

High Frequency of Large Intragenic Deletions in the Fanconi Anemia Group A Gene

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Summary

Fanconi anemia (FA) is an autosomal recessive disorder exhibiting chromosomal fragility, bone-marrow failure, congenital abnormalities, and cancer. At least eight complementation groups have been described, with group A accounting for 60%–65% of FA patients. Mutation screening of the group A gene (*FANCA*) is complicated by its highly interrupted genomic structure and heterogeneous mutation spectrum. Recent reports of several large deletions of *FANCA*, coupled with modest mutation-detection rates, led us to investigate whether many deletions might occur in the heterozygous state and thus fail to be detected by current screening protocols. We used a two-step screening strategy, in which small mutations were detected by fluorescent chemical cleavage of the *FANCA* transcript, and heterozygosity for gross deletions was detected by quantitative fluorescent multiplex PCR. We screened 26 cell lines from FA complementation group A for *FANCA* mutations and detected 33 different mutations, 23 of which were novel. Mutations were observed in all 26 cell lines and included 43 of a possible 52 mutant alleles (83%). Of the mutant alleles, 40% were large intragenic deletions that removed up to 31 exons from the gene, indicating that this may be the most prevalent form of mutation in *FANCA*. Several common deletion breakpoints were observed, and there was a highly significant correlation between the number of breakpoints detected in a given intron and the number of Alu repeats that it contained, which suggests that Alu-mediated recombination may explain the high prevalence of deletions in *FANCA*. The dual screening strategy that we describe may be useful for mutation screening in other genetic disorders in which mutation-detection rates are unexpectedly low.

Introduction

Fanconi anemia (FA; MIM 227650) is an autosomal recessive disorder associated with a wide range of clinical features, including progressive bone-marrow failure, diverse developmental abnormalities, and a high incidence of acute myeloid leukemia (Auerbach et al. 1993). FA cells are hypersensitive to DNA cross-linking agents, such as diepoxybutane (DEB) and mitomycin C (MMC), and this provides a valuable laboratory test to support the clinical diagnosis. The disorder is genetically heterogeneous, with at least eight complementation groups described to date (Joenje et al. 1997). This indicates that FA may be caused by a defect in at least eight different genes, which have been denoted “*FANCA*”–“*FANCH*.” The *FANCA*, *FANCC*, and *FANCG* genes have been cloned (Strathdee et al. 1992; Fanconi Anemia Breast Cancer Consortium 1996; Lo Ten Foe et al. 1996; de Winter et al. 1998), and the *FANCD* and *FANCE* genes have been mapped to chromosomes 3p and 6p, respectively (Whitney et al. 1995; Waisfisz et al. 1999). *FANCA* is the most commonly mutated FA gene, accounting for 60%–65% of all cases.

Mutation screening of the *FANCA* gene is a difficult task, since the 4.3-kb coding sequence is interrupted by 43 exons (Ianzano et al. 1997), and the mutational spectrum is very heterogeneous. More than 100 different *FANCA* mutations have been described to date and include almost all classes of mutations (Fanconi Anemia Breast Cancer consortium 1996; Lo Ten Foe et al. 1996; Levran et al. 1997; Savino et al. 1997; Tachibana et al. 1999; Wijker et al. 1999). Mutation-detection rates in these studies are ~30%–70%, and there have been several recent reports of intragenic deletions in *FANCA* (Centra et al. 1998; Levran et al. 1998; Wijker et al. 1999). This led us to postulate that the low mutation-detection rates might be caused by a high frequency of intragenic deletions, which would not be detected by the majority of screening protocols if they occurred in compound heterozygotes whose other mutant allele was a point mutation. We therefore developed a two-step fluorescence-based mutation-screening strategy that would detect both small mutations in the coding sequence of

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Table 1**Primer Pairs for Six Overlapping RT-PCR Fragments of FANCA Gene**

