Neurofibromatosis type 1 (NF1): a protein truncation assay yielding identification of mutations in 73% of patients

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Abstract

Neurofibromatosis type 1 (NF1) is caused by mutations in a tumour suppressor gene located on chromosome 17 (17q11.2). Disease causing mutations are dispersed throughout the gene, which spans 350 kilobases and includes 59 exons. A common consequence of NF1 mutations is introduction of a premature stop codon, and the majority of mutant genes encode truncated forms of neurofibromin. We used a protein truncation assay to screen for mutations in 15 NF1 patients and obtained positive results in 11 of them (73%). Sequencing of cDNA and genomic DNA yielded identification of 10 different mutations, including four splicing errors, three small deletions, two nonsense mutations, and one small insertion. Nine mutations were predicted to cause premature termination of translation, while one mutation caused in frame deletion as a result of exon skipping. In one other case involving abnormal splicing, five different aberrantly spliced transcripts were detected. One germline nonsense mutation (R1306X, 3916C>T) corresponded to the same base change that occurs by mRNA editing in normal subjects. The second nonsense mutation (R2496X) was the sole germline mutation that has been previously described. The subjects studied represented typically affected NF1 patients and no correlations between genotype and phenotype were apparent. A high incidence of ocular hypertelorism was observed.

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NF1 is a common genetic disorder characterised by multiple neurofibromas, café au lait spots, axillary freckling, and pigmented iris hamartomas (Lisch nodules). Affected subjects often exhibit additional complications including various kinds of osseous lesions, optic glioma, learning disabilities, and malignancy.¹ NF1, an autosomal dominant disorder, is notable for its variable expressivity, with clinical presentations that may vary from mild to severe within a single family. The NF1 gene exhibits a high mutation rate, and approximately 50% of index cases represent new mutations.²

Because of the large size of the NF1 gene, traditional methods of mutation screening have

tended to focus on one portion of the gene at a time rather than on the entire coding region. Since disease causing mutations are dispersed throughout the gene, these studies have detected mutations in a relatively small proportion of subjects. For example, one study used chemical mismatch cleavage to screen 70% of the coding region in 25 NF1 patients and identified potentially disease causing mutations in 28% of subjects.³ Other studies focused on smaller regions of the gene and detected mutations in a correspondingly smaller fraction of subjects.⁴⁻⁹ Other than protein truncation testing, the only reported method developed to screen the entire coding region used long RT-PCR to amplify the entire 8.5 kb open reading frame and full length RT-PCR products were digested with restriction enzymes.¹⁰ By this method, mutations associated with deletion or insertion within the mRNA altered the size of the RT-PCR product and the restriction digest improved the resolution with which size changes could be detected. Still, the authors predicted the method to yield positive results in only 30% of previously reported NF1 mutations.

Compared to other methods, the protein truncation test (PTT) provides improved efficiency in detecting NF1 mutations.¹¹ First developed for use in Duchenne muscular dystrophy (DMD)¹² and familial adenomatous polyposis (APC),¹³ PTT has been applied to mutation screening in numerous genes¹⁴⁻¹⁸ including NF1.^{11 19 20} A primary advantage of the method is its ability to screen an entire coding region for any type of sequence alteration that affects the size of the encoded protein. This is accomplished by using one or more amplified segments of the gene as substrate for coupled in vitro transcription and translation. Mutations causing premature termination of translation within a given segment yield protein products of less than the expected size. Such truncating mutations are common in NF1, where approximately 80% of fully characterised mutations detected by methods other than PTT are predicted to lead to severe truncation of neurofibromin.² In the current study, mutations were identified in 11 of the 15 patients screened (73%), which is the highest rate of mutation detection reported in a study of typical NF1 patients.

