequencing reaction mix (Applied Biosystems), 1.05 µl 5×sequencing diluent (Applied Biosystems), 0.35 µl primer at 10 pmol/µl, 5% (v/v) betaine (Sigma) and ddH₂O to 7 µl. Initial denaturation was at 95°C for 5 min, followed by 25 cycles with annealing at 50°C for 15 s, elongation at 60°C for 4 min, and denaturation at 95°C for 30 s. For batches D02 and D04, completed sequencing reactions were purified using Dynapure™ Dye Terminator Removal Ver.2 (DynaL AS, Oslo, Norway) according to the manufacturer's protocol. Products were resuspended in 1.5 µl formamide and 0.8 µl dotted on 96 well RapidLoad™ membrane combs (Web Scientific Crewe, UK). The ABI 377 was loaded using the "water protocol" as described by Web Scientific. For batch D05, four plates of sequencing reactions were transferred to a single Multiscreen 384 well filter plate (Millipore, Bedford, UK) and purified according to the manufacturer's protocol. The samples were then resuspended in 15 µl Hi-Di formamide (Applied Biosystems) with 0.05 µl GeneScan™ 500 LIZ size standard (Applied Biosystems) for each reaction and transferred to a 384 MicroAmp optical plate (Applied Biosystems) for loading on the ABI 3100.

Analysis

Analysis was carried out using an automated comparative sequence analysis (CSA) program, AutoCSA12. This will be described fully elsewhere. In brief, the technique involves horizontal alignment of each of the four base traces separately with a control trace. The program then calculates peak position and height for each base and will highlight all differences. No electrophoretograms were examined directly unless variations were called by the program. Called variations were examined in GeneScan V3 (Applied Biosystems) by non-automated CSA.²⁴ For batches D02 and D04, horizontal alignment was achieved by generating an artificial size standard that applied arbitrary numbers to the peaks in one of the four traces (usually the red C trace). Batch D05 was horizontally aligned using the GeneScan™ 500 LIZ size standard. Sequence variations were confirmed by resequencing using a new aliquot of DNA. Fig 1 shows an example of CSA.

RESULTS

We have studied 131 unrelated subjects referred for NF1 screening. Of the 91 subjects who fulfilled the NIH criteria for NF1 we found putative mutations in 81 subjects (89.0%). In the possibly affected group of 13 patients we found putative mutations in 8 patients (61.5%). No mutations were found in any of the patients classified as unaffected. Thus, of the 104 patients with possible or definite NF1 we detected a total of 89 putative mutations (85.6%). Altogether, 77 different potentially significant sequence variations were found, of which 57 (74%) were novel. Sequence variants were classified as pathogenic mutations if they fulfilled one of the following criteria: a) they resulted in the introduction of a stop codon that truncated the protein—for example, frame shifts—or an amino acid alteration to a stop codon, b) they affected an invariant splice consensus sequence, c) they could be demonstrated to be de novo in the patient (that is, not present in the parents), d) they were previously reported as pathogenic in the literature and fulfilling any of the above or with supporting data to show that function was impaired. Table 2 provides a summary of the NF1 mutations and sequence variations found.

Table 1 Primer sequences, annealing temperatures, and buffers used for PCR reactions