FRAGMENT	1° AND 2° PCR PRIMER PAIRS (5'→3')	NUCLEOTIDE POSITION ^a	EXONS SPANNED	PRODUCT SIZE (bp)	PCR CONDITIONS	
					Annealing Temperature (°C)	[Mg ²⁺] of PCR (mM)
1	Forward, CCA AGG CCA TGT CCG ACT CG	13–595	1–6	581	60	1.5
	Reverse, CAG AAA GCA TGG CCC TGG CGA CG				55	1.5
2	Forward, GGG TCC CGA ACT CCG CCT CGG GCC	393–1079	4–12	686	66	1.5
	Reverse, TTT CCA GCA GCT CTT GCA GG				64	1.5
3	Forward, GCG CCT CCT GCG AAG CCA TCA G	893–1627	11–18	734	60	1.5
	Reverse, CAG CTG GCA GCT CTC GAA TGC				60	1.5
4	Forward, CAG TCA CCC TGT GCT GCT GAC	1100–1900	13–21	800	60	5.0
	Reverse, CGG TAC AGT GAG GTG AGC AG				60	4.0
5	Forward, GGT TCG GAG TGT TCA GTG GAC	1724–3308	19–33	1,584	60	6.7
	Reverse, GCT CAG TAA TGT CCC CAG CTG				60	6.7
6	Forward, GTG CAG AGG AGT TGG TTG G	3093–4486	32–3' UTR	1,327	60	6.7
	Reverse, CTT CTG GCT TCT CTT CAG CAG				60	6.7
	Forward, CAT CAT GGT GTT TGA GCA TAC G				60	6.7
	Reverse, GAA GTC GAC CAT CAG GGA GGG				60	6.7
	Forward, GGA GGC CTT ACT ACG TGT CC				60	6.7
	Reverse, GCA GTG ATG GGC TGT TCT GCC TG				60	6.7
	Forward, GCA AGA CCT CAT AGT GCC TC				60	6.7
	Reverse, CGC AAA CGC TGA GTG ACT CG				60	6.7
	Forward, CAG ACG GCT CCA GGC TCT GAC				60	6.7
	Reverse, CCC ACT AAA GCA GTC GAG G				60	6.7

^a Numbering, from the first nucleotide of the initiation codon.

FANCA and heterozygous deletions of one or more exons. The method was then used to screen the FANCA gene for mutations in 26 cell lines from patients with FA who were classified as group A by complementation analysis. Mutations were observed in all 26 cell lines and included a high frequency of intragenic deletions, some of which removed >30 exons from the FANCA gene. This strategy may have general application for mutation screening in other disorders.

Patients and Methods

Patients

Patients were ascertained by EUFAR (European Concerted Action on Fanconi Anemia Research), and blood samples were obtained with informed consent. Twenty-six unrelated FA patients had been diagnosed previously, on the basis of their clinical presentation and their cellular hypersensitivity to mitomycin C (MMC) or di-epoxybutane (DEB). Lymphoblastoid cell lines were established and tested by complementation analysis to identify group A patients, as described elsewhere (Joenje et al. 1995).

Methods

RNA Extraction and Reverse-Transcriptase PCR (RT-PCR).—Total RNA was extracted from lymphoblasts, as described elsewhere (Chomczynski and Sacchi 1987). From each cell line, 2.5 µg of total RNA was reverse transcribed by use of 25 ng each of the following specific primers, in order to ensure transcription of the entire coding sequence: 1727R (5'-GCCTCCATGACGGTGA-CTGG-3'), 3' R1 (5'-GCTCCAGGTCAGCTACCATC-TCCTGC-3'), 3494R (5'-GAAGTCGACCATCAGGG-AGGG-3'), and RU4955R (5'-CAGAGCTGGACTTT-GCCTGT-3').

These primers were multiplexed in a single reaction and heated for 10 min at 65°C. Then, to each patient sample, 1 × reverse transcriptase buffer, 10 mM DTT, 500 µM each dNTP, 40 U RNase inhibitor, and 200 U MMLV reverse transcriptase (Gibco BRL) was added, incubated at 42°C for 1 h, and heated at 95°C for 3 min to destroy the enzyme. The full coding sequence was amplified in six overlapping fragments, with ≥200 bp of overlapping sequence between each pair of fragments. A primary PCR was carried out for each of the six fragments, with 0.4 µM each of forward and reverse primer (for primer sequences and conditions, see table 1), 500