Subjects, material, and methods

SUBJECTS

All study participants were recruited from the clinic population of the University of Tennessee, Memphis NF Clinic according to an IRB

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Table 1 Summary of PTT results and clinical descriptions

PTT Segment	cDNA mutation	Encoded Protein (aa)	DNA mutation	Case ID	Age (y)	Sex	Ethnicity	Sporadic (S) familial (F)	CAL spots	Freckling	Flex neurofib
1	480-586del +others	1-163	?	95-15	6	F	White	Adopted	+	+	—
1	587-654del	1-206	587-3C>A	96-62	24	F	White	F	+	+	+
2	1393-1527del	1-464// 510-2818	K505E (1513A>G)	96-21	7	F	White	S	+	+	—
2	1756-1759del	1-602	1756-1759del	96-77	9	F	Af Am	F	+	+	—
3	2320-2321insA	1-774	2320-2321insA	96-53	5	F	White	S	+	+	—
4	3151-3152delGG	1-1057	3151-3152delGG	96-45	24	F	White	F	+	+	+
4	3151-3152delGG	1-1057	3151-3152delGG	96-65	22	F	White	F	+	+	—
5	R1306X	1-1305	R1306X (3916C>T)	96-43	8	F	Af Am	S	+	+	—
7	5453delT	1-1840	5453delT	96-70	7	F	White	F	+	—	—
8	6580-6641del	1-2198	6641+1G>T	96-42	4	M	White	S	+	—	—
9	R2496X	1-2495	R2496X (7486C>T)	96-41	3	M	Af Am	S	+	+	+
Neg	—	—	—	96-73	7	F	White	S	+	+	+
Neg	—	—	—	96-51	6	M	White	S	+	+	—
Neg	—	—	—	96-55	8	M	Af Am	F	+	+	—
Neg	—	—	—	96-84	7	M	White	F	+	+	—

aa = amino acids, MRI UBS = hyperintense T2 signal on MRI, ND = not determined, LD = learning disabilities, ADHD = attention deficit/hyperactivity disorder. Key to "Other": (A) Malignant peripheral nerve sheath tumour. (B) Low grade cerebral astrocytoma. (C) Partial heterochromia of iris. (D) Pulmonary stenosis. (E) Mental retardation with autistic features. (F) Cerebral palsy. (G) Severe behavioural problem. (H) Eyelid ptosis. (I) Strabismus.

approved research protocol. The clinical diagnosis of NF1 was based on the NIH Consensus Conference criteria.²¹ Clinical descriptions are given in table 1. None of the participants had the Noonan-NF phenotype. In some cases, blood specimens were obtained from other affected family members or from unaffected parents in addition to the index case. Informed consent was obtained from all study participants. Mutation screening was performed on 15 affected subjects who were presumed to be unrelated based on family history (including names of all first and second degree relatives).

RT-PCR

Peripheral blood from subjects was used to prepare total cellular RNA and genomic DNA. For RNA, leucocytes were isolated by ficoll gradient centrifugation followed by acid guanidinium thiocyanate-phenol-chloroform extraction.²² RNA was recovered following alcohol precipitation. Genomic DNA was prepared using a high salt precipitation method (Gentra Systems Inc, Minneapolis, MN).

Total cellular RNA was treated with DNase I and then used to prepare cDNA. Reverse transcriptase reactions included 5-10 µg RNA, random hexamers, and Superscript II reverse transcriptase (Gibco BRL, Gaithersburg, MD) in a final volume of 60 µl. PCR reactions contained 2 µl cDNA, 10 mmol/l Tris-HCl, pH 8.3, 50 mmol/l KCl, 2 mmol/l MgCl₂, 0.2 mmol/l each dATP, dTTP, dCTP, and dGTP, 0.4 µmol/l each forward and reverse primer (table 2), 2.5 units AmpliTaq Gold polymerase (Perkin Elmer, Foster City, CA), and 0.02 units Vent polymerase (New England Biolabs, Beverly, MA). All forward primers included a leader sequence consisting of a T7 transcription promoter plus eukaryotic translation initiation signals (table 2). PCR parameters were 40 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 90 seconds. The initial cycle was preceded by nine minutes at 94°C to activate AmpliTaq Gold polymerase in addition to denaturing the template, and the

last cycle was followed by five minutes at 72°C. An aliquot of each PCR reaction was analysed by agarose gel electrophoresis to monitor yield and integrity of the product. Typical yield was 0.5-1.0 µg per 50 µl PCR reaction.

