 Human Molecular Genetics, 2000, Vol. 9, No. 2 237–247

Mutations affecting mRNA splicing are the most common molecular defects in patients with neurofibromatosis type 1

Elisabet Ars, Eduard Serra, Judit García, Helena Kruyer, Antonia Gaona, Conxi Lázaro and Xavier Estivill+

Medical and Molecular Genetics Center—IRO, Hospital Duran i Reynals, Avia. Castelldefels, Km 2.7, L'Hospitalet de Llobregat, 08907 Barcelona, Spain

Received 6 September 1999; Revised and Accepted 16 November 1999

Neurofibromatosis type 1 (NF1) is one of the most common inherited disorders in humans and is caused by mutations in the *NF1* gene. To date, the majority of the reported *NF1* mutations are predicted to result in protein truncation, but very few studies have correlated the causative *NF1* mutation with its effect at the mRNA level. We have applied a whole *NF1* cDNA screening methodology to the study of 80 unrelated NF1 patients and have identified 44 different mutations, 32 being novel, in 52 of these patients. Mutations were detected in 87% of the familial cases, but in 51% of the sporadic ones. At least 15 of the 80 NF1 patients (19%) had recurrent mutations. The study shows that in 50% of the patients in whom the mutations were identified, these resulted in splicing alterations. Most of the splicing mutations did not involve the conserved AG/GT dinucleotides of the splice sites. One frameshift, two nonsense and two missense mutations were also responsible for alterations in mRNA splicing. The location and type of mutation within the *NF1* gene, and its putative effect at the protein level, do not indicate any relationship to any specific clinical feature of NF1. The high proportion of aberrant spliced transcripts detected in NF1 patients stresses the importance of studying mutations at both the genomic and RNA level. It is possible that part of the clinical variability in NF1 could be due to mutations affecting mRNA splicing, which is the most common molecular defect in NF1.

INTRODUCTION

Neurofibromatosis type 1 (NF1) (MIM 162200) is one of the most common inherited disorders in humans with a prevalence of ~1 in 3000 individuals. NF1 is an autosomal dominant disorder fully penetrant at the age of 5 years, but with a variable clinical expression, even among members of the same family. The main clinical features of NF1 are *café au lait* spots (CLSs), cutaneous neurofibromas and Lisch nodules (1,2). The *NF1* gene was mapped to chromosome 17q11.2 and was positionally cloned in 1990 (3–5). It spans ~350 kb of genomic DNA, contains 60 exons (6,7) and is transcribed ubiquitously to an mRNA of 11–13 kb that encodes for a protein, neurofibromin, of 2818 amino acids (8). The only region of neurofibromin with a well-defined function and structure is a central domain with a high similarity to ras-specific GTPase-activating proteins (GAPs), called the GAP-related domain (GRD), which downregulates ras activity (9–11).

The *NF1* gene has one of the highest mutation rates described for any human disorder (~1×10^{-4}/gamete/generation), with the result that ~30–50% of NF1 cases represent new mutations (1,2). Despite the fact that the *NF1* gene was identified ~9 years ago, information on the spectrum of mutations that cause NF1 is still very limited. So far, 255 different mutations (NNFF International NF1 Mutation Analysis Consortium, 29 March 1999; http://www.nf.org/nf1gene/nf1gene.home.html) have been reported in the *NF1* gene. The search for *NF1* mutations has probably been hampered by: (i) the large size of the gene; (ii) the existence of homologous pseudogenes (12); and (iii) the lack of mutational hot spots. Several methods have been employed to search for *NF1* mutations, but they have mainly been used for the analysis of a few exons (13–17). In addition, approaches that allow a rapid screening of the entire *NF1* coding region have been employed (18–20). The protein truncation test (PTT) has been applied with an efficiency of ~70% (18,20), but PTT is a radioactive technique and is better performed using RNA from lymphoblastoid cell lines.

It has been described that a significant fraction (15%) of disease-causing mutations in mammalian genes affect mRNA splicing (21). Moreover, aberrations in *NF1* pre-mRNA processing have been suggested to play an important role in the pathogenesis of NF1 (22). To date, the majority (60–70%) of *NF1* mutations reported to the NNFF Consortium (29 March 1999) are predicted to result in protein truncation. Unfortunately, most of these *NF1* mutations have been studied only in genomic DNA and their effect at the mRNA level has not been reported.

To gain knowledge on the spectrum of *NF1* mutations in NF1 patients, we have developed a rapid and efficient strategy to screen the entire *NF1* coding region. The method combines single-strand conformation polymorphism (SSCP) with

+To whom correspondence should be addressed. Tel: +34 93 2607775; Fax: +34 93 2607776; Email: estivill@iro.es

heteroduplex (HD) analysis of *NF1* cDNA (cDNA-SSCP/HD) fragments, followed by DNA sequencing. This method allows mutation analysis at both the genomic and mRNA level. By applying cDNA-SSCP/HD analysis to 80 unrelated NF1 patients, we have identified 44 different mutations and found that in 26 patients (50%) the disease is due to aberrant spliced transcripts. We have also tried to discern any possible correlation between genotype and phenotype.

RESULTS

NF1 mutations detected by cDNA-SSCP/HD analysis

We have analyzed 80 unrelated patients with NF1, representing 61% sporadic and 39% familial cases. Mutations in the entire *NF1* coding region were identified by RT–PCR followed by SSCP/HD analysis (Figs 1 and 2). Abnormal SSCP/HD mobility patterns were detected in 52 (65%) unrelated samples. In addition, several SSCP/HD patterns, which did not segregate with the disease or which were present in several subjects, were identified; these were considered as potential polymorphisms and were not characterized in this study. The abnormal SSCP/HD patterns were analyzed in the parents of the proband when available, allowing the detection of co-segregation with the disease. Mutations were identified for 87% of the familial cases, but for 51% of the sporadic ones. After identifying the *NF1* alteration at the cDNA level, the underlying genomic mutation was characterized for each of the 52 unrelated patients. Forty-four different mutations were identified in these 52 patients, 32 of which have not been reported previously (Tables 1 and 2). Expression studies of the two *NF1* alleles were performed in the RT–PCR fragment II1, ranging from exon 4b to exon 7, by analysis of an expressed polymorphic *Rsa*I restriction site in exon 5 of the *NF1* gene (23). This analysis was applied to nine NF1 individuals in whom the mutation was not detected and who were heterozygous at this polymorphic site. All the patients studied expressed the two *NF1* alleles (data not shown).

Eighteen different frameshift mutations were identified, all causing an altered reading frame probably leading to truncated proteins (Tables 1 and 2). Six different nonsense mutations were detected: R304X (910C→T); W599X (1797G→A); R681X (2041C→T), in two unrelated patients; S1140X (3419C→G); R1513X (4537C→T); Y2264X (6792C→A), in two other unrelated patients.

Five missense mutations were detected: I117S (350T→G); Y489C (1466A→G), in three unrelated patients; G922S (2764G→A); R1204W (3610C→T); and L1425P (4274T→C). R1204W and L1425P are located in the GRD domain. Mutations Y489C and G922S produce abnormal splicing of *NF1* (see below). We rejected the hypothesis that the remaining three missense mutations found in this study were polymorphisms as they co-segregate with the disease and they were not detected in 200 control samples (unrelated unaffected relatives of the NF1 patients studied). Amino acids I117, R1204 and L1425 are conserved in mouse neurofibromin (24) and in *Drosophila* NF1 protein (25). L1425 is also conserved in RasGAP of *Caenorhabditis elegans*, but neither L1425 nor R1204 are conserved in RasGAPs of *Bos taurus*, *Mus musculus*, *Rattus norvegicus*, *D.melanogaster*, *Sus scrofa* and *Saccharomyces cerevisiae* (26).