μM each dNTP, $1 \times$ *Taq* DNA polymerase buffer (67 mM Tris-HCl, pH 8.8, 16.6 mM $(\text{NH}_4)_2\text{SO}_4$, X mM MgCl_2 , where X is 1.5–6.7, 170 μg BSA/ml, 10 mM β -mercaptoethanol), 1 μl cDNA, and 1.5 U (Promega) *Taq* polymerase. PCR conditions were an initial denaturation step at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 10 s, annealing for 30 s, and extension for 1 min at 72°C for each kilobase of fragment size. A final extension for 5 min at 72°C was added. Nested PCR was then carried out by addition of 1 μl of the primary PCR product to a secondary PCR mix, as before (see table 1), but with the inclusion of 0.3 μl of one of three different fluorescently-labeled dUTP solutions: TAMRA-yellow, R6G-green, or R110-blue (PE Biosystems). Secondary products were then electrophoresed in a 1.5% agarose gel to check for aberrantly sized bands, which were GeneClean-purified and sequenced by use of the ABI 377 DNA sequencer. Full-size fragments were used directly for fluorescent chemical cleavage of mismatch (FCCM) analysis (see below). Probes used for FCCM were amplified from the *FANCA* cDNA (Lo Ten Foe et al. 1996), as for patient cDNA, but with use of biotinylated primers as well as fluorescent dUTPs.

Solid-Phase FCCM Detection.—This procedure was based on the use of chemical cleavage to detect mismatched bases in heteroduplexes formed between wild-type and patient DNA (Cotton et al. 1988; Ellis et al. 1998). The original method has been modified to a solid-phase, fluorescence-based protocol that allows rapid and sensitive detection of point mutations and other small lesions (Rowley et al. 1995). Two microliters of biotinylated, fluorescently labeled probe was gel-purified and added to 2 μl fluorescently labeled target in $1 \times$ hybridization buffer (0.3 M NaCl, 100 mM Tris-HCl, pH 8.0) in a total volume of 30 μl . This was denatured for 5 min at 95°C, then held for 60 min at 65°C to form heteroduplexes, which could be stored at 4°C for several hours. Streptavidin-coated beads (Dynal) were treated by taking 10 μl of beads for every patient sample DNA and washing twice in $1 \times$ binding and washing buffer (5 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 1 M NaCl), before resuspension in 20 μl $2 \times$ binding and washing buffer. This 20- μl bead suspension was then added to the hybrid DNA and resuspended. After 15 min the supernatant was removed from the beads by use of a magnet (Promega). The beads with the bound DNA were then resuspended in 20 μl hydroxylamine solution (4 M hydroxylamine hydrochloride, 2.3 M diethylamine) and incubated at 37°C for 2 h or were resuspended in osmium tetroxide solution (0.4% OsO_4 , 2% pyridine) and incubated at room temperature for 15 min. The supernatant was removed from the beads as before, and the beads were washed in binding and washing buffer before resuspension in 5 μl piperidine solution (2 M piperidine

in formamide plus dextran blue dye) and heating at 90°C for 15 min. Two microliters of the supernatant was loaded on a 6% acrylamide denaturing gel and electrophoresed for 5 h on an ABI 377 DNA sequencer. Data were collected and analyzed by means of Genescan 2.0 ABI 377 software (see electrophoretogram, in fig. 1).