IN VITRO TRANSCRIPTION AND TRANSLATION
Approximately 75 ng of each RT-PCR product was used in 12.5 µl coupled transcription and translation reactions according to recommendations of the supplier of the reticulocyte lysate system (Promega Corp, Madison, WI). Reactions included ³⁵S-methionine (Amersham, Arlington Heights, IL). Proteins were analysed by Laemmli SDS polyacrylamide gel electrophoresis using 15% acrylamide (BioRad, Hercules, CA) and 20 cm gels. Specimens were batched by segment to facilitate comparison of segment specific band patterns. Gels were treated with Entensify (Dupont NEN, Boston, MA), dried, and exposed to ray film. The sizes of protein bands observed by fluorography were estimated by comparison to prestained protein molecular weight standards (Gibco BRL, Gaithersburg, MD) on the gels.

DNA SEQUENCING

Segments yielding positive results by PTT were sequenced. PCR products were cloned into a plasmid vector by either TA cloning or TOPA TA cloning (both Invitrogen, San Diego, CA). DNA sequencing was performed by dye terminator cycle sequencing (Perkin-Elmer, Applied Biosystems Inc, Foster City, CA) and analysed either at the Molecular Resource Center, University of Tennessee, Memphis or at the Center for Biotechnology, St Jude Children's Research Hospital. Sequencing primers were either the appropriate PTT reverse primer for a given segment or a primer internal to the abnormal segment. The respective PTT primers (table 2) used for the following segments were: 95-15, PTT-1R; 96-77, PTT-2R; 96-53, PTT-3R; 96-45 and 96-65, PTT-4R; and 96-41, PTT-9R. The following internal primers were used for sequencing, where primer sequences are designated by nucleotide positions and primer

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Table 1 continued

Cut neurofib	Optic pathway tumour	MRI UBS	Cognitive function	Hyper-telorism	Macro-cephaly	Short stature	Other
+	+	+	+LD +ADHD	—	—	+	E
+	—	—	+LD	—	—	+	A C H
+	+	+	+LD +ADHD	+	—	+	D F H I
+	ND	ND	ND	—	+	—	
+	—	—	ND	+	—	—	D
+	ND	ND	ND	+	—	—	H
+	—	ND	+LD	+	—	—	B
+	—	+	+LD	+	—	—	
—	—	+	+LD	+	—	—	
+	+	—	+ADHD	+	+	—	C
+	—	+	ND	+	—	+	H
—	—	—	+LD	+	—	—	
+	—	—	+LD	+	+	—	G
			+ADHD				
+	ND	ND	+LD	+	—	+	
+	—	—	+LD	+	+	—	I
			+ADHD				

orientation (F or R) as in table 2: 95-15 and 96-62, CCAAGTCAAATAGCTTTTCTGC (736-757-R); 96-21, TGTGTATTGTCACTCGG (1293-1309-F); 96-45 and 96-65, CTTGCCTGGTCCAAATC (3199-3215-R); 96-43, AGGGAAACGCTGGCTAAC (4114-4131-R); 96-70, CCTTAAATGGCATAGTGTTTTGT (5376-5394-F) and CATTATGCTTGCAAAAACGAAC (5806-5827-R); and 96-42, CACTCTCTGTGTACTTG (6328-6344-F). Some abnormal segments were sequenced using more than one primer, and all abnormalities were identified in at least two independent clones. Mutations identified in cDNA were confirmed by direct sequencing of PCR products generated using genomic DNA and exon specific primers²³ with one of the PCR primers used as a sequencing primer. Mutation names are according to recommendations of the Nomenclature Working Group, with the start site of translation denoted as nucleotide 1 for both cDNA and genomic DNA lesions.²⁴