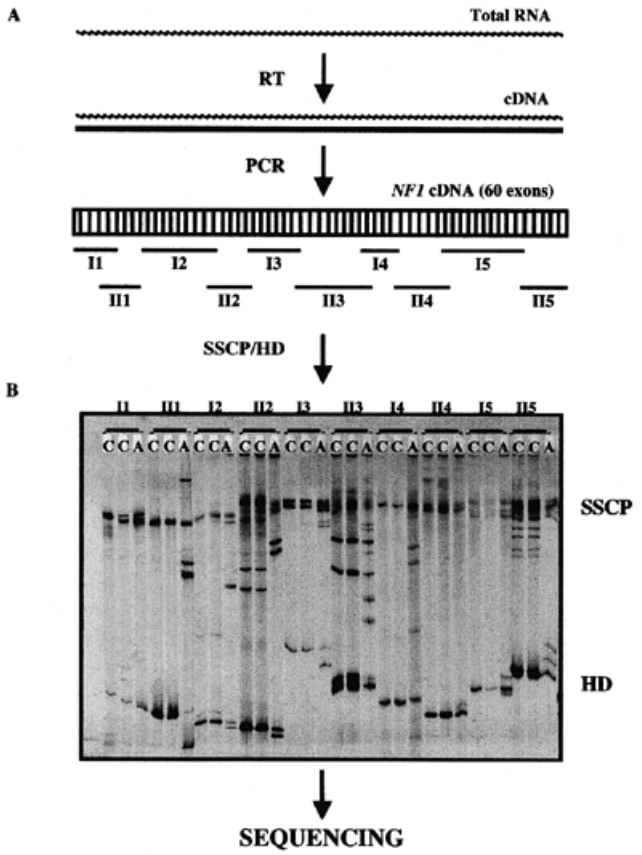

Figure 1. Strategy for mutation analysis of the *NF1* gene by cDNA-SSCP/HD. (**A**) Schematic representation of mutation identification at the cDNA level by the cDNA-SSCP/HD method. The entire *NF1* cDNA was amplified in 10 overlapping fragments (from I1 to II5) (53). (**B**) SSCP/HD analysis of the 10 RT–PCR fragments of the *NF1* mRNA for 10 NF1 patients (A) and two controls (C) for each fragment. One abnormal pattern for each fragment is shown.

A previously described (27) small in-frame deletion, 7096delAACTTT, which removed two amino acids (asparagine and phenylalanine) was identified in exon 39 of patient 97-113.

NF1 mutations producing splicing defects

Fourteen different mutations involving the consensus splice sites were identified (Tables 1–3). Of these, seven mutations inactivated the 5′ splice site (5′ ss), producing skipping of the corresponding exon [IVS4b+5G→A, skipping 4b; IVS8+1G→A, skipping 8; IVS10b+1G→A, skipping 10b; IVS18+1G→A, skipping 18; IVS19a+1G→A, skipping 19a; 5546G→A (R1849Q), skipping 29; and IVS37+2T→G, skipping 37]; and one affected the 3′ splice site (3′ ss), also producing exon skipping (IVS16-1delGGTTT, skipping 17). The remaining six splice site mutations implied the utilization of cryptic splice sites, two resulting in the production of *NF1* mRNAs lacking a portion of the coding sequence (IVS27b+1del9, 4704del69; IVS28+1G→A, 5152del54), and four causing the insertion of intronic nucleotides in the coding sequence (IVS10a-9T→A, 1392insUUUUUAG; IVS11-3C→G, 1721ins42; IVS15-16A→G, 2409ins15; IVS30+332A→G, 5749ins177).

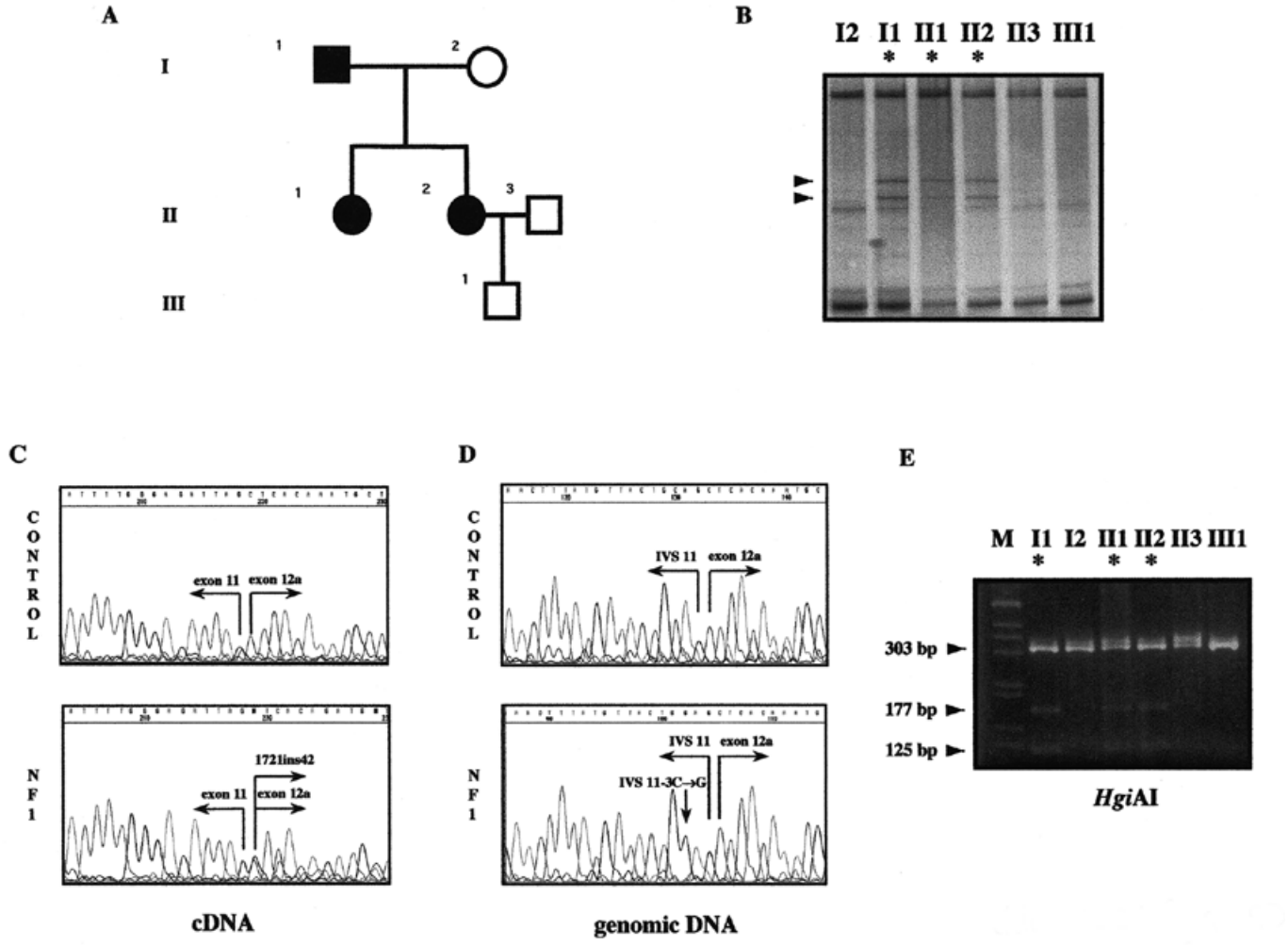

Figure 2. Example of *NF1* mutation characterization. (**A**) Pedigree of an NF1 family with mutation IVS11-3C→G. Filled symbols, NF1 patients; unfilled symbols, healthy individuals; squares, males; circles, females. (**B**) SSCP/HD analysis of the I2 RT–PCR fragment (from exon 8 to 12b) showing an abnormal pattern (arrowheads) for the affected individuals (*). (**C**) Partial sequence of the I2 RT–PCR fragment of a control and of patient 96-255 showing the start of the insertion of 42 nucleotides at cDNA position 1721 (exon 12a). (**D**) Single-strand genomic sequence of the intron–exon boundaries of exon 12a of a control and of patient 96-255; a point mutation (IVS11-3C→G) was detected in intron 11 of the *NF1* gene. (**E**) Mutation IVS11-3C→G generates a new *Hgi*AI restriction site. The digestion of exon 12a of *NF1* and its intron boundaries resulted in two fragments of 177 and 125 bp in the affected individuals in addition to the normal fragment of 303 bp.

Three unrelated patients presented the above-mentioned splice site mutation 5546G→A, which is also a missense (R1849Q) change with respect to the amino acid sequence. However, this mutation alters the last nucleotide of exon 29 of *NF1*, which has been considered highly conserved in the 5′ ss (28), and causes skipping of exon 29.

Interestingly, one frameshift, two missense and two nonsense mutations were responsible for defects in mRNA processing. Frameshift mutation 3214del11 caused the utilization of a cryptic 5′ ss, resulting in a deletion of 104 exonic nucleotides from cDNA position 3211. Two missense mutations introduced novel 5′ ss inside the exon, producing the deletion of exonic nucleotides [1466A→G (Y489C), causing 1466del62 as described (29), found in three unrelated patients; and 2764G→A (G922S), causing 2761del90] (Fig. 3). Finally, exon skipping was induced by two nonsense mutations [910C→T (R304X), skipping 7; and 6792C→A (Y2264X), skipping 37, in two unrelated NF1 patients], as described (30,31).