Exon	Forward primer	Reverse primer	Length	T _a	Sequence Buffer primer	Orientation
1	1F.A AGACCTCTCCTTGCCTCTT	1R.0 M13RX-ATGAGGGTTCGGAGGCTG	458	55.0	D M13R	Reverse
2	2F.1 TGGCAAGTAAAGTTATTATGGTC	2R.1 M13F-AATCAAAAAGAAAAGAAAAGCAA	288	55.0	B M13F	Reverse
3	3F.1 GTTTCCTTACACTTTAGTTTT	3R.1 M13F-CATCTGTACTTTGGGACATAA	368	55.0	B M13F	Reverse
4a	4aF.0 TTTGAAAAATTTTATAATAGAAAAATGT	4aR.0 M13RX-GGTCAAAGCTGCTGTGAG	435	55.0	C M13R	Reverse
4b	4bF.0 M13FX-TCCTGGCCTCAAGTGGTC	4bR.0 M13RX-TTATAAAAANCCAGATTGGTGTC	386	57.0	B M13F	Forward
4c	4cF.0 M13FX-TCCTAGCAGACAACATATCGA	4cR.0 AAAAAAAAATCAATCGTATCCTTA	582	55.0	C M13F	Forward
6	6F.0 M13FX-CATGTTTATCTTTAAAAATGTGGC	6R.0 ATAATGGAAATAATTTTGCCTCC	323	55.0	C M13F	Forward
7	7F.0 M13FX-TGCTATAATATTAGTACATCTGG	7R.0 CCTATGAACTTATCAACGAAGAG	395	55.0	B M13F	Forward
8	8F.0 M13FX-TGTGTGGGTAATGTGTGA	8R.0 AAATATAGTTAGATAAAAAACCAATG	270	55.0	B M13F	Forward
9	9F.1 TAITTCTGCTCTTTTGG	9R.1 M13F-AAATTAAGCAATACCTTTTGG	204	55.0	B M13F	Reverse
9a	9aF.0 TCCGCTGTGGCTCAGAACAC	9aR.0 M13RX-AGTAGAAGAGGATGCACAGCC	315	60.0	B M13R	Reverse
10a	10aF.0 M13FX-GATAAACAGAGCATACAACTCA	10aR.0 AAATGCAATAGAAAAGGAGGTGA	270	55.0	B M13F	Forward
10b	10bF.A AACTATTGAGTGTCTACTATACC	10bR.0 M13RX-TTGGCATTACGCTAAACC	374	55.0	G M13R	Reverse
10c	10cF.1 M13F-CGTCCAGCCTAGTTCTAGAA	10cR.1 ACCCTCTCTTCTCCAT	444	55.0	C M13F	Forward
11	11F.0 M13FX-ATAAGTACTCCAGTGTATGT	11R.0 TAAAGTTGAAATTTAAAAAATTAAGTAC	179	52.0	C M13F	Forward
12a	M13FX- AAACCTTACAAGAAAACTAAGCT	12aR.0 ATTACCATCCAAATATTCTCCA	325	55.0	C M13F	Forward
12b	12bF.0 M13FX-TTCTAGTAAATCTCCTTCAAGT	12bR.0 ATGAAATTTACCAAAATTCATTTCAG	404	55.0	C M13F	Forward
13	13F.1 AAGATATTTGGGGTTTGAA	13R.1 M13R-GCCATGTGCTTTGAGG	508	55.0	B M13R	Reverse
14	14F.0 M13FX-AGCTTATCAGGTTCTCCATTGG	15R.0 M13R- AGTTAACAGACAAAAAGTCAACTTTACAG	535	55.0	D M13F	Forward
15	14F.0 M13FX-AGCTTATCAGGTTCTCCATTGG	15R.0 M13R- AGTTAACAGACAAAAAGTCAACTTTACAG	535	55.0	D M13R	Reverse
16	16F.0 M13FX-TGGATAAAGCATAAATTTGTCAAGT	16R.0 TAGAGAAAAGGTGAAAAATAAGAG	571	55.0	B M13F	Forward
17	17F.0 CTGTGTGTTTATAGTCACTCA	17R.0 M13RX-TTTATCAATTAACCAGTATCAG	339	55.0	B M13R	Reverse
18	18F.0 AGAAGTGTGTACGTTCTTTTCT	18R.0 M13RX-TCTTCTACCAATAACCGC	388	55.0	I M13R	Reverse
19a	19aF.0 M13FX-ATGTCACCTAGGTTATCTGG	19aR.0 TGTAATTAAGTATGATAACTCTC	290	60.0	B M13F	Forward
19b	19bF.1 M13F-ATTTGAGGGGAAGTGAA	19bR.2 M13R-ATGGGTATTATTCTTTGCT	312	55.0	E M13R	Reverse
20	20F.0 M13FX- CATTACACCATGCACATATGATTG	21R.0 M13R-GATTGTATGTGCCAGGCAC	703	55.0	E M13R	Forward
21	20F.0 M13FX- CATTACACCATGCACATATGATTG	21R.0 M13R-GATTGTATGTGCCAGGCAC	703	55.0	E M13R	Reverse
22	22F.0 M13FX-TGCTACTCTTTAGCTTCTAC	22R.0 ACAGCGTCTATGTGAAAAG	537	55.0	B M13F	Forward
23-2	23F.0 M13FX-CTTAATGTCTGTATAAGAGTCTC	23R.0 ACITTAGAITTAATAATGGAATCTC	290	55.0	C M13F	Forward