Table 2 Primer pairs for PTT

Segment	Exons*	Nucleotides†	Sequence (5'→3')‡§
1	1→8	(1-1094)	F: (L)-ATGCCCGCGCACAGGCCGGTGAAT R: GAGAATGGCTTACTTGGATTAAA
2	6/7→12a/12b	(879-1868)	F: (L)-ATGAATAAGAACTTATTTCTGGACAG R: TGACAGGAACCTTCTATCTGCCTGCTTA
3	10b→16	(1484-2580)	F: (L)-ATGGTGAAACTAATTCATGCAGAT R: CAGGCCAGAATTGCTTCTCTG
4	14→20	(2278-3489)	F: (L)-ATGGCACTGCTGAGGCGCATT R: GTGCATGAGACCACTGTCTACG
5	19b→25	(3216-4364)	F: (L)-ATGGAAGCAGTAGTTTCACTT R: CGTGCCTGCATCAAAGTTGC
6	24→29	(4160-5256)	F: (L)-ATGTTTCCTCAGATTTATCA R: TAGGACTTTTGTTCGCTCTGCTGA
7	28→32/33	(4998-6100)	F: (L)-ATGGAGTACACCAAGTATCATGAG R: ACATCCTTCCAATAACCTTGC
8	31→38	(5878-6987)	F: (L)-ATGACCATCAATGAAAACAGATG R: TATACGGAGACTATCTAAAGTATGCAG
9	34/35→43	(6573-7596)	F: (L)-ATGGAGGCATGCATGAGAGATATTC R: AGCCTTTGTGTCTGATATCAAG
10	41→49	(7354-8404)	F: (L)-ATGGATACATATCCATTATCA R: TCTGCACTTGGCTTGCGGAT

*Primers are either located within the exons given or span the junction of two adjacent exons (indicated by /).
†Nucleotides are numbered from the start site of translation according to gb/M82814/HUMNF1AB.
‡Leader sequence (L) for forward (F) primers: GGATCCTAATACGACTCACTATAGGGCCGCCACC-.
§The following primer sequences are from Heim *et al*¹¹: 1F, 2R, 3F, 5F, 6R, 7F, 8R, 9F, 10R. In that paper, the primers are named 1F, 1R, 2F, 3F, 3R, 4F, 4R, 5F, and 5R, respectively.

Results

LOCALISATION OF MUTATIONS BY PROTEIN TRUNCATION TEST

Mutation screening was performed by using RT-PCR and coupled in vitro transcription and translation to assess the coding potential of NF1 RNA. The normal mRNA includes 8.5 kb of coding sequence and encodes a protein product of 2818 amino acid residues. The approach used was similar to that of Heim *et al*,¹¹ but the sizes of individual segments were reduced. Additional primers were designed to divide the NF1 coding region into 10 segments of approximately 1.1 kb each (table 2) rather than the five 2 kb segments used in the previous study. The region of overlap between adjacent segments ranged in length from 205 bp (segments 5 and 6) to 414 bp (segments 8 and 9). This corresponded to a minimum overlap of 8 kDa between adjacent protein segments.

For each subject, RT-PCR was used to amplify all 10 overlapping segments. While most PCR reactions yielded a single band, several patient samples (95-15, 96-21, 96-62) showed abnormalities at the RT-PCR step (fig 1). Samples yielding RT-PCR products of reduced length subsequently were shown to skip exons (described below). In all subjects analysed, segment 5 yielded two closely spaced bands (data not shown), corresponding to alternative splicing of exon 23a.²⁵ In addition, for segment 7, all subjects yielded products of reduced size corresponding to alternative processing (skipping) of exons 29 and 30.²⁶

RT-PCR products were subjected to coupled transcription and translation followed by protein gel electrophoresis. The normal protein banding pattern for each segment consisted of a predominant band corresponding to the expected full length product (range of approximately 38-43 kDa for different segments) plus a predictable pattern of background bands (fig 2). As expected for subjects heterozygous for a dominant disorder, all specimens yielded full length product for each segment. In addition,

Figure 1 Representative RT-PCR products for segments 1 and 2. (A) Segment 1, normal control and subjects 95-15, 96-21, and 96-62. (B) Segment 2, normal control and subjects 95-15, 96-21, and 96-62. Each normal control illustrates the expected full length RT-PCR product for that segment. Cases of aberrant splicing yielded both full length product and deleted product, as seen for segment 1 in (A) 95-15 and 96-62 and for segment 2 in (B) 96-21. RT-PCR products were resolved on 1% agarose gels.