Quantitative Fluorescent Multiplex PCR.—Genomic DNA was prepared from lymphoblastoid cell lines by salt/chloroform extraction, as described elsewhere (Gibson et al. 1996). DNA samples were then incubated at 55°C for 1 h; half of this sample was taken to determine the concentration, and the remainder was diluted to 25 ng/ μl for the PCR. Three fluorescently labeled multiplex PCR assays were used to amplify exons from the *FANCA* and *FANCC* genes. The *FANCC* gene was used as an external control, since cell lines were classified as FA-A and no deletions have been described in extensive screening of the *FANCC* gene (Verlander et al. 1994; Gibson et al. 1996). Multiplex assay 1 amplified exons 10–12, 31, and 32 from the *FANCA* gene and exons 5 and 6 from the *FANCC* gene. Multiplex assay 2 amplified exons 5, 17, 35, and 43 from the *FANCA* gene and exons 5 and 6 from the *FANCC* gene. Multiplex assay 3 amplified exons 21 and 27 from the *FANCA* gene and exons 5 and 6 from the *FANCC* gene. All forward primers were labeled with either the fluorescent phosphoramidite 6-FAM or HEX dyes (PE Biosystems). Primer sequences, fluorescent dyes used, and sizes of products are shown in table 2. PCR amplifications were performed in 25 μl reactions with 125 ng DNA, $1 \times$ *Taq* DNA polymerase buffer (as used for RT-PCR), and 200 μM each dNTP. Of each of the primer pairs, 0.2 μM was used for each of the three multiplexes, apart from 0.4 μM for *FANCA* exons 5, 11, 12, and 31. After an initial denaturation at 94°C for 3 min, 1.5 U *Taq* DNA polymerase (Promega) was added (hot start), and 18 PCR cycles of 93°C for 1 min, annealing for 1 min at either 60°C (multiplex 1 and 3) or 58°C (multiplex 2), and extension for 2 minutes at 72°C were performed, followed by a final extension for 5 min at 72°C. Only 18 PCR cycles were performed, to keep the reactions within the exponential phase of PCR (see Yau et al. 1996). An aliquot of the PCR product (4 μl) was added to 3.5 μl formamide loading buffer (95% formamide in $1 \times$ TBE and 5 mg dextran blue/ml) and 0.5 μl internal lane size standard (Genescan-500 Rox; PE Biosystems). The samples were denatured for 5 min at 94°C and electrophoresed on a 5% denaturing polyacrylamide gel at 45 W for 6 h on an ABI 373 fluorescent DNA sequencer. Data were then analyzed by means of Genescan and Genotyper software, to obtain electrophoretograms for each sample. The position of the peaks indicates the size (in bp) of the exons amplified, and the areas under the peaks indicate the amount of fluorescence from the product. Fig-

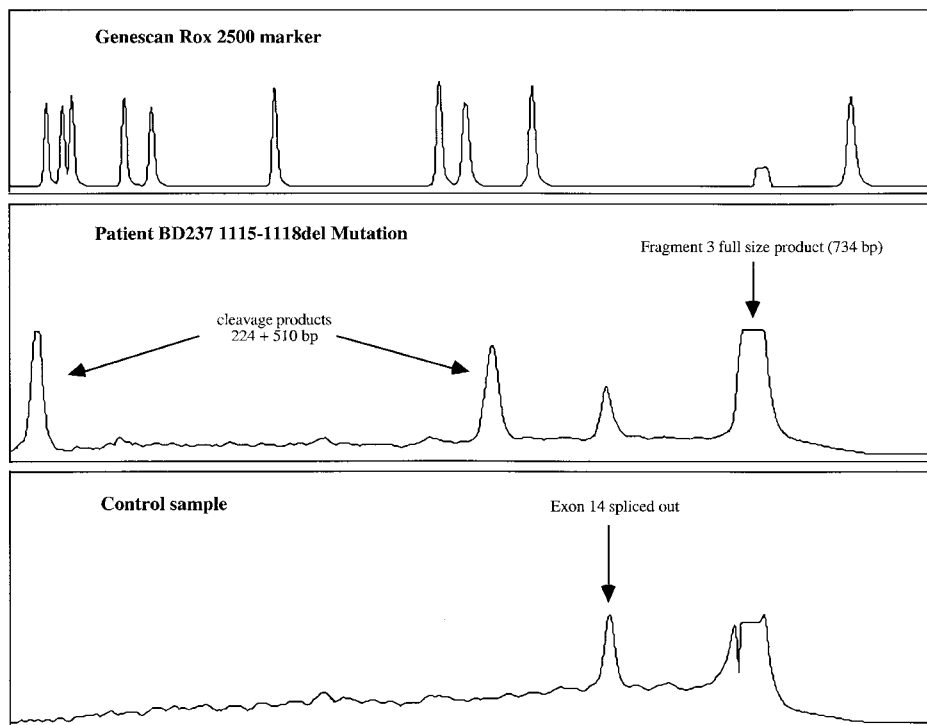


Figure 1 Electrophoretogram showing FCCM analysis of fragment 3 in patient BD237. Both this sample and a control show alternative splicing of exon 14. Rox2500 = MW marker.