Overall, 10 of the 19 mutations producing splicing defects induced exon skipping (53%), whereas the remaining nine mutations involved the utilization of cryptic splice sites (47%) in the vicinity of or within the exon, producing an exon of altered size or transcripts with an abnormal supplementary exon. Twelve splicing mutations were due to nucleotide changes occurring at intronic regions (six being nucleotide substitutions of the conserved AG/GT dinucleotides of the splice sites), six alterations were found in the respective exon, and one deleted one intronic and four exonic nucleotides. Twelve of these mutations should lead to in-frame amino acid deletions or insertions, with a protein length of between 2760 and 2877 amino acids (Table 1). We have calculated the consensus values (CVs) for the splice sites involved in splicing mutations, which reflect the similarity of any one splice site to the consensus sequences (28) (Table 3). In all cases of skipping of *NF1* exons, the CVs for the mutated splice sites (CVMs) were lower than those for the wild-type splice site sequences (CVNs) as found by Krawczak *et al*. (21). In the five cases

Table 1. *NF1* mutations and their effect on mRNA in 52 unrelated patients with neurofibromatosis type 1

Fragment	Patient	F/S	E/I	Mutation	mRNA level	Type/effect	Protein (aa)	Reference
I1	97-13	F	E 4a	I117S (350T→G)	350U→G	Missense	2818	This report
I1	98-337	S	E 4a	446delA	446delA	Frameshift	163/trunc	This report
I1	96-273	F	I 4b	IVS4b+5G→A	Inact. 5′ ss/skipping E 4b	Splice	163/trunc	This report
I1	97-8	S	E 4b	580delC	580delC	Frameshift	203/trunc	This report
II1	98-397	S	E 4b	527delAT	527delAU	Frameshift	198/trunc	This report
II1	96-16	S	E 5	723insA	723insA	Frameshift	243/trunc	This report
II1	98-1370	S	E 5	717insTCCCACAG	717insUCCCACAG	Frameshift	282/trunc	This report
II1	95-89	F	E 7	910C→T (R304X)	Skipping E 7	Nonsense/splice	2760/IF(–58)	31
I2	94-177	S	E 8	IVS8+1G→A	Inact. 5′ ss/skipping E 8	Splice	2777/IF(–41)	NNFF Consortium
I2	95-137	F	I 10a	IVS10a-9T→A	Inact. 3′ss/cryptic 3′ ss/1392insUUUUUAG	Splice	470/trunc	This report
I2	93-20	S	I 10b	IVS10b+1G→A	Inact. 5′ ss/skipping E 10b	Splice	2773/IF(–45)	55[a]
I2	94-26	S	E 10b	1466A→G (Y489C)	Cryptic 5′ ss/1466del62	Missense/splice	488/trunc	29
I2	98-1333	S	E 10b	1466A→G (Y489C)	Cryptic 5′ ss/1466del62	Missense/splice	488 trunc	29
I2	97-55	F	E 10b	1466A→G (Y489C)	Cryptic 5′ ss/1466del62	Missense/splice	488/trunc	29
I2	98-1338	S	E 10b	1465insT	1465insU	Frameshift	489/trunc	This report
I2	96-255	F	I 11	IVS11-3C→G	Inact. 3′ ss/cryptic 3′ ss/1721ins42	Splice	2832/IF(+14)	This report
I2	97-105	S	E 12a	W599X (1797G→A)	W599X (1797G→A)	Nonsense	598/trunc	This report
I2	98-362	S	E 12a	1758delTA	1758delUA	Frameshift	590/trunc	This report
II2	92-152	S	E 13	R681X (2041C→T)	R681X (2041C→U)	Nonsense	680/trunc	This report
II2	96-216	F	E 13	R681X (2041C→T)	R681X (2041C→U)	Nonsense	680/trunc	This report
II2	95-141	F	I 15	IVS15-16A→G	Cryptic 3′ ss/2409ins15	Splice	807/trunc	This report
II2	96-81	F	I 15	IVS15-16A→G	Cryptic 3′ ss/2409ins15	Splice	807/trunc	This report
II2	96-284	F	E 16	2764G→A (G922S)	Cryptic 5′ ss/2761del90	Missense/splice	2788/IF(–30)	This report
I3	94-3	S	I 16/E 17	IVS16-1delGGTTT	Inact. 3′ ss/skipping E 17	Splice	972/trunc	This report
I3	98-1320	S	I 18	IVS18+1G→A	Inact. 5′ ss/skipping E 18	Splice	2777/IF(–41)	56
I3	97-81	S	I 19a	IVS19a+1G→A	Inact. 5′ ss/skipping E 19a	Splice	2790/IF(–28)	This report
I3	97-78	S	E 19b	3214del11	Cryptic 5’ ss/3211del104	Frameshift/splice	1080/trunc	This report
I3	95-18	F	E 20	S1140X (3419C→G)	S1140X (3419C→G)	Nonsense	1139/trunc	This report
I3	98-313	S	E 20	3456delACTC	3456delACUC	Frameshift	1155/trunc	57
I3	98-369	S	E 21	R1204W(3610C→T)	3610C→U	Missense	2818	This report
II3	97-26	F	E 22	3759delCTACC	3759delCUACC	Frameshift	1260/trunc	This report
II3	98-1324	S	E 22	3818delCT	3818delCU	Frameshift	1281/trunc	This report
II3	98-315	F	E 25	L1425P (4274T→C)	4274U→C	Missense	2818	58
II3	97-89	F	E 27a	R1513X (4537C→T)	4537C→U	Nonsense	1512/trunc	NNFF Consortium
I4	96-203	F	I 27b	IVS27b+1del9	Inact. 5′ ss/cryptic 5′/4704del69	Splice	2795/IF(–23)	This report
I4	95-200	F	I 28	IVS28+1G→A	Inact. 5′ ss/cryptic 5′/5152del54	Splice	2800/IF(–18)	This report
I4	96-226	S	E 29	5546G→A (R1849Q)	Inact. 5′ ss/skipping E 29	Splice (missense)	1739/trunc	This report
I4	98-1327	F	E 29	5546G→A (R1849Q)	Inact. 5′ ss/skipping E 29	Splice (missense)	1739/trunc	This report
I4	98-1367	S	E 29	5546G→A (R1849Q)	Inact. 5′ ss/skipping E 29	Splice (missense)	1739/trunc	This report
II4	97-65	S	E 30	5617/5618del16	5617/5618del16	Frameshift	1897/trunc	This report
II4	96-238	S	I 30	IVS30+332A→G	Cryptic 5′ ss/5749ins177	Splice	2877/IF(+59)	59
II4	98-1309	F	I 30	IVS30+332A→G	Cryptic 5′ ss/5749ins177	Splice	2877/IF(+59)	59
II4	97-106	F	E 31	5887/5888delA	5887/5888delA	Frameshift	1989/trunc	This report
II4	95-115	F	E 34	6395del10	6395del10	Frameshift	2174/trunc	This report
II4	97-88	F	E 35	6593insT	6593insU	Frameshift	2219/trunc	This report
I5	90-21	F	E 37	6792C→A (Y2264X)	Skipping E 37	Nonsense/splice	2784/IF(–34)	16,30,31
I5	95-66	S	E 37	6792C→A (Y2264X)	Skipping E 37	Nonsense/splice	2784/IF(–34)	16,30,31
I5	97-32	F	I 37	IVS37+2T→G	Inact. 5′ ss/skipping E 37	Splice	2784/IF(–34)	This report
I5	97-113	F	E 39	7096delAACTTT	7096delAACUUU	Amino acid deletion	2816/IF(–2)	27
I5	96-269	F	E 41	7337delC	7337delC	Frameshift	2466/trunc	This report
II5	96-205	F	E 46	8042insA	8042insA	Frameshift	2680/trunc	60[a]
II5	96-178	F	E 48	8134delAA	8134delAA	Frameshift	2713/trunc	This report

Fragment, RT–PCR fragment (see Materials and Methods); F/S, familial/sporadic case; E/I, exon/intron; aa, amino acid; trunc, truncated protein; IF, inframe; inact. 5′ ss or 3′ ss, inactivation of 5′ or 3′ splice site; cryptic 5′ or 3′ ss, activation/creation of a novel 5′ or 3′ splice site.
[a]Patients previously reported.

where a novel splice site is created due to the mutation, the scores for the new site (CVAs) were higher or similar to the CVNs. Finally, in four cases in which the mutation altered the normal splice site, the cryptic site that was utilized had in general similar values to the mutated site. We have also calculated the CVs for the splice sites of exons 7 and 37, since mutations R304X and Y2264X produce skipping of exons 7 and 37, respectively. As also calculated by Hoffmeyer *et al*. (31), all

Table 2. Forty-four different mutations in the *NF1* gene detected in 52 unrelated patients with neurofibromatosis type 1

Mutation type	*n*	%
Splice[a]	14	32
Frameshift	18	41
Nonsense	6	14
Missense	5	11
Amino acid deletions	1	2
Total	44	100

Mutation type refers to the identification of the defects at the genomic level.
[a]The splice site mutation 5546G→A could also be considered an amino acid substitution (R1849Q), but here it has only been counted as a splice site mutation.

the splice site CVs were within a normal range [exon 7: CVN (5′ ss) = 80.1, CVN (3′ ss) = 79.4; exon 37: CVN (5′ ss) = 85.6, CVN (3′ ss) = 87.5].