23a	23aF.0 CAGAAATAGTATACATGATTGGGT	23aR.0 M13RX-CTATTTTCTGCCAGAATTAGTAGA	465	55.0	C M13R	Reverse
24	24F.0 M13FX-TTGAACCTTTTGTTCATGTCTT	24R.0 GGAATTTAAGATAGCTAGATTATC	289	55.0	C M13F	Forward
25	25F.0 M13FX- AATATAATAATTATATTTGGGAAGGT	25R.0 GAAAATATTTGATTCAAAACAGAGC	360	55.0	B M13F	Forward
26	26F.1 M13F-CCCTCATAITTTGTAATCTT	26R.1 TTAACCGAGAGAGTTCAC	324	58.0	B M13F	Forward
27a	27aF.1 AGGGCATTATAATCTTTAT	27aR.1 M13F-GCAAACCTCTCTTCTCAAC	406	55.0	F M13F	Reverse
27b	27bF.1 M13R-GCCAAATACCCTTTAGAATG	27bR.1 AACCAAACCTGCCCATCTCT	348	55.0	C M13R	Forward
28F*	28F.1 M13F-GACTTTGAAGAATTGTTTTATA	28R.0 M13RX-TTATAGGGGTAGGACACC	693	55.0	E M13F	Forward
28R*	28R.1 M13F-GACTTTGAAGAATTGTTTTATA	28R.0 M13RX-CTATAGGGGTAGGACACC	693	55.0	E M13R	Reverse
29F*	29F.1 M13F-TACAATGGTGGGAACCTTCCCTTA	29R.0 M13RX-ACCAACACTGCATACCTTCCAAT	635	55.0	E M13F	Forward
29R*	29R.1 M13F-TACAATGGTGGGAACCTTCCCTTA	29R.0 M13RX-ACCAACACTGCATACCTTCCAAT	635	55.0	E M13R	Reverse
30F.0	GCCTCACAGTGTCTTATGG	30R.0 M13RX-CCTTCAGAGTTCCTTCAGTTAC	628	55.0	C M13R	Reverse
31	31F.0 TGTTGATGTGATTTTTCATTGACCA	31R.0 M13R-CCAATGTGGCACCAGATAAATAT	337	55.0	B M13R	Reverse
32	32F.0 M13FX-ATCTAGTATTTTGGAGCCCTCAG	32R.0 CAGATATGCTATGACAGAAAGG	334	55.0	C M13F	Forward
33	33F.0 M13FX-TATCTGTTTATCATCAGGAGG	33R.1 M13R-TAAATGGAGAAAAGAACTGG	497	55.0	E M13R	Reverse
34	34F.1 M13F-AAAATGAAACATGGAACCTTAG	34R.0 M13RX- TAAGCATTAAAGTACAAAATAGCAC	439	55.0	E M13F	Forward
35	35F.0 ATGTATTAGAGTATCCCTT	35R.0 M13RX-ATTTAAACGCTTTTAGAAAAATG	458	55.0	C M13R	Reverse
37	37F.0 M13FX-ATCCGAGATTCAGTTAGGAG	37R.0 AGTAACATTCACACTGATACCC	256	55.0	H M13F	Forward
38	38F.1 ACATGGGTAAATTTAGGAAGATAAG	38R.1 M13F-CAACAAGAAAAGATGGAAGA	335	55.0	C M13F	Reverse
39	39F.0 TCACTGTGTGAACCTCATCAACC	39R.0 M13RX- GTAAGACATAAGGGCTAACTTACTTC	306	55.0	I M13R	Reverse
40	40F.0 GGAAGTAGAACCTCAGCAGAT	40R.0 M13RX-AACTTCTGCTCTGCCACGCA	345	55.0	I M13R	Reverse
41	41F.0 TTCATCCTGTTTAAAGTCACACTTG	41R.0 M13RX-TTGCCTCCATTAGTTGGAAAAATG	295	55.0	H M13R	Reverse
42	42F.0 GAAGGAGCAAACGATGGTTG	42R.0 M13RX-AAACTTTGCTACACTGACATGG	371	55.0	C M13R	Reverse
43	43F.0 GTATTAGACTTCTTTGAGTC	43R.0 M13RX-GAATGGTAGAGTTTATCTTTG	421	55.0	C M13R	Reverse
44	44F.2 M13R-GGTGAAGTGAATTCCAGGTG	44R.2 TGTATCTGTTGGGTGAGAAAG	328	55.0	E M13R	Forward
45	45F.0 M13R-AGATAACAATTAGCCACAAG	45R.0 TAAAGACAGGCACGAAGGT	225	55.0	F M13R	Forward
46	46F.1 M13R-ATTTTGGCACATTATCTGG	46R.1 GCGCATGTGCAAGTT	316	55.0	C M13R	Forward
47	47F.0 CTGTTACAATTAAGAGATACCTTGC	47R.0 M13RX-GTGTCTTTTAAAGCAGGCATAC	204	55.0	H M13R	Reverse
48	48F.0 TTTTGGCTCAGATGGGGATTAC	48R.0 M13RX-AAGGAAATTCCTAATGTGGTGTG	373	55.0	B M13R	Reverse
48a	48aF.0 ATCTAGTACTAATTGTATTTACCC	48aR.0 M13RX-AGACTGAGCTACAGGGAC	265	55.0	A M13R	Reverse
49	49F.0 M13FX-CTGGAGAAACAGGCTATAC	49R.0 CACTTCTTTCAGATTGTCTG	639	58.0	E M13F	Forward