Figure 2 shows an example of the electrophoretograms obtained. On one gel, ≤ 24 samples could be amplified and electrophoresed. At least four control DNAs were always included on each gel. The copy number of each exon amplified was established by importing the peak area values into an Excel spreadsheet and calculating a dosage quotient for each exon relative to all the other amplified exons in patients and controls (for an example see table 3). Peak areas from two control samples with approximately equal values were chosen, and the dosage quotient was calculated from them; values are typically within the range 0.77–1.25 (Yau et al. 1996). An average peak area of these controls was taken and compared with the peak area of the exon from the patient samples. As an example, in patient EUFA444 the dosage quotient for *FANCA* exon 10 and *FANCC* exon 5 is given by: $DQ_{FANCA \text{ exon } 10 / FANCC \text{ exon } 5}$ and is calculated by: $[\text{sample } FANCA \text{ exon } 10 \text{ peak area} / \text{sample } FANCC \text{ exon } 5 \text{ peak area}] [\text{control } FANCA \text{ exon } 10 \text{ peak area} / \text{control } FANCC \text{ exon } 5 \text{ peak area}] = [1857/5301] / [6034/8180] = 0.47$. After assessment of which exons were most frequently deleted from *FANCA*, multiplex 4 was developed (see table 2), with exon 1 of the myelin protein zero gene as the external control.

Results

A two-step mutation-screening strategy was devised to detect both small mutations—such as single-base substitutions—and heterozygous deletions of one or multiple exons of the *FANCA* gene. In the first step, the entire coding region was amplified by RT-PCR, and the fragments were sized on agarose gels. Aberrantly sized fragments were characterized by automated sequencing. The loss of an exon or the incorporation of intronic sequence led to the investigation of genomic DNA for splice-site mutations, whereas the loss of multiple exons revealed the presence of intragenic deletions. Full-length transcripts were screened by FCCM for nucleotide substitutions or small deletions and insertions. In the second step, genomic DNA was used to screen for deletions of one or more exons, by a fluorescent quantitative multiplex dosage assay that was based on an approach that we had originally developed to test for deletion and duplication carriers in Duchenne muscular dystrophy (Yau et al. 1996). In this assay, several exons of the *FANCA* gene were coamplified with two control exons from another locus, by use of fluorescently labeled primers, and the products were analyzed on an ABI 373 DNA-frag-

Table 2**PCR Primer Sets for Multiplex Dosage Assays**

Exon	Primers (5'→3')	Size (bp)
Multiplex 1:		
<i>FANCA</i> :		
Exon 10	Forward, GAT TGT AGA AGT CTT GAT GGA TGT G Reverse, ATT TGG CAG ACA CCT CCC TGC TGC	259
Exon 11	Forward, GAT GAG CCT GAG CCA CAG TTT GTG Reverse, AGA ATT CCT GGC ATC TCC AGT CAG	301
Exon 12	Forward, CCA CAA CTT TTT GAT CTC TGA CTT G Reverse, GTG CCG TCC ACG GCA GGC AGC ATG	224
Exon 31	Forward, CAC ACT GTC AGA GAA GCA CAG CCA Reverse, CAC GCG GCT TAA ATG AAG TGA ATG C	205
Exon 32	Forward, CTT GCC CTG TCC ACT GTG GAG TCC Reverse, CTC ACT ACA AAG AAC CTC TAG GAC	369
<i>FANCC</i> :		
Exon 5	Forward, CTG ATG TAA TCC TGT TTG CAG CGT G Reverse, TCC TCT CAT AAC CAA ACT GAT ACA	186
Exon 6	Forward, GTC CTT AAT TAT GCA TGG CTC TTA G Reverse, CCA ACA CAC CAC AGC CTT CTA AG	293
Multiplex 2:		
<i>FANCA</i> :		
Exon 5	Forward, ACC TGC CCG TTG TTA CTT TTA Reverse, AGA ACA TTG CCT GGA ACA CTG	250
Exon 17	Forward, CCC TCC ATG CCC ACT CCT CAC ACC Reverse, AAA AGA AAC TGG ACC TTT GCA T	207
Exon 35	Forward, GAT CCT CCT GTC AGC TTC CTG TGA G Reverse, GCA TTT TCC CTG AGA TGG TAA CAC C	315
Exon 43	Forward, GCC TGG CTG GCA ATA CAA CTC GAC Reverse, GGC AGG TCC CGT CAG AAG AGA TGA G	223
<i>FANCC</i> :		
Exon 5	Forward, CTG ATG TAA TCC TGT TTG CAG CGT G Reverse, TCC TCT CAT AAC CAA ACT GAT ACA	186
Exon 6	Forward, GTC CTT AAT TAT GCA TGG CTC TTA G Reverse, CCA ACA CAC CAC AGC CTT CTA AG	293
Multiplex 3:		
<i>FANCA</i> :		
Exon 21	Forward, CAG GCT CAT ACT GTA CAC AG Reverse, CAC CGG CTT GAG CTG GCA CAG	335
Exon 27	Forward, CAG GCC ATC CAG TTC GGA ATG Reverse, CCT TCC GGT CCG AAA GCT GC	285
<i>FANCC</i> :		
Exon 5	Forward, CTG ATG TAA TCC TGT TTG CAG CGT G Reverse, TCC TCT CAT AAC CAA ACT GAT ACA	186
Exon 6	Forward, GTC CTT AAT TAT GCA TGG CTC TTA G Reverse, CCA ACA CAC CAC AGC CTT CTA AG	293
Multiplex 4:		
<i>FANCA</i> :		
Exon 5	Forward, ACC TGC CCG TTG TTA CTT TTA Reverse, AGA ACA TTG CCT GGA ACA CTG	250
Exon 11	Forward, GAT GAG CCT GAG CCA CAG TTT GTG Reverse, AGA ATT CCT GGC ATC TCC AGT CAG	301
Exon 17	Forward, CCC TCC ATG CCC ACT CCT CAC ACC Reverse, AAA AGA AAC TGG ACC TTT GCA T	207
Exon 21	Forward, CAG GCT CAT ACT GTA CAC AG Reverse, CAC CGG CTT GAG CTG GCA CAG	335
Exon 31	Forward, CAC ACT GTC AGA GAA GCA CAG CCA Reverse, CCC AAA GTT CTG GGA TTA CAG GCG TG	268
Myelin protein zero:		
Exon 1	Forward, CAG TGG ACA CAA AGC CCT CTG TGT A Reverse, GAC ACC TGA GTC CCA AGA CTC CCA G	389