Recurrent mutations in the *NF1* gene

Twenty-one of the 80 patients had mutations that were detected more than once in this study and/or were reported previously by other investigators (Table 1). Mutation 1466A→G (Y489C) was identified in two sporadic and in one familial case; this mutation was recently found by Osborn and Upadhyaya (29). Mutation 5546G→A (R1849Q) was identified in one familial and two sporadic cases, being described for the first time. Three other mutations [IVS30+332A→G, R681X (2041C→T) and 6792C→A (Y2264X)] have each been detected in one familial and one sporadic case of NF1. However, mutation IVS15-16A→G was identified in two unrelated families with the same *NF1* haplotype, suggesting that they could be identical by descent. Finally, three additional mutations, previously described by other investigators, were found in three sporadic cases of NF1. Overall, at least 15 of the 80 NF1 patients (19%) had a total of eight different mutations that are recurrent.

Genotype/phenotype analysis

Our data suggest that there is no clear relationship between specific *NF1* mutations and clinical features. However, several interesting findings were observed.

CLSs. No relationship was found between the number of CLSs and the identified mutations in patients of similar age. For example, mutation 1466A→G was found in patients with a wide variation in the number of CLSs (from 6 to >50) (Table 4). Similarly, mutations causing the same effect at the mRNA level (6792C→A and IVS37+2T→G, both producing skipping of exon 37) were present in patients with different numbers of CLSs (9 and >50) and similar ages (>40 years). However, we have also found patients with the same mutation (8134delAA) and a similar number of CLSs (Table 4).

Table 3. Consensus values (CVs), as defined by Shapiro and Senapathy (28), for splice sites involved in splicing mutations in the *NF1* gene

Mutation	Splice site/mutation	CVN	CVM	CVA	Comment
Mutations at 5′ ss					
IVS4b+5G→A	GG gtaa(g→a)t	92.15	77.74	–	Skipping E 4b
IVS8+1G→A	GT(g→a)tgaga	91.06	72.81	–	Skipping E 8
IVS10b+1G→A	GT(g→a)taagt	79.38	61.13	–	Skipping E 10b
IVS18+1G→A	AG(g→a)tgagt	96.72	78.47	–	Skipping E 18
IVS19a+1G→A	AG(g→a)taaa	79.93	61.68	–	Skipping E 19a
IVS27b+1del9	AG(del9)actatg	81.93	34.49	57.48	Cryptic, 69 nt upstream
IVS28+1G→A	AA(g→a)aagt	87.59	69.34	68.43	Cryptic, 54 nt upstream
5546G→A(R1849Q)	C(G→A)gtaggt	81.02	68.61	–	Skipping E 29
IVS37+2T→G	AGg(t→g)aatt	85.58	67.33	–	Skipping E 37
Mutations at 3′ ss					
IVS10a-9T→A	tgttt(t→a)gtttttagAG	87.83	82.99	77.76	Cryptic, 7 nt upstream
IVS11-3C→G	ttatgttactg(c→g)agCT	85.5	73.78	75.88	Cryptic, 42 nt upstream
IVS16-1delGGTTT	tgttctttcttta(delgGTTT)tAT	89.01	69.62	–	Skipping E 17
Creation of novel splice sites					
5′ ss					
1466A→G(Y489C)	CT(a→g)taagt	79.38	–	79.00	Cryptic, 62 nt upstream
2764G→A(G922S)	TGgtg(g→a)gt	81.02	–	88.87	Cryptic, 90 nt upstream
3214del11	AGgca(del11)agt	73.17	–	81.75	Cryptic, 104 nt upstream
IVS30+332A→G	AC(a→g)taagt	–	–	86.49	Cryptic exon
3′ ss					
IVS15-16 A→G	catgctttgcaca(a→g)AA	79.5	–	84.60	Cryptic, 16 nt upstream

CVN, consensus value normal; CVM, consensus value mutant; CVA, consensus value of activated cryptic splice site; ss, splice site; E, exon; –, not applicable. Upper case letters denote nucleotides within exons and lower case letters nucleotides within introns.

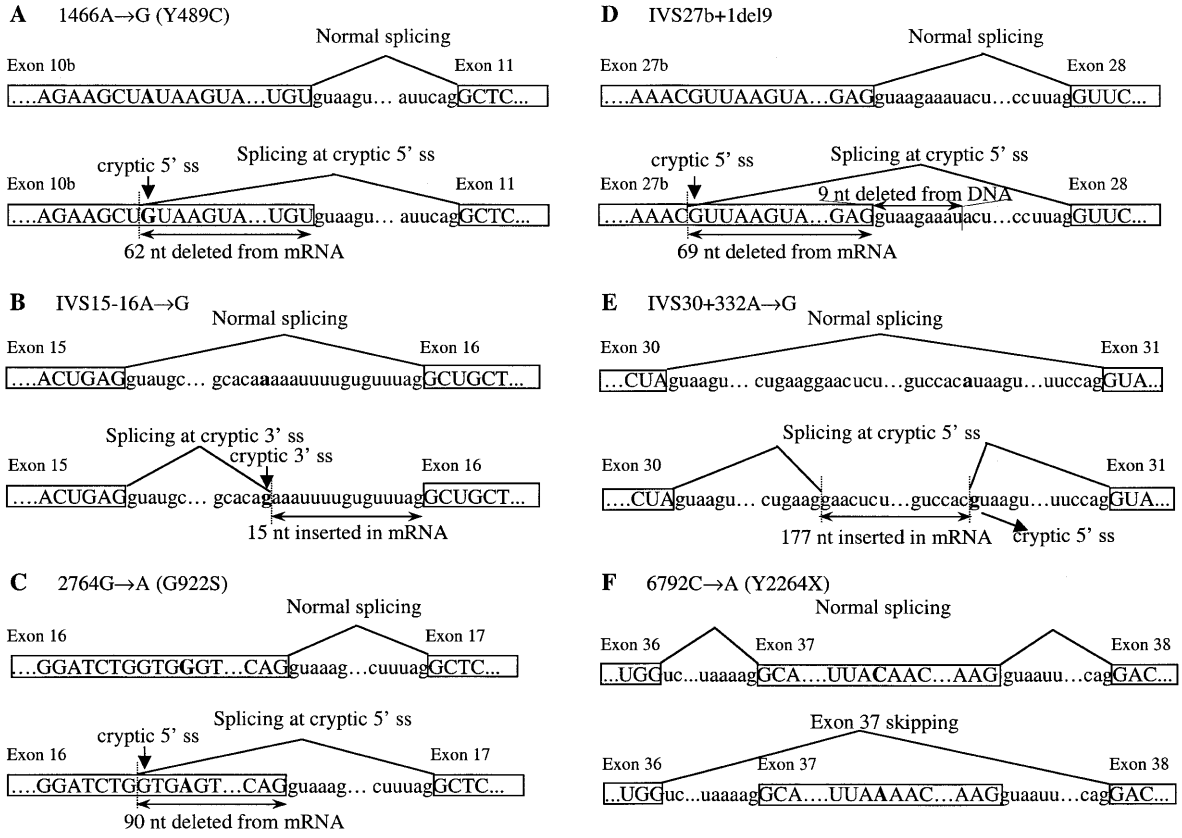

Figure 3. Schematic diagram of the splicing mechanism involved in some *NF1* mutations. (**A**) Missense mutation 1466A→G (Y489C) creates a novel 5′ splice site (5′ ss) producing a deletion of 62 nucleotides (nt) at cDNA position 1466. (**B**) Mutation IVS15-16A→G creates a novel 3′ ss that leads to a 15 nt insertion at cDNA position 2409. (**C**) Missense mutation 2764G→A (G922S) creates a novel 5′ ss within exon 16 that gives rise to a deletion of 90 nt at cDNA position 2761. (**D**) Mutation IVS27b+1del9 inactivates the 5′ wild-type ss, leading to the utilization of a cryptic 5′ ss located in exon 27b and producing a deletion of 69 nt at position 4704. (**E**) Mutation IVS30+332A→G activates a cryptic 5′ ss in intron 30 and results in the introduction of a supplementary exon of 177 nt in the mRNA between exons 30 and 31. (**F**) Nonsense mutation 6792C→A (Y2264X) in exon 37 induces skipping of this exon in the *NF1* mRNA. Upper case letters indicate exonic sequences, lower case letters indicate intronic sequences. The nucleotides bearing point mutations are in bold. Arrows indicate the starting points of deletions or insertions.