M13F, TGTA AACAGCAGCCAGT; M13FX, GTGGGTAAAACGACGGCCAGT; M13R, CAGGAAACAGCTATGACC; M13RX, GTGGCAGGAAACAGCTATGACC

* PCR product sequenced in both directions for single coverage

Mutation spectrum

By far the largest group of variations were single base pair substitutions, which were detected in 61 cases (67%). Of these, 43 (72%) were transitions and 17 (28%) were transversions, proportions corresponding closely with those

observed by Ars et al.²² Mutation at CpG dinucleotides account for 20/60 (33%) of the single base pair substitutions. Of these single base pair substitutions, the most frequently detected single group of sequence variants was that of missense substitutions, which were found in 25 subjects

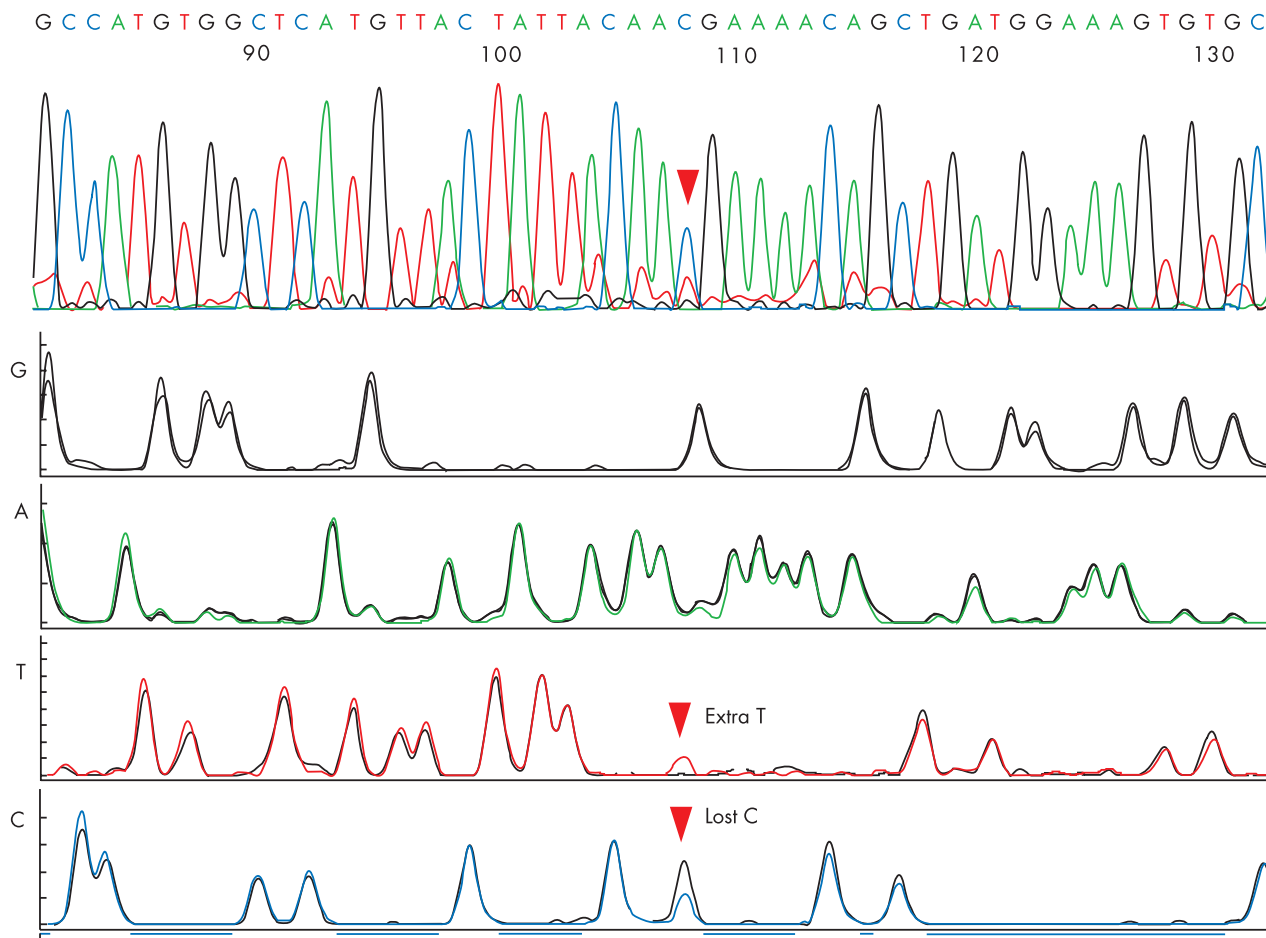


Figure 1 Example of mutation detected by CSA. Normal sequence indicated in black, test sample indicated in colour. The base represented by each track is indicated in the top left of each panel. A heterozygote point mutation is seen C>T. The half height peak in the C trace and the extra peak in the T trace can be easily seen. These differences can be read by the software program ACSA.

(28%). Of these, 18 were found in patients who fulfilled the NIH criteria while seven were found in possibly affected patients. Only five of these have been previously described but a further 11 have closely related variations (affecting the same codon) in the literature. There were two mutations of note. First, the variant of codon 844 (2531T>C L844P), which was found twice in this study, and is one of at least four known variations of this codon, which was found in conjunction with a second variant affecting codon 838 (2514 C>G I838M). It is not known if these two variants occurred in *trans* or *cis*. Second, the missense variant 1722 C>A S574R, which affects the first base of exon 12a and may exert an effect by amino acid substitution or by interfering with normal splicing.

We also detected 22 nonsense mutations within the single base pair substitutions, of which 16 (73%) have been described by previous investigators. In 13/22 (59%) of the nonsense mutations the mutation occurs at a CpG dinucleotide and alters the primary base of the codon to a T (HGA>TGA), forming a termination signal.

Ten of the missense mutations affected bases potentially involved in splicing. These included five substitutions altering splice acceptors and one altering a splice donor. In addition we found two intronic deletions, which directly alter invariant splice site dinucleotides. 2991-4 del ATA changes the intron 17 acceptor site AG>TG, while 6641+1 del G appears to shift the intron 35 donor site 1 bp upstream. Three other potentially significant intronic sequence variations

were found: 3113+3 ins A, 4111-8 del GTT, and 288+5 G>C. None of these has been previously reported.

A second class of mutations, frame shifts, was detected in 21 patients. Fourteen were small deletions ranging from 1 bp to 23 bp in length. In this group only 499 del TGTT and 1756 del ACTA have been previously reported by other investigators. However, four other deletions had closely related mutations described in the literature, such as 3758 to 3762 del TCTAC related to 3759 to 3763 del CTACC, which was reported by Ars et al.²² All deletions were entirely exonic except 1-14 to 7 del 21bp which deletes 14 bp of non-coding sequence upstream of exon 1 together with 7 bp of exon 1, thereby removing the start codon.

Together, frameshift and nonsense mutations that would be expected to truncate the reading frame constituted 44 of the 89 mutations detected (49.4%) in patients who fulfilled the NIH criteria. The distribution of these mutations appears to be evenly spread within the gene, with 27/44 (61%) predicted to terminate the protein in or upstream of the GRD.

A third class of mutations, insertions and deletions, was detected in 11 patients. Seven insertions were detected comprising five 1 bp insertions and two 2 bp insertions. None of these insertions has been previously described. Deletions detected included four in frame deletions; two removing a single amino acid and two removing two consecutive amino acids. Only 2970 del AAT Δ M991 and 7096 del 6 Δ NF2366-7 have been previously reported.