Figure 2 Composite of protein gels showing positive PTT results. The position of each abnormal, truncated protein is indicated by an arrow. A normal control (nl) is shown to the left of each patient sample, and the segment (Seg) is identified below each panel. Migration of protein molecular weight standards is indicated (in kDa) to the left of each panel.

truncated products were observed in a single segment from 11 of the 15 subjects analysed. Truncated products ranged in size from approximately 18 to 34 kDa. The 11 positives were distributed throughout the gene, with one

or two truncating mutations identified in each segment, except for segments 6 and 10 where no positives were identified in this series.

Within the families reported here, mutation screening by PTT was reliable and reproducible. In two families, multiple affected subjects were tested and consistent results were obtained (fig 2, seg 4 (96-65, 96-45, 96-47) and seg 7 (96-70, 96-71)). In all cases, mutations subsequently identified by sequencing were consistent with the observed sizes of truncated protein products.

IDENTIFICATION OF MUTATIONS BY DNA SEQUENCING

The 11 segments yielding positive results by PTT were sequenced (table 1, fig 3). Ten different mutations were identified, including four splice errors, three small deletions, two nonsense mutations, and one 1 bp insertion.

The four splice aberrations (96-21, 96-62, 96-42, and 95-15) all involved exon skipping and all but 96-21 introduced a change in reading frame consistent with premature termination of translation. In 96-21, the skipped exon (10b) caused an in frame deletion of 135 bp (1393-1527del) corresponding to elimination of 45 amino acids; this was consistent with the size shift of approximately 5 kDa observed by PTT. Cases 96-62 and 96-42 skipped exon 4b (587-654del) and exon 35 (6580-6641del) respectively. A complex pattern of aberrant splicing was observed in 95-15 (described below).

To investigate the genomic basis of exon skipping, specific exons were amplified from genomic DNA and used for direct sequencing (exons 4b and 4c of 95-15, exon 4c of 96-62, exon 10b of 96-21, and exon 35 of 96-42). Observations of possible heterozygosities were followed by cloning the appropriate PCR products and sequencing multiple individual clones. Two patients (96-42 and 96-62) were heterozygous for splice site mutations. Subject 96-42 carried a G to T substitution of the first base of intron 35 (6641+1G>T). In 96-62, a C to A substitution was observed in the splice acceptor site of intron 4b (587-3C>A). In subject 96-21, no splice site abnormalities were found to explain the in frame deletion of exon 10b. However, a missense mutation was identified near the 3' end of exon 10b. Substitution of G for A at nucleotide 1513 corresponded to non-conservative amino acid substitution of glutamic acid for lysine (K505E). Both unaffected parents of subject 96-21 were studied and neither exhibited either the 1513A>G substitution in genomic DNA or skipping of exon 10b in cDNA (data not shown). The parental studies are consistent with 1513A>G being a de novo disease causing mutation in subject 96-21. Additional studies are in progress to evaluate the relationship between the observed missense mutation and the aberrant splicing of exon 10b. In the fourth patient (95-15), no genomic defect was detected in either exon 4b or 4c.

The other mutations identified were sequence changes involving one to four base pairs. An identical deletion of 2 bp (3151-

Figure 3 Mutations identified by PTT. Mutations are aligned above a map of the NF1 cDNA, with each exon represented by a small box (not drawn to scale). Brackets represent mutations that skip exons, as indicated. Relative positions of the 10 segments used for PTT are indicated below the map. Abbreviations: del (deletion), R (arginine), X (stop codon). Nucleotides are numbered from the start site of translation.