Cutaneous neurofibromas. In general, there was no relationship between the number of neurofibromas and the identified mutations in individuals of similar age. However, none of the seven patients aged >20 years and harboring mutations causing in-frame deletions or insertions at the mRNA level (IVS10b+1G→A; IVS11-3C→G; 6792C→A, in two related and two unrelated patients; IVS37+2T→G) had more than seven cutaneous neurofibromas.

Plexiform neurofibromas. Of 45 patients, for whom this clinical information was available, 7 had plexiform neurofibromas, with mutations located either within or outside the GRD (IVS4b+5G→A; IVS10b+1G→A; 1466A→G; 3818delAT; IVS30+332A→G; 8042insA). Two unrelated patients out of five with mutation 1466A→G had plexiform neurofibromas (Table 4).

Scoliosis. Of 40 patients with scoliosis data, 13 were affected but none had mutations within the GRD (IVS4b+5G→A; IVS10b+1G→A; 1466A→G, in four unrelated patients; R681X; S1140X; IVS30+332A→G; 5887/5888delA, in two related patients; IVS37+2T→G; 8134delAA). Four patients with scoliosis had mutations involving exon 10b (1466A→G; IVS10b+1G→A) (Table 4).

Optic glioma. This complication was found in 3 of 31 patients with information about this feature. All three patients had mutations that lead to a putative truncated NF1 protein (580delC; IVS10a-9T→A; 5546G→A).

Mental retardation. Only one patient (92-152) was reported as having mental retardation. In contrast, another patient with the same mutation, R681X, had a good academic level.

DISCUSSION

A large number of the mutations identified in this study cause alterations of the splicing of the *NF1* mRNA. Whereas in 37% of the patients the identified mutation at the DNA level was predicted to affect mRNA splicing, this proportion was significantly increased to 50% when the effect of the mutation was studied at the mRNA level (Fig. 4). The putative consequences at the protein level of the detected mutations are NF1 proteins of abnormal size in 94% of patients. This high rate of detection

Table 4. Main clinical features of 12 NF1 patients carrying three different *NF1* mutations

Genotype	Patient	Family	Sex	Age (years)	F/S	CLSs (*n*)	CNF (*n*)	Lisch nodules
1466A→G (Y489C)	94-26	140	Female	27	S	+ (20–50)	+ (10–50)	–
1466A→G (Y489C)	98-1333	409	Female	16	S	+ (>50)	–	+
1466A→G (Y489C)	97-55	347	Male	58	F (f)	+ (15)	+ (>50)	+
1466A→G (Y489C)	97-58	347	Male	27	F (b)	+ (6)	+ (10)	–
1466A→G (Y489C)	97-60	347	Female	31	F (p)	+ (>50)	+ (9)	+
R681X	92-152	98	Male	19	S	+ (20–50)	–	–
R681X	96-215	311	Female	38	F (m)	+ (20–50)	+ (10–50)	ND
R681X	96-216	311	Female	14	F (p)	+ (20–50)	+ (10–50)	+
8134delAA	96-189	301	Male	77	F (g)	+ (8)	+ (>100)	–
8134delAA	96-177	301	Female	39	F (a)	+ (6)	+ (10)	–
8134delAA	96-178	301	Female	45	F (f)	+ (5)	+ (8)	–
8134delAA	96-179	301	Male	9	F (p)	+(18)	–	–

F, familial case; S, sporadic case; p, propositus; a, aunt of propositus; b, brother of propositus; f; father of propositus; g, grandfather of propositus; m, mother of propositus; CLSs, café au lait spots; CNF, cutaneous neurofibroma; PNF, plexiform neurofibromas (number); ND, not determined.

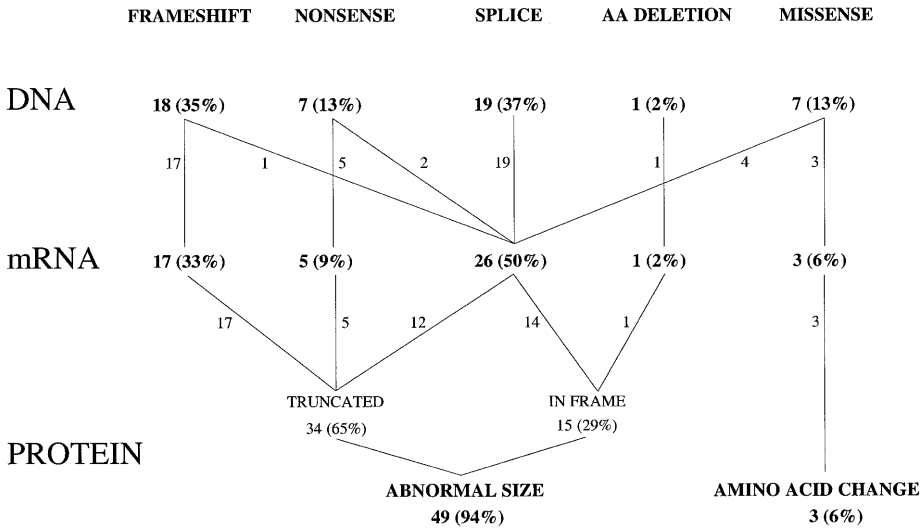

Figure 4. Types of mutation identified in 52 patients with NF1. The number of patients bearing each type of mutation and their percentages are indicated for alterations detected at the DNA and mRNA levels, and for their putative consequences on the NF1 protein length.

of mutations that cause abnormally spliced transcripts is probably biased by the use of cDNA-SSCP/HD. Only 9% of the mutations reported to the NNFF International NF1 Genetic Analysis Consortium (29 March 1999) occur in *NF1* intronic sequences, compared with 29% in our study. Osborn and Upadhyaya (29) have recently reported the characterization of 19 *NF1* mutations at the genomic and RNA level and have found that ~20% cause splicing abnormalities. Interestingly, one frameshift (3214del11), two nonsense (R304X and Y2264X) and two missense (Y489C and G922S) mutations resulted in defective splicing of the *NF1* transcript (Fig. 3), some of them described previously (29–31). Thus, it is likely that other reported mutations categorized as one mutation type could be considered splicing mutations if they were analyzed both at the mRNA and genomic levels. Similar findings have recently been reported for the *ATM* gene (32). Therefore, these results stress the importance of studying mutations at the genomic and RNA levels so as to clarify the primary effect of the mutation on mRNA processing, which could be different to that predicted solely at the genomic level.

Of the 23 different single base pair substitutions identified here, 74% (17/23) correspond to transitions and 26% (6/23) to transversions. These proportions are similar to those reported previously (33). This higher rate of transitions compared with transversions can partially be attributed to the hypermutability of the CpG dinucleotide. Accordingly, five of these transitions occur within CpG dinucleotides, of which four are C→T and one G→A.

Some of the mutations involving small deletions and insertions could be explained by the model of 'slipped mispairing' that occurs during DNA replication, mediated by direct or indirect repeats as determinants of secondary structures, which act as mutation intermediates (33). For instance, mutation 717insTCCCACAG creates a tandem duplication; 1465insT

creates an inverted repeat (CTtATAAG); another inverted repeat overlaps the deletion 6395del10 (TTTTTTGAGACTcagtctgacaGAGTTCT); 6593insT could be mediated by a symmetric element (GAGAtTATTCC). Slipped mispairing between two AACTTT repeats in tandem probably also accounts for the 7096delAACTTT mutation (27).

The identification of mutations in 52 of 80 patients (65%) using the cDNA-SSCP/HD method indicates that this approach is very powerful in the search for mutations in the *NF1* gene and is extremely useful for the molecular diagnosis of NF1, especially for sporadic cases. Approximately 20% of the patients studied here have mutations that are recurrent; although mutation recurrence has previously been described in NF1 (34), the high proportion detected here was unexpected.