Table 2 Summary of NF1 mutations and sequence variations found. A total of 77 different potentially significant sequence variations were found, of which 57 (74%) are novel

Sample	NIH	Exon	Mutation	Type	Predicted effect	Accession	CpG
D05-54	NF	1	1-14 to 7 del 21 bp	Frameshift	Truncated protein		
D04-07	NF	2	127 del CT V65X	Frameshift	Truncated protein		
D05-16	NF	3	271 G>A E91X	Nonsense	Truncated protein		
D05-69	NF	4a	311 T>A L104X	Nonsense	Truncated protein		
D02-34	NF	4b	499 del TGTT D176X	Frameshift	Truncated protein	CD995532	
D02-65	NF	4c	597/8 del 23 V207X	Frameshift	Truncated protein		
D05-53	NF	6	787 A>T K263X	Nonsense	Truncated protein		
D02-16	NF	7	916 del G L316X	Frameshift	Truncated protein		
D02-49	NF	7	889-2 A>G	Splice acceptor	Truncated protein	CS991467	
D05-48	NF	9	1246 C>T R416X	Nonsense	Truncated protein	CM992366	Y
D02-11	NF	10a	1274 G>A W425X	Nonsense	Truncated protein	CM000779	
D02-03	NF	10a	1318 C>T R440X	Nonsense	Truncated protein	CM950845	Y
D02-41	NF	10a	1318 C>T R440X	Nonsense	Truncated protein	CM950845	Y
D02-72	NF	10a	1318 C>T R440X	Nonsense	Truncated protein	CM950845	Y
D02-21	NF	10b	1527+1 G>C	Splice donor	Truncated protein		
D02-56	NF	11	1642-2 A>G	Splice acceptor	Truncated protein		
D02-23	NF	12a	1756 del ACTA I603X	Frameshift	Truncated protein	CD982825	
D05-11	NF	13	2124 del CT E715X	Frameshift	Truncated protein		
D05-43	NF	13	2034 ins C D699X	Frameshift	Truncated protein		
D05-10	NF	13	2180 C>G S727X	Nonsense	Truncated protein		
D02-12	NF	16	2851-2 A>G	Splice acceptor	Truncated protein		
D05-66	NF	18	2991-4 del ATA	Splice acceptor	Truncated protein		
D02-02	NF	19a	3163 C>T Q1055X	Nonsense	Truncated protein		
D05-74	NF	21	3528 del A L1183X	Frameshift	Truncated protein		
D05-45	NF	21	3546 del T L1183X	Frameshift	Truncated protein		
D02-53	NF	22	3758 to 3762 del TCTAC F1261X	Frameshift	Truncated protein		
D05-73	NF	22	3721 C>T R1241X	Nonsense	Truncated protein	CM000799	Y
D05-78	NF	22	3721 C>T R1241X	Nonsense	Truncated protein	CM000799	Y
D02-09	NF	23.1	3916 C>T R1306X	Nonsense	Truncated protein	CM981381	Y
D02-17	NF	23.2	3982 ins A E1333X	Frameshift	Truncated protein		
D02-61	NF	23.2	4045 ins T S1373X	Frameshift	Truncated protein		
D04-08	NF	23.2	4095 ins TG	Frameshift	Truncated protein		
D05-90	NF	24	4265 C>A S1422X	Nonsense	Truncated protein		
D02-45	NF	27a	4537 C>T R1513X	Nonsense	Truncated protein	CM941093	Y
D05-87	NF	27a	4537 C>T R1513X	Nonsense	Truncated protein	CM941093	Y
D05-76	NF	27a	4515-1 G>A	Splice acceptor	Truncated protein		
D05-47	NF	28	4913 del TCTCT C1661X	Frameshift	Truncated protein	CD972357	
D05-57	NF	28	4950 ins A Y1650X	Frameshift	Truncated protein		
D05-62	NF	31	5898 del GA D1987X	Frameshift	Truncated protein		
D05-01	NF	31	5943+1 G>T	Splice donor	Truncated protein		
D02-31	NF	32	5944-1 G>C	Splice acceptor	Truncated protein		
D05-04	NF	33	6181del 8bp D2074X	Frameshift	Truncated protein		
D04-02	NF	35	6641+1 del G	Splice donor	Truncated protein		
D04-05	NF	36	6709 C>T R2237X	Nonsense	Truncated protein	CM000815	Y
D05-70	NF	36	6709 C>T R2237X	Nonsense	Truncated protein	CM000815	Y
D02-62	NF	37	6792 C>A Y2264X	Nonsense	Truncated protein	CM981382	
D02-36	NF	37	6792 C>G Y2264X	Nonsense	Truncated protein	CM981382	
D05-65	NF	39	7096 ins A N2400X	Frameshift	Truncated protein		
D02-35	NF	40	7206 del CA C2405X	Frameshift	Truncated protein		
D05-09	NF	41	7285 C>T R2429X	Nonsense	Truncated protein	CM000818	Y
D02-22	NF	42	7427 ins TC S2502X	Frameshift	Truncated protein		
D02-30	NF	42	7486 C>T R2496X	Nonsense	Truncated protein	CM941096	Y
D05-55	Q	12b	1994 C>T S665F	Missense	Amino acid substitution	CM000785	
D02-40	NF	12b	1885 G>A G629R	Missense	Amino acid substitution	CM961026	Y
D02-68	NF	12b	1885 G>A G629R	Missense	Amino acid substitution	CM961026	Y
D02-78	Q	16	2530 C>T L844F	Missense	Amino acid substitution	CM002379	
D02-38	Q	17	2970 del AAT ΔM991	In frame deletion	Amino acid deletion	CD931025	
D05-72	Q	26	4402 A>G S1468G	Missense	Amino acid substitution	CM971049	
D02-06	NF	2	92 A>G H31R	Missense	Amino acid substitution		
D02-54	NF	3	288+5 G>C	Intronic	Truncated protein		
D02-48	NF	4a	434 T>C L145P	Missense	Amino acid substitution		
D05-34	Q	4b	528 T>A D176E	Missense	Amino acid substitution		
D05-25	NF	6	846 G>A Q282Q	Same sense	Truncated protein		
D02-39	NF	6	846 G>A Q282Q	Same sense	Truncated protein		
D05-71	NF	7	1010 A>T E337V	Missense	Amino acid substitution		
D05-60	NF	7	970 T>C C324R	Missense	Amino acid substitution		
D02-18	NF	10b	1466 A>G Y489C	Missense	Amino acid substitution		
D05-39	NF	10b	1466 A>G Y489C	Missense	Amino acid substitution		
D02-50	NF	10b	1466 A>G Y489C	Missense	Amino acid substitution		
D02-10	NF	10c	1595 T>C L532P	Missense	Amino acid substitution		
D02-15	NF	12a	1722 C>A S574R	Missense	Truncated protein		
D05-64	NF	13	2040 C>T C680C	Same sense	Truncated protein		
D05-58	NF	16	2514 C>G I838M, 2531 T>C L844P	Missense	Amino acid substitution		
D02-08	NF	16	2531 T>C L844P	Missense	Amino acid substitution		
D04-12	NF	16	2617 C>T R873C	Missense	Amino acid substitution		Y
D02-05	NF	18	3113+3 ins A	Intronic	Truncated protein		
D02-52	Q	19b	3217 A>G M1073V	Missense	Amino acid substitution		
D05-85	Q	21	3587 T>G L1196R	Missense	Amino acid substitution		
D05-42	NF	22	3826 C>G R1276G	Missense	Amino acid substitution		Y
D05-56	NF	22	3826 C>G R1276G	Missense	Amino acid substitution		Y