3152delGG) was identified in two patients (96-45 and 96-65). More extensive family histories showed that the people tested were members of a common lineage (probands were second cousins). Other small deletions of 1 bp (5453delT) and 4 bp (1756-1759del) were observed in patients 96-70 and 96-77, respectively. An insertion of 1 bp (2320-2321insA) was detected in 96-53. All four mutations altered the reading frame, and in each case the new reading frame contained a stop codon shortly downstream of the mutation site (table 1). Two nonsense mutations, R1306X and R2496X, were identified in patients 96-43 and 96-41, respectively. Both mutations were C to T transitions, converting arginine codons to stop codons. R1306X, observed as a constitutional mutation in 96-43, is identical to a previously reported mRNA edit in NF1^{27 28} (see Discussion). The mutation R2496X, found in an affected subject with no family history of NF1, was previously reported in a Scottish patient.³ Other than R2496X, the mutations reported here have not been previously described.²⁹ Following identification in cDNA clones, all six mutations were confirmed in genomic DNA by direct sequencing of the appropriate PCR amplified exon (data not shown).

COMPLEX ABERRANT SPLICING WITH MULTIPLE SKIPPED EXONS AND AN INSERTION

Patient 95-15 exhibited protein truncation within segment 1. RT-PCR analyses showed the presence of at least two products, the full length segment 1 product (1.1 kb) and a shorter product of approximately 0.5 kb (fig 1A). PTT showed a truncated protein of approximately 18 kDa (fig 2, segment 1). Subsequent studies of cloned RT-PCR products showed five different splicing errors (fig 4A), each observed in two independent clones. Four of the five errors included skipping of exon 4b. The first error was skipping of exon 4b only (480-586del). The second class of aberrant transcripts skipped four contiguous exons, 2 to 4b (61-586del), whereas the third skipped exons 4b and 7 while retaining the intervening exons 4c, 5, and 6 (480-586del; 889-1062del). The last two classes of splice errors involved both a novel insertion and exon skipping. One class skipped exon 4b and contained a novel 31 bp sequence in its place (9479-480ins31bp; 480-586del) (fig 4B). Another class contained the same 31 bp sequence between exons 4a and 4b and then skipped exons 4c, 5, and 6 (479-480ins31bp; 587-888del). A search of sequence databases failed to show homologies to the novel 31 bp sequence.

Figure 4 Multiple splice errors in patient 95-15. (A) Exons normally present in segment 1 are shown at the top with the observed aberrant splice products indicated below. Hatched bars represent the exons skipped in each abnormal transcript. An inverted triangle indicates the presence of a novel 31 bp sequence. (B) Sequence of the 31 bp insertion, represented as an inverted triangle above.

Four of the five aberrant transcripts (all except 61-586del) introduced stop codons just downstream of exon 4a. These four were predicted to encode protein products of similar size (approximately 19 kDa), which was in good agreement with the observed truncated protein band of approximately 18 kDa. Although the 61-586del transcript also altered the reading frame, the encoded peptide of 28 amino acids (3.4 kDa) would be below the resolving power of the gel system and was not observed. Interestingly, the shortened band of 0.5 kb seen by RT-PCR (fig 1A) corresponded in size with 61-586del, whereas the other aberrantly spliced RT-PCR products were only marginally visible by agarose gel electrophoresis. All abnormalities observed in this patient predicted severe protein truncation and probably complete loss of function *in vivo*.

Discussion

Mutation screening in NF1 has been hindered by the large size of the gene and by the random distribution of disease causing mutations. We used a protein truncation test to screen the entire coding region in 15 NF1 patients and detected 10 different mutations in 11 patients. By reducing the sizes of the segments screened from approximately 2 kb¹¹ to approximately 1.1 kb, we achieved consistently high yields of RT-PCR product, which were associated with good resolution and low background on protein gels. The newly reported mutations were distributed throughout the gene and included nine truncating mutations (seven frameshift and two nonsense) and one in frame deletion. Four splice errors were detected and all involved exon skipping. The current study adds to the growing body of reports showing the usefulness of PTT for mutation screening in disorders where disease causing mutations are heterogeneous and loss of function mutations are common.