The efficiency of cDNA-SSCP/HD in mutation detection is similar to that previously obtained using the PTT (67%) (18). There are several advantages of cDNA-SSCP/HD over the PTT system, such as that it allows the detection of missense mutations and small in-frame insertions/deletions, and that it is a non-radioactive technique. Neither PTT nor cDNA-SSCP/HD detect deletions that span the regions of overlap of any two consecutive cDNA segments and deletions spanning the whole *NF1* gene. However, putative deleted regions can potentially be identified by haplotype analysis using markers that may detect hemizygosity (35) or by FISH analysis using genomic clones that span the whole *NF1* gene (36–39). Although deletions were partially excluded in the patients studied here by loss of heterozygosity (LOH) analysis, 8 of the 28 NF1 individuals in which the mutation was not detected by cDNA-SSCP/HD were homozygous for all the *NF1* polymorphic markers analyzed and they could possibly bear intragenic *NF1* deletions, although these could not be demonstrated due to sample limitations. Another problem in RNA methodology is that in some cases the mutation can result in mRNA instability. We have confirmed the expression of the two *NF1* alleles in the nine patients where an *NF1* mutation was not detected and who were heterozygous for the *Rsa*I polymorphic site in exon 5 (23), but we cannot be sure that the allelic messages were equally represented since a quantitative analysis was not performed. Another possibility is that some of the unidentified mutations were located in the 3′-untranslated region (3′-UTR) and/or in the promoter (24,40). To date, no mutations have been reported in the promoter and only one in the *NF1* 3′-UTR (41).

One additional difficulty in mutation analysis of *NF1* studying genomic regions is due to other sequences related to the *NF1* gene, which are present in several human chromosomes (12). Mutation analysis using *NF1* mRNA partially avoids this problem as most of these loci represent non-processed pseudogenes. When some of the mutations described here were characterized at the genomic level, we found that for some exons the primers designed by Purandare *et al*. (42) produced the co-amplification of the homologous pseudogene. To solve this problem, specific *NF1* primers were designed (see Materials and Methods).

The different efficiency in the detection of mutations in familial cases (87%) versus sporadic ones (51%) could be attributed to mosaicism. Mosaicism seems to be present in the *de novo* cases of neurofibromatosis, as has been reported for NF1 (43–45) and NF2 (46). Thus, it is possible that some sporadic NF1 patients could be somatic mosaics with a low proportion or absence of the *NF1* mutation in their lymphocytes. To overcome this problem, in the sporadic cases that are negative for mutation analysis in lymphocytes, it would be useful to search for mutations in NF1 target tissues such as neurofibromas, where the first- and second-hit mutations should be present (47).

The only genotype–phenotype correlation described so far in NF1 associates large deletions encompassing the whole *NF1* gene with mental retardation and/or learning disabilities, mild facial dysmorphology and a large number of early-onset cutaneous neurofibromas (36–39,48). We have identified the *NF1* mutation in only one patient with mental retardation. This low proportion of cases is probably due to the fact that patients bearing *NF1* large deletions were already excluded before mutation analysis was performed (see Materials and Methods).

The information on the clinical features of the NF1 patients in whom mutations were identified suggests that the location and type of mutation within the *NF1* gene and its putative effect at the protein level do not indicate any relationship to any specific clinical feature of NF1. However, since CLSs and neurofibromas are highly age dependent (49), their putative relationship with *NF1* mutations should be analyzed in a large number of patients to allow comparisons between patients of similar ages. Notable findings detected here involve scoliosis with mutations in exon 10b, optic glioma with mutations causing putative truncated NF1, and a low number of neurofibromas in patients >20 years old with in-frame mutations. These findings will need further investigation, but the lack of relationship between phenotype and genotype strongly suggests that modifier genes probably play an important role in NF1 (49).

A large proportion of *NF1* mutations have consequences for the correct splicing of the *NF1* mRNA. In cystic fibrosis, it has been described that disease variability among patients carrying the same splicing mutation was associated with variable levels of aberrantly spliced cystic fibrosis transmembrane regulator (CFTR) transcripts (50). It is hypothesized that variations in the RNA splicing mechanism may lead to differential expression of the splicing mutation, and hence to different levels of the aberrantly spliced mRNA. Thus, it is tempting to postulate that splicing mutations, which we show here are predominant in NF1, could account for part of the clinical intrafamilial and interfamilial variability that is observed in NF1 patients carrying the same mutation.

MATERIALS AND METHODS

Patients and families

Eighty unrelated NF1 patients were studied, 31 being familial cases and 49 sporadic. Patients with large deletions in the *NF1* gene, previously detected by LOH analysis (35), were excluded from this study. The NF1 diagnosis was based on the NIH Consensus Conference criteria (51). When available, blood samples of other family members were also obtained. All the participants were informed about the study and consent was obtained from all patients and their relatives.

RNA/DNA extraction

Total RNA was extracted from peripheral blood lymphocytes using the Tripure isolation reagent (Boehringer Mannheim,

Mannheim, Germany), according to the manufacturer's instructions. DNA was extracted by the 'salting out' method (52).

Reverse transcription and amplification of the *NF1* coding region

RNA (2–5 μg) was reverse transcribed using 500 ng of random hexamers and 200 U of Superscript II reverse transcriptase (Gibco BRL, Life Technologies, Paisley, UK) in a 20 μl reaction volume using conditions recommended by the manufacturer. The entire *NF1* cDNA was amplified in 10 overlapping fragments, ranging from 634 to 1262 bp, using primer sets designed by Hoffmeyer *et al*. (53). PCR reactions were performed with denaturation at 94°C for 3 min, 40 cycles of 94°C for 60 s, 58°C for 60 s and 74°C for 90 s, and a final extension at 74°C for 10 min under standard conditions, except for 3 mM $MgCl_2$. The size of the PCR products was verified by electrophoresis in 2% agarose gels.

cDNA-SSCP/HD mutation analysis

RT–PCR products (1–4 μl of each) were mixed with 4 μl of denaturing buffer and heated at 95°C for 2 min. The samples were loaded on 10% polyacrylamide gels (CleanGel DNA Analysis kit; Pharmacia, Uppsala, Sweden). RT–PCR products, ranging from 634 to 996 bp (fragments I1, II1, I2, I3 and II5), were run for 2 h, and those ranging from 1034 to 1262 bp (fragments II2, II3, I4, II4 and I5) for 3 h, all at 600 V/15°C. SSCP/HD patterns were visualized by silver staining (Fig. 1B). When an abnormal pattern was detected, the corresponding RT–PCR was repeated to confirm that the anomalous pattern was reproducible, always comparing with controls (unaffected relatives of NF1 patients). Finally, the abnormal product was analyzed using an automatic sequencer (ABI PRISM 377; Perkin Elmer, Foster City, CA).

Characterization of *NF1* mutations at the genomic level

Sequence changes identified at the cDNA level were confirmed at the genomic level by amplification and direct sequencing of the specific exon containing the mutation. When a skipped exon or the insertion or deletion of several nucleotides were detected at the cDNA level, then the corresponding exon and flanking intron boundaries were analyzed for potential point mutations. In most cases, the primers used for amplification and sequencing of individual exons were those described by Purandare *et al*. (42), except for exons 10b, 19b and 21 that also amplify pseudogenes being substituted for:
10bD2, 5′-CTACTATACCACACATTGGTAG-3′, and
10bR2, 5′-TCAGGTATGATCAGAAAGG-3′, for exon 10b;
19bD2, 5′-AACTTGAAAGATTCATGGTCTC-3′, and
19bR2, 5′-TTTATGTTTTTTGGTGACTG-3′, for exon 19b;
21D2, 5′-CATGTTAGTAAATTTGCATCTG-3′, and
21R2, 5′-ATTTGCTATGTGCCAGGCAC-3′, for exon 21.
Mutations that created or abolished a restriction site were confirmed by restriction enzyme analysis as described (54) (Fig. 2). Segregation analysis of the identified mutations verified the familial or sporadic nature of the mutation.

Expression analysis of the two *NF1* alleles

RT–PCR fragment II1, which contains the *Rsa*I polymorphic site in exon 5 (23), was analyzed in the nine samples that were heterozygous at this site and that were also negative for *NF1* mutations, showing that the *NF1* mRNA was stable.

Genotype/phenotype analysis

We compared the causative *NF1* mutations with the clinical features of the patients. We analyzed the patients according to age, numbers of CLSs and neurofibromas, Lisch nodules, freckling, plexiform neurofibroma, scoliosis, optic glioma, mental retardation, hypertension, and the type/effect and location of the *NF1* mutations.