Table 2 continued

Sample	NIH	Exon	Mutation	Type	Predicted effect	Accession	CpG
D05-19	NF	22	3827 G>A R1276Q	Missense	Amino acid substitution		Y
D05-27	NF	22	3827 G>A R1276Q	Missense	Amino acid substitution		Y
D04-21	NF	24	4111-8 del GTT	Intronic	Truncated protein		
D05-33	Q	24	4255 A>G K1419E	Missense	Amino acid substitution		
D02-27	NF	25	4312 del GAAΔE1438	In frame deletion	Amino acid deletion		
D05-75	NF	28	4973 del 6 ΔIY1658-9	In frame deletion	Amino acid deletion		
D02-69	NF	28	5172 G>A K1724K	Same sense	Truncated protein		
D05-91	NF	29	5427 C>T R1808R	Same sense	Truncated protein		
D02-14	NF	39	7096 del 6 ΔNF2366-7	In frame deletion	Amino acid deletion		

NF, fulfils diagnostic criteria; Q, does not fulfil diagnostic criteria.

Four different conservative base substitutions were found in five unrelated, definitely affected patients. No other variations were detected in any of these patients and none of these conservative base substitutions were found in 260 normal chromosomes. Sequence variations 2040 C>T C680C and 5427 C>T R1808R were found in two patients in whom no other family members were available for testing at the time of writing. Another patient was found to have 5172 G>A K1724K and, on testing this patient's parents, the affected mother was found to carry the same variation while the father was found to be normal at this position. Finally, the variation 846 G>A Q282Q was found in two unrelated patients of eastern decent.

Recurrent mutations

Twenty of the 77 novel variations were detected in more than one unrelated individual or have been previously reported by other investigators (table 2). Ten variants were observed more than once in this study. All of these variations were single base pair substitutions and seven of them occurred at hypermutable CpG dinucleotides. In this study eight sequence variations were each found in two unrelated cases; 1885 G>A G629R, 2530 C>T L844F, 3721 C>T R1241X, 4537 C>T R1513X, and 6709 C>T R2237X have also been previously reported by other investigators, whereas 3826C>G R1276G, 3827 G>A R1276Q, and 846 G>A Q282Q have not. Two sequence variations were detected in three unrelated patients; the nonsense mutation 1318 C>T R440X has been previously reported while the missense variation 1466 A>G Y489C is novel.

Two pairs of mutations were found to be very closely related, affecting a different base in the same codon. Codon 844 was alternatively mutated CTT>TTT (L844F), which was found twice and has been previously described, and CTT>CCT (L844P), which is novel and was only found in one patient. Neither 3826C>G R1276G, 3827 G>A R1276Q have been previously described and both were found in two unrelated cases in this study. However two other mutations of this codon, 3826 C>T R1276X, 3827 G>C R1276Q have been previously reported.^{26 27} Twenty other mutations found in this study were also found to have closely related mutations in the literature. Of particular note is 1466 A>G Y489C, which was found in three cases in this study but has not been previously reported although Fahsold et al²¹ reported an identical mutation of an identical codon 2 bp downstream of Y489C (1472 A>G Y491C).

Although there seems to be a general spread of mutations found throughout the gene, we found no sequence changes in exons 1, 5, 15, 20, 27b, 29, 30, 34, 35, 43, 44, 45, 47, 48, or 49.