PROTEIN TRUNCATION ASSAY

In spite of its apparent strengths, protein truncation screening has limitations. Missense mutations are not detected, as these do not affect the size of the encoded protein. When used as an RNA based assay, PTT cannot detect mutant alleles associated with very rare or absent mRNA. Although no systematic evaluations have been reported, 10-20% of normal mRNA levels has been suggested as a lower detection limit.¹⁴ Thus, large gene deletions and mutations associated with low mRNA abundance will be missed. In NF1, large deletions and missense mutations are associated with disease²⁹ and allelic instability of NF1 mRNA has been reported.³⁰ These factors, which are beyond the capabilities of PTT, limit the sensitivity of the method in screening affected subjects.

Technical limitations of the assay itself may also account for false negative results. A truncating mutation may be missed if it causes segment specific amplification failure. When one or more segments from an abnormal allele fail to amplify, the observed product represents only the normal allele rather than both gene

copies. Since PTT is not quantitative, an apparently normal result is obtained. Segment specific amplification failure may occur because a mutation destroys one or more primer annealing sites or because of the presence of a large insertion that increases the size of a particular segment beyond the length that can be amplified. The overlap between adjacent segments provides partial protection against false negatives caused by amplification failure since a mutation that destroys one priming site should be picked up as a truncating event within the overlapping segment. Two mutations reported here illustrate successful use of this safeguard. The mutation 1393-1527del skips exon 10b, which contains the forward priming site for segment 3, and 6580-6641del skips exon 35, which contains the forward priming site for segment 9. Each mutation was detected as a result of truncation within the respective upstream segment (2 or 8). However, a somewhat larger deletion, encompassing the region of overlap between two adjacent segments, would be missed since one primer annealing site from each segment would be destroyed.¹⁰ Truncating mutations may also be missed if the size of the truncated product falls outside the resolving ability of the protein gel system (for example, 61-586del in 95-15).

No accurate estimate is available of the relative contributions of various factors in limiting the sensitivity of PTT. Of particular interest is the percentage of truncating mutations that are missed. In one review,² 80% of fully characterised mutations detected by methods other than PTT were associated with a significant change in protein size. In other studies using PTT for mutation screening in NF1, detection rates were 67% (14 mutations in 21 subjects),¹¹ 54% (seven mutations in 13 subjects),²⁰ and 44% (eight mutations in 18 subjects).¹⁹ In combination with the present study, PTT has identified 40 NF1 mutations in 67 affected subjects (60%). Thus, an approximation of the sensitivity of PTT in detecting truncating mutations is 75% (60/80). Interestingly, the study with the lowest detection rate¹⁹ evaluated children with myeloid malignancies and used affected tissue (bone marrow) for mutation screening in the majority of cases. As suggested by those authors, destabilisation of RNA may be a significant factor limiting the efficiency of PTT.

MUTATIONS

The mutations described here are consistent with previous reports in failing to identify mutational hotspots within the NF1 gene. While there is no apparent predominant mechanism for NF1 mutations, as a group the mutations are representative of naturally occurring mutations in the human genome.³¹⁻³² The three short deletions and single insertion all occurred within some type of short sequence repeat and presumably arose through the common mutational mechanism of slipped strand mispairing.³¹⁻³³ The single base pair insertion, 2320-2321insA, occurred within the sequence "AACAC", changing it to a direct repeat, "AACAAAC". The 2 bp deletion, 3151-3152delGG, removed two of three consecutive

G residues. 5453delT involved the deletion of a thymine adjacent to five C residues. The mutation 1756-1759del (deletion of the 4 bp sequence "ACTA") removed one flanking 2 bp repeat ("TA") plus the sequence between repeats ("AC") from the normal sequence "TAACTA". The other sequence changes observed in genomic DNA were either transitions (1513A>G, 3916C>T, 7486C>T) or transversions (587-3C>A and 6641+1G>T).