ACKNOWLEDGEMENTS

The authors wish to thank all the patients and family members who participated in this study, and all the clinicians for referring the families. We are indebted to M. Morell for technical help, to Y. Espinosa for advice with the technique and the manuscript, to A. Puig for sequencing and to A. Aviñó for synthesis of PCR primers. E.A. is a fellow of the Comissió Interdepartamental de Recerca i Innovació Tecnològica (CIRIT) of the Generalitat de Catalunya. This work was supported by grants from the Fondo de Investigaciones Sanitarias de la Seguridad Social (95-368 and 98-0992), the European Union (BIOMED BMH-4-CT96-1364), the Institut Català de la Salut and the Fundació Agust Pi i Sunyer/Marató de TV3.

REFERENCES

1. Riccardi, V.M. (1992) *Neurofibromatosis: Phenotype, Natural History and Pathogenesis*, 2nd edn. Johns Hopkins University Press, Baltimore, MD.
2. Huson, S.M. and Hughes, R.A.C. (1994) *The Neurofibromatoses: A Pathogenetic and Clinical Overview*. Chapman & Hall, London, UK.
3. Cawthon, R.M., Weiss, R., Xu, G., Viskochil, D., Culver, M., Stevens, J., Robertson, M., Dunn, D., Gesteland, R., O'Connell, P. and White, R. (1990) A major segment of the neurofibromatosis type 1 gene: cDNA sequence, genomic structure, and point mutations. *Cell*, **62**, 193–201.
4. Viskochil, D., Buchberg, A.M., Xu, G., Cawthon, R.M., Stevens, J., Wolff, R.K., Culver, M., Carey, J.C., Copeland, N.G., Jenkins, N.A. *et al*. (1990) Deletions and a translocation interrupt a cloned gene at the neurofibromatosis type 1 locus. *Cell*, **62**, 187–192.
5. Wallace, M.R., Marchuk, D.A., Andersen, L.B., Letcher, R., Odeh, H.M., Saulino, A.M., Fountain, J.W., Brereton, A., Nicholson, J., Mitchell, A.L. *et al*. (1990) Type 1 neurofibromatosis gene: identification of a large transcript disrupted in three NF1 patients. *Science*, **249**, 181–186.
6. Danglot, G., Regnier, V., Fauvet, D., Vassal, G., Kujas, M. and Bernheim, A. (1995) Neurofibromatosis 1 (NF1) mRNAs expressed in the central nervous system are differentially spliced in the 5′ part of the gene. *Hum. Mol. Genet.*, **4**, 915–920.
7. Li, Y., O'Connell, P., Breidenbach, H.H., Cawthon, R., Stevens, J., Xu, G., Neil, S., Robertson, M., White, R. and Viskochil, D. (1995) Genomic organization of the neurofibromatosis 1 gene (NF1). *Genomics*, **25**, 9–18.
8. Marchuk, D.A., Saulino, A.M., Tavakkol, R.L., Swaroop, M., Wallace, M.R., Andersen, L.B., Mitchell, A.L., Gutmann, D.H., Boguski, M. and Collins, F. (1991) cDNA cloning of the type 1 neurofibromatosis gene: complete sequence of the *NF1* gene product. *Genomics*, **11**, 931–940.
9. Ballester, R., Marchuk, D., Boguski, M., Saulino, A., Letcher, R., Wigler, M. and Collins, F.S. (1990) The NF1 locus encodes a protein functionally related to GAP and yeast IRA proteins. *Cell*, **63**, 851–859.
10. Martin, G.A., Viskochil, D. and Bollag, G. (1990) The GAP-related domain of the NF1 gene interacts with ras p21. *Cell*, **63**, 843–849.
11. Xu, G., Lin, B., Tanaka, K., Dunn, D., Wood, D., Gesteland, R., White, R., Weiss, R. and Tamanoi, F. (1990) The catalytic domain of the neurofibromatosis type 1 gene product stimulates *ras* GTPase and complements IRA mutants of *S. cerevisiae*. *Cell*, **63**, 835–841.