Polymorphisms

Twenty sequence variations were found either in a normal subject or in an affected patient who already had a mutation characterised (table 3). Of these 14 had a rare allele frequency of less than 0.05 and eight were only found in a single subject in the entire study (167 subjects). Eight of the polymorphisms found were in the coding sequence and the remaining 12 all fell within 40 bases of an exon. All but three of the polymorphisms were single base pair substitutions, six being transversions and 11 being transitions, of which four

Table 3 Polymorphisms found during the mutation analysis process. Twenty such sequence variations were recorded in a normal subject or in an affected patient who had already had a mutation characterised

Exon Base	P	Q	Amino acid change	p	q	Found with:		
						Unaffected	Definite	Others
1 1-22 G>C	G	C		0.984	0.016	Y	7486 C>T R2496X	Y
2 61-4 del T	9T	8T		0.996	0.004		1642-2 A>G	
2 168 C>T	C	T	S56S	0.959	0.041	Y	4045 ins T S1373X	Y
4b 528 T>A	T	A	D176E	0.996	0.004	Y		
5 702 A>G	A	G	L234L	0.728	0.364	Y	many	Y
7 889-31 del ATTAT				0.996	0.004		1885 G>A G629R	
10b 1393-32 C>T	C	T		0.707	0.403	Y	many	Y
10c 1528-29 del T	8T	7T		0.694	0.320	Y	many	Y
11 1642-25 T>C	T	C		0.973	0.027	Y	7096 del 6	Y
12a 1810 T>C	T	C	L604L	0.993	0.007		4255 A>G K1419E	
16 2617 C>T	C	T	R873C	0.996	0.004		1-14 to 7 del 21bp	
16 2531 G>A	G	A	G844G	0.996	0.004		1994C>T S665F	
17 2851-16 T>C	T	C		0.977	0.023	Y	3528 del A L1183X	
22 3867C>T	C	T	F1289F	0.996	0.004		1885 G>A G629R	
29 5546-19 T>A	T	A		0.598	0.471	Y	many	Y
32 6084+8 C>G	C	G		0.996	0.004		1318 C>T R440X	
33 6173 C>A	C	A	A2058D	0.995	0.005		6181 del 8bp D2074X	
39 7126+37 G>C	G	C		0.937	0.063	Y	many	Y
42 7395-29 A>G	A	G		0.666	0.356	Y	many	Y
46 8050+20 G>A	G	A		0.989	0.011	Y		

occurred at CpG dinucleotides. The remaining three polymorphisms comprised two deletions involving a single T and one deletion of 5 bp.

DISCUSSION

In this study we used direct sequencing and automated comparative sequence analysis to study 167 subjects including 91 unrelated definitely affected NF1 patients. Putative mutations were found in 81 of these affected patients (89%), which means that our study achieved the highest recorded mutation detection rate using a single technique for this gene. Given that the current estimate of whole gene deletions in this disorder is approximately 10%, this technique may therefore pick up all the remaining classes of mutations and potentially replaces all existing technologies, used either alone or together, with advantages of increased specificity and sensitivity associated with decreased cost and considerably reduced analysis times. When 96 well plates are used, an approximate cost for sequencing the entire NF1 coding region for 1 patient is £220. Although commercial sequencing centres exist that may be able to match these prices the level of quality assurance, follow up, and guarantees needed for diagnostic laboratory purposes cannot be assured. A class of mutations that would not be detected with this assay are large gene deletions, as the test is not dosage sensitive. We plan to set up a multiplex ligation dependent probe amplification assay to look for whole exon and whole gene deletions in the patients in whom we have not found pathogenic mutations by sequence analysis.

Many new mutations have been identified in our study, with 74% being novel. This confirms that there are few recurrent mutations in NF1. While we find that mutations are evenly distributed along the NF1 coding region, the scale of our present study allows further analysis of possible mutation hot spots and functional domains. The RasGAP activity of the central GAP related domain (GRD) and the structure of the GRD from neurofibromin have already been well characterised.^{1,28} Indeed clustering of mutations has been reported in this region, and much attention has since been concentrated on this area. The GRD is not shown to be a significant mutation hot spot in this study, with only 22% of mutations falling within this region. A second possible functional domain upstream of GRD has also been reported around exons 11–17.²¹ When our mutations were analysed by type and a weighted distribution was calculated for each exon, the number of missense and in frame deletions found to be clustered in this region was assessed visually and by means of a χ^2 analysis on mutation percentage. This is shown in fig 2, confirming that exons 11–17 represent an important area of the NF1 protein. A similar cluster, which was termed a hot spot, was seen in the study of Fahsold et al²¹ although we detected more mutations in upstream exons. This region is known to be a domain rich in cysteine and serine domain, as defined by Izawa et al. This domain has three cysteine pairs suggestive of ATP binding as well as three potential cAMP-dependent protein kinase recognition sites, which are subject to phosphorylation by protein kinase.²⁹ As abnormalities of cAMP signalling have been demonstrated in