The most common abnormalities detected in cDNA were splice errors. Two examples of exon skipping associated with splice site abnormalities were found (splice donor site mutation in 96-42 and splice acceptor site mutation in 96-62). In a third case (96-21), the only mutation found in the skipped exon (10b) was a missense mutation located 15 bp from the 3' end of exon 10b and encoding a non-conservative amino acid substitution (Lys to Glu). Although we have not proved such a relationship in this case, other examples of exon based mutations affecting exon recognition have been reported. These have included cases where exon skipping was induced by silent mutations,³⁴ missense mutations,^{35,36} and nonsense mutations.³⁷⁻³⁹ Aberrantly spliced NF1 transcripts, including skipped exons, were also observed in other reports of RNA based mutation screening.^{11,19} Aberrant splicing appears to be a frequent cause of protein truncation.

The fourth case of aberrant splicing (95-15) exhibited multiple abnormal transcripts involving both exon skipping and a small insertion of unknown origin (fig 4). Based on the position of the insertion, it most probably consisted of sequences from intron 4a, which has not yet been completely sequenced. Although the most straightforward explanation for the insertion is inclusion of 31 bp of intron 4a through the use of cryptic splice sites, more complex possibilities, such as a germline insertion, cannot be ruled out. Additional studies to identify the genomic basis of the aberrant splicing in this patient are in progress.

In subject 96-43 a nonsense mutation was identified which is identical to a reported edit site in exon 23-1 of NF1 mRNA.^{27,28} In the mRNA edit, post-transcriptional base modification converts the normal cytosine at position 3916 to a uracil, thus changing an arginine to a stop codon. The edited transcript accounts for approximately 2% of NF1 mRNA in normal subjects.²⁷ In subject 96-43, sequencing showed the presence of a C to T transition (3916C>T) in genomic DNA, thus confirming the presence of a bona fide nonsense mutation, rather than increased mRNA editing (data not shown). Subject 96-43 had typical NF1 and is an apparent new mutation (table 1). Absence of the 3916C>T mutation was shown in blood from the subject's unaffected mother, but the reportedly unaffected father was not available for testing. Assuming that this is indeed the patient's disease causing mutation, it suggests a pathogenic potential of the sequence change, and this is consistent with the increased level of edited mRNA seen in some tumours.^{27,40} The

role of the mRNA edit in normal subjects is unknown.

PHENOTYPIC FEATURES

Clinical features of the study population were typical with respect to NF1 phenotype (table 1), although an unexpected incidence of orbital and ocular manifestations of NF1 was observed, including hypertelorism, ptosis, strabismus, and partial iris heterochromia. Twelve of 15 study participants (80%) exhibited hypertelorism. Hypertelorism has been noted as a component of the facial dysmorphism seen in some severely affected patients with large gene deletions.^{41,42} However, none of our study participants displayed the Noonan-NF phenotype.^{43,44} In typical NF1 patients, the incidence of hypertelorism has been reported as approximately 25% (eight of 34 patients).⁴⁵ We have determined interpupillary distances in 44 patients (including the 15 described in this report) and found hypertelorism in 61% (27 of 44).⁴⁶ Hypertelorism may be relatively common in NF1 patients, representing one aspect of mildly abnormal orbital development.⁴⁷

Interestingly, partial iris heterochromia was observed in two unrelated subjects (96-62, 96-42). Both carried truncating mutations caused by exon skipping, but the mutations were at opposite ends of the gene (exons 4c and 35, respectively), and no common mechanism was apparent. Although heterochromia of the iris has been reported in association with NF1⁴⁸ and may be another manifestation of NF1 associated neurocristopathy, its significance is unknown.

A primary goal of mutation screening, correlations between genotype and disease phenotype, continues to be difficult in NF1. In addition to efficient methods of mutation identification, more studies of gene function, protein function, and the genetic basis of disease progression are needed to understand the pathogenesis of this complex disease.

Note added in proof

The 31 bp cDNA insertion observed in patient 95-15 (fig 4B) is from intron 4a (gb/AC004222/AC004222). Cryptic splicing of this sequence has been observed by others in some NF1 tumours (M R Wallace, unpublished results).

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