12. Cummings, L.M., Trent, J.M. and Marchuk, D.A. (1996) Identification and mapping of type 1 neurofibromatosis (NF1) homologous loci. *Cytogenet. Cell. Genet.*, **73**, 334–340.
13. Ainsworth, P.J., Rodenhiser, D.I. and Costa, M.T. (1993) Identification and characterization of sporadic and inherited mutations in exon 31 of the neurofibromatosis (NF1) gene. *Hum. Genet.*, **91**, 151–156.
14. Shen, M.H., Harper, P.S. and Upadhyaya, M. (1993) Neurofibromatosis type 1 (NF1): the search for mutations by PCR-heteroduplex analysis on Hydrolink gels. *Hum. Mol. Genet.*, **2**, 1861–1864.
15. Valero, M.C., Velasco, E., Moreno, F. and Hernandez-Chico, C. (1994) Characterization of four mutations in the neurofibromatosis type 1 gene by denaturing gradient gel electrophoresis (DGGE). *Hum. Mol. Genet.*, **3**, 639–641.
16. Robinson, P.N., Boddrich, A., Peters, H., Tinschert, S., Buske, A., Kaufmann, D. and Nurnberg, P. (1995) Two recurrent nonsense mutations and a 4 bp deletion in a quasi-symmetric element in exon 37 of the NF1 gene. *Hum. Genet.*, **96**, 95–98.
17. Colman, S.D., Abernathy, C.R., Ho, V.T. and Wallace, M.R. (1997) Four frameshift mutations in neurofibromatosis type 1 caused by small insertions. *J. Med. Genet.*, **34**, 579–581.
18. Heim, R.A., Kam-Morgan, L.N., Binnie, C.G., Corns, D.D., Cayouette, M.C., Farber, R.A., Aylsworth, A.S., Silverman, L.M. and Luce, M.C. (1995) Distribution of 13 truncating mutations in the neurofibromatosis 1 gene. *Hum. Mol. Genet.*, **4**, 975–981.
19. Martinez, J.M., Breidenbach, H.H. and Cawthon, R. (1996) Long RT–PCR of the entire 8.5-kb NF1 open reading frame and mutation detection on agarose gels. *Genome Res.*, **6**, 58–66.
20. Park, V.M. and Pivnick, E.K. (1998) Neurofibromatosis type 1 (NF1): a protein truncation assay yielding identification of mutations in 73% of patients. *J. Med. Genet.*, **35**, 813–820.
21. Krawczak, M., Reiss, J. and Cooper, D.N. (1992) The mutational spectrum of single base-pair substitutions in mRNA splice junctions of human genes: causes and consequences. *Hum. Genet.*, **90**, 41–54.
22. Skuse, G.R. and Cappione, A.J. (1997) RNA processing and clinical variability in neurofibromatosis type I (NF1). *Hum. Mol. Genet.*, **6**, 1707–1712.
23. Hoffmeyer, S., Assum, G., Griesser, J., Kaufmann, D., Nurnberg, P. and Krone, W. (1995) On unequal allelic expression of the neurofibromin gene in neurofibromatosis type 1. *Hum. Mol. Genet.*, **4**, 1267–1272.
24. Bernards, A., Snijders, A., Hannigan, G.E., Murthy, A.E. and Gusella, J.F. (1993) Mouse neurofibromin type 1 cDNA sequence reveals high degree of conservation of both coding and non-coding mRNA segments. *Hum. Mol. Genet.*, **2**, 645–650.
25. The, I., Hannigan, G.E., Cowley, G.S., Reginald, S., Zhong, Y., Gusella, J.F., Hariharan, I.K. and Bernards, A. (1997) Rescue of a *Drosophila* NF1 mutant phenotype by protein kinase A. *Science*, **276**, 791–794.
26. Scheffzek, K., Ahmadian, M.R., Wiesmuller, L., Kabsch, W., Stege, P., Schmitz, F. and Wittinghofer, A. (1998) Structural analysis of the GAP-related domain from neurofibromin and its implications. *EMBO J.*, **17**, 4313–4327.
27. Abernathy, C.R., Colman, S.D., Kousseff, B.G. and Wallace, M.R. (1994) Two NF1 mutations: frameshift in the GAP-related domain, and loss of two codons toward the 3′ end of the gene. *Hum. Mutat.*, **3**, 347–352.
28. Shapiro, M.B. and Senapathy, P. (1987) RNA splice junctions of different classes of eukaryotes: sequence statistics and functional implications in gene expression. *Nucleic Acids Res.*, **15**, 7155–7174.
29. Osborn, M.J. and Upadhyaya, M. (1999) Evaluation of the protein truncation test and mutation detection in the NF1 gene: mutational analysis of 15 known and 40 unknown mutations. *Hum. Genet.*, **105**, 327–332.
30. Messiaen, L., Callens, T., De Paepe, A., Craen, M. and Mortier, G. (1997) Characterisation of two different nonsense mutations, C6792A and C6792G, causing skipping of exon 37 in the NF1 gene. *Hum. Genet.*, **101**, 75–80.
31. Hoffmeyer, S., Nurnberg, P., Ritter, H., Fahsold, R., Leistner, W., Kaufmann, D. and Krone, W. (1998) Nearby stop codons in exons of the neurofibromatosis type 1 gene are disparate splice effectors. *Am. J. Hum. Genet.*, **62**, 269–277.
32. Teraoka, S.N., Telatar, M., Becker-Catania, S., Liang, T., Onengut, S., Tolun, A., Chessa, L., Sanal, O., Bernatowska, E., Gatti, R.A. and Concannon, P. (1999) Splicing defects in the ataxia-telangiectasia gene, ATM: underlying mutations and consequences. *Am. J. Hum. Genet.*, **64**, 1617–1631.
33. Cooper, D.N. and Krawczak, M. (1993) *Human Gene Mutation.* BIOS Scientific, Oxford, UK.
34. Estivill, X., Lazaro, C., Casals, T. and Ravella, A. (1991) Recurrence of a nonsense mutation in the NF1 gene causing classical neurofibromatosis type 1. *Hum. Genet.*, **88**, 185–188.
35. Lazaro, C., Gaona, A., Ravella, A., Volpini, V., Casals, T., Fuentes, J.J. and Estivill, X. (1993) Novel alleles, hemizygosity and deletions at an Alu-repeat within the neurofibromatosis type 1 (NF1) gene. *Hum. Mol. Genet.*, **2**, 725–730.
36. Wu, B.L., Austin, M.A., Schneider, G.H., Boles, R.G. and Korf, B.R. (1995) Deletion of the entire NF1 gene detected by the FISH: four deletion patients associated with severe manifestations. *Am. J. Med. Genet.*, **59**, 528–535.
37. Leppig, K.A., Viskochil, D., Neil, S., Rubenstein, A., Johnson, V.P., Zhu, X.L., Brothman, A.R. and Stephens, K. (1996) The detection of contiguous gene deletions at the neurofibromatosis 1 locus with fluorescence *in situ* hybridization. *Cytogenet. Cell. Genet.*, **72**, 95–98.
38. Upadhyaya, M., Roberts, S.H., Maynard, J., Sorour, E., Thompson, P.W., Vaughan, M., Wilkie, A.O. and Hughes, H.E. (1996) A cytogenetic deletion, del(17)(q11.22q21.1), in a patient with sporadic neurofibromatosis type 1 (NF1) associated with dysmorphism and developmental delay. *J. Med. Genet.*, **33**, 148–152.
39. Wu, B.L., Schneider, G.H. and Korf, B.R. (1997) Deletion of the entire NF1 gene causing distinct manifestations in a family. *Am. J. Med. Genet.*, **69**, 98–101.
40. Hajra, A., Martin-Gallardo, A., Tarle, S.A., Freedman, M., Wilson-Gunn, S., Bernards, A. and Collins, F.S. (1994) DNA sequences in the promoter region of the NF1 gene are highly conserved between human and mouse. *Genomics*, **21**, 649–652.
41. Upadhyaya, M., Maynard, J., Osborn, M., Huson, S.M., Ponder, M., Ponders, B.A.J. and Harper, P.S. (1995) Characterisation of germline mutations in the neurofibromatosis type 1 (NF1) locus. *Hum. Mol. Genet.*, **1**, 735–740.
42. Purandare, S.M., Huntsman Breidenbach, H., Li, Y., Zhu, X.L., Sawada, S., Neil, S.M., Brothman, A., White, R., Cawthon, R. and Viskochil, D. (1995) Identification of neurofibromatosis 1 (NF1) homologous loci by direct sequencing, fluorescence *in situ* hybridization, and PCR amplification of somatic cell hybrids. *Genomics*, **30**, 476–485.
43. Lazaro, C., Ravella, A., Gaona, A., Volpini, V. and Estivill, X. (1994) Neurofibromatosis type 1 due to germ-line mosaicism in a clinically normal father. *N. Engl. J. Med.*, **331**, 1403–1407.
44. Colman, S.D., Rasmussen, S.A., Ho, V.T., Abernathy, C.R. and Wallace, M.R. (1996) Somatic mosaicism in a patient with neurofibromatosis type 1. *Am. J. Hum. Genet.*, **58**, 484–490.
45. Wu, B.L., Boles, R.G., Yaari, H., Weremowicz, S., Schneider, G.H. and Korf, B.R. (1997) Somatic mosaicism for deletion of the entire NF1 gene identified by FISH. *Hum. Genet.*, **99**, 209–213.
46. Evans, D.G., Wallace, A.J., Wu, C.L., Trueman, L., Ramsden, R.T. and Strachan, T. (1998) Somatic mosaicism: a common cause of classic disease in tumor-prone syndromes? Lessons from type 2 neurofibromatosis. *Am. J. Hum. Genet.*, **63**, 727–736.
47. Serra, E., Puig, S., Otero, D., Gaona, A., Kruyer, H., Ars, E., Estivill, X. and Lazaro, C. (1997) Confirmation of a double-hit model for the NF1 gene in benign neurofibromas. *Am. J. Hum. Genet.*, **61**, 512–519.
48. Kayes, L.M., Burke, W., Riccardi, V.M., Bennett, R., Ehrlich, P., Rubenstein, A. and Stephens, K. (1994) Deletions spanning the neurofibromatosis 1 gene: identification and phenotype of five patients. *Am. J. Hum. Genet.*, **54**, 424–436.
49. Easton, D.F., Ponder, M.A., Huson, S.M. and Ponder, B.A.J. (1993) An analysis of variation in expression of NF1: evidence for modifying genes. *Am. J. Hum. Genet.*, **53**, 305–313.
50. Chiba-Falek, O., Kerem, E., Shoshani, T., Aviram, M., Augarten, A., Bentur, L., Tal, A., Tullis, E., Rahat, A. and Kerem, B. (1998) The molecular basis of disease variability among cystic fibrosis patients carrying the 3849+10 kb C→T mutation. *Genomics*, **53**, 276–283.
51. Mulvihill, J.J., Parry, D.M., Sherman, J.L., Pikus, A., Kaiser-Kupfer, M.L. and Eldridge, R. (1990) NIH conference: Neurofibromatosis 1 (Recklinghausen disease) and neurofibromatosis 2 (bilateral acoustic neurofibromatosis). *Ann. Intern. Med.*, **113**, 39–52.
52. Miller, S.A., Dykes, D.D. and Polesky, H.F. (1988) A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res.*, **16**, 1215.
53. Hoffmeyer, S., Assum, G., Kaufmann, D., Schwenk, K. and Krone, W. (1994) A deletion in the 5′-region of the neurofibromatosis type 1 (NF1) gene. *Hum. Genet.*, **94**, 97–100.

54. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
55. Ars, E., Kruyer, H., Gaona, A., Serra, E., Lazaro, C. and Estivill, X. (1999) Prenatal diagnosis of sporadic neurofibromatosis type 1 (NF1) by RNA and DNA analysis of a splicing mutation. *Prenat. Diagn.*, **19**, 739–742.
56. Purandare, S.M., Lanyon, W.G., Arngrimsson, R. and Connor, J.M. (1995) Characterisation of a novel splice donor mutation affecting position +1 in intron 18 of the NF-1 gene. *Hum. Mol. Genet.*, **4**, 767–768.
57. Upadhyaya, M., Osborn, M.J., Maynard, J., Kim, M.R., Tamanoi, F. and Cooper, D.N. (1997) Mutational and functional analysis of the neurofibromatosis type 1 (NF1) gene. *Hum. Genet.*, **99**, 88–92.
58. Peters, H., Hess, D., Fahsold, R. and Schulke, M. (1999) A novel mutation L1425P in the GAP-region of the NF1 gene detected by temperature gradient gel electrophoresis (TGGE). Mutation in brief no. 230. Online. *Hum. Mutat.*, **13**, 337.
59. Perrin, G., Morris, M.A., Antonarakis, S.E., Boltshauser, E. and Hutter, P. (1996) Two novel mutations affecting mRNA splicing of the neurofibromatosis type 1 (NF1) gene. *Hum. Mutat.*, **7**, 172–175.
60. Ars, E., Kruyer, H., Gaona, A., Casquero, P., Rosell, J., Volpini, V., Serra, E., Lazaro, C. and Estivill, X. (1998) A clinical variant of neurofibromatosis type 1: familial spinal neurofibromatosis with a frameshift mutation in the NF1 gene. *Am. J. Hum. Genet.*, **62**, 834–841.