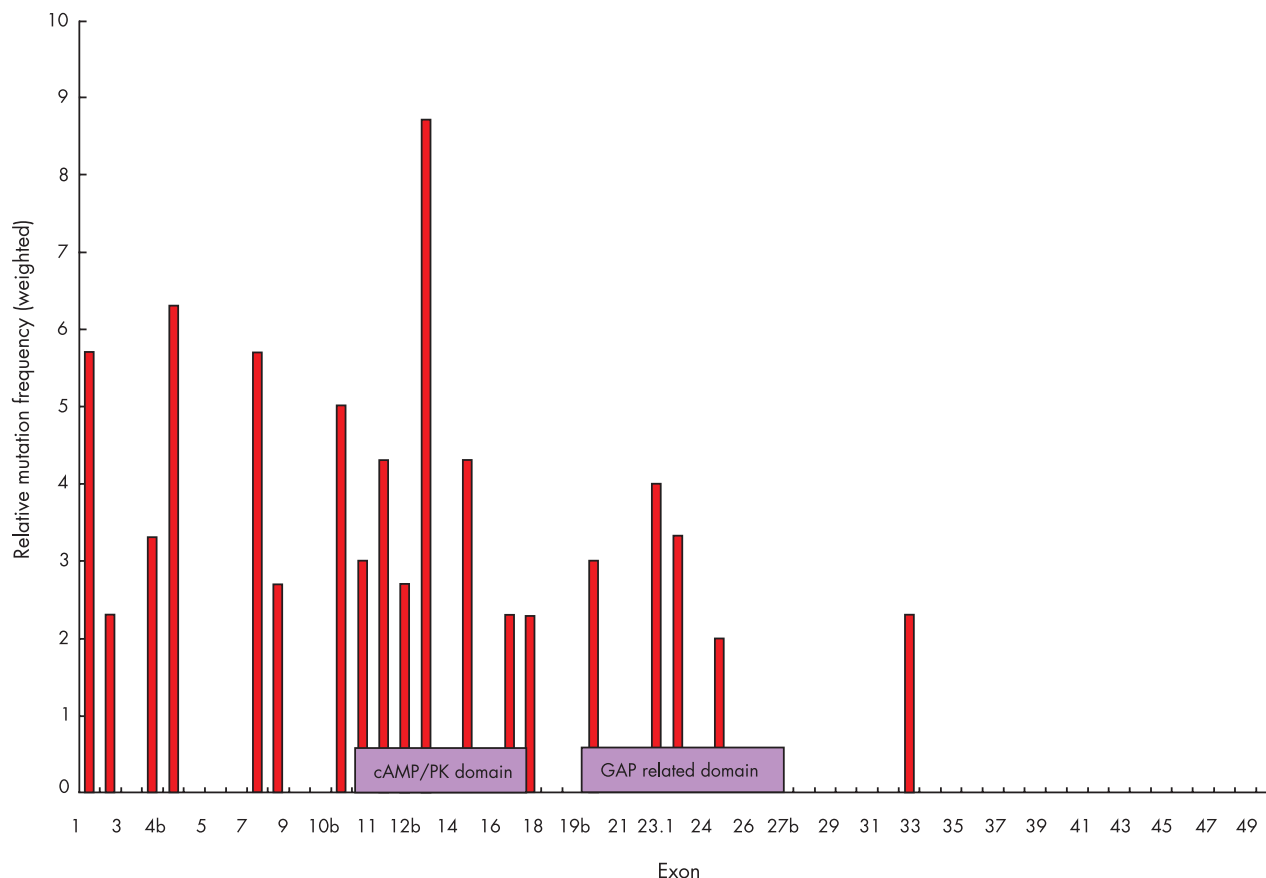


Figure 2 Weighted distribution of missense mutations and single base changes in the NF1 gene. The relative mutation frequency was calculated by dividing the number of missense or single base changes by the number of base pairs per exon. The value is then shown as a ratio between the exon specific mutation densities and the average mutation density for the whole gene (30/8473 bp). A clustering can be seen between exons 10–17. Between exon group and number of mutations present $\chi^2 = 20.85$, df 3, $p = 0.0001$. Mutation rate is greatest between exons 1–20: $\chi^2 = 11.74$, df 2, $p < 0.005$, compared with exons 21–49: $\chi^2 = 9.1$, df 2, $p < 0.01$.

Drosophila neurofibromin mutations, it will be important to clarify further the link between cAMP and the Ras signalling pathway in NF1.

In previous studies that examined the effect of mutations at the RNA level it was found that 50% resulted in mutations causing splicing alterations. Splicing defects therefore appear to be the most common molecular defect in NF1.²² We have developed a functional splicing assay using a minigene system,³⁰ which will allow us to clarify the significance of point mutations including missense or silent changes. One mutation found in this study has already been studied in this way. The 288+5 G>C was found to exclude exon 3 and further investigation showed that this aberrant splicing could be rescued by co-expression of an altered U1-snRNA complementary to the mutation, proving that the mutation induces exon 3 skipping by preventing binding of U1 snRNA and thus causes the disease. This assay will be of great value in the analysis of mutations such as the previously described 6792 C>G (Y2264X), which affects splicing.²²

In addition to clinical utility, the availability of a powerful mutation detection technique for the NF1 gene will allow us to address two long standing questions in NF1. First, do phenotype-genotype correlations exist? This could be studied in a large number of patients where the whole gene is sequenced and attention paid to the type of mutation and clinical sign or symptom. Second, what is the contribution of the NF1 gene to some of the rare related disorders, such as segmental NF, gastrointestinal NF, familial spinal NF and familial café au lait spots? For example, using this technique we have recently examined the rare syndrome Noonan-Neurofibromatosis where patients exhibit features of both disorders. Mutation analysis found Noonan-Neurofibromatosis to be a subset of NF1 in some cases, with mutations in the NF1 gene.³¹ The high sensitivity of automated CSA makes analysis of the other rare related disorders feasible.

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