ment analyzer. Heterozygous deletions were detected by a reduction in peak area of *FANCA* exons relative to those in controls. The two-step strategy was used to screen for mutations in RNA and DNA from 26 FA-A cell lines.

Aberrant *FANCA* Transcripts

RT-PCR analysis of the *FANCA* coding region in six overlapping fragments detected abnormally sized transcripts, in seven patients, that were found to be either splice-site mutations or intragenic deletions (all of the mutation data in this study are summarized in table 4). Patients EUFA123 and EUFA137 had reductions in size for fragments 2 and 4, respectively; sequencing of their genomic DNA revealed the splice-site mutations IVS6-2A→G and IVS16+3A→C. In patient EUFA779, fragment 4 contained an insertion of 90 bp of intron 15 sequence upstream of exon 16, and sequencing of genomic DNA around exon 16 from this patient revealed the splice-site mutation IVS15-1G→T. The intronic insertion resulted from the use of a cryptic splice-site in intron 15 and contained a stop codon (TGA) that would cause premature termination of translation. In patient EUFA755, fragment 4 showed no full-length product but did show two smaller products, which were revealed by sequencing to be two different deletions, of exons 15-17 and exons 16-20. PCR analysis of genomic DNA from this patient showed homozygous deletions of exons 16 and 17, which resulted from the overlap of the two intragenic deletions. In patients EUFA006 and EUFA417, reduced-size products in fragment 4 were shown, by se-

Table 3

Statistical Profile of *FANCA* Dosage Multiplex 1

EXON	PEAK AREA IN		DOSAGE QUOTIENT IN	
	Control	EUFA444 ^a	<i>FANCC</i> Exon 5	<i>FANCC</i> Exon 6
Control:				
<i>FANCC</i> exon 5	8180	530199
<i>FANCC</i> exon 6	4490	2949	1.01	...
Test:				
<i>FANCA</i> exon 10	6034	1857	.47	.47
<i>FANCA</i> exon 11	16967	5392	.49	.48
<i>FANCA</i> exon 12	9068	3256	.55	.55
<i>FANCA</i> exon 31	5466	3110	.88	.87
<i>FANCA</i> exon 32	5838	3887	1.03	1.01

^a Heterozygous for a deletion of exons 10-12. For a sample calculation, see Methods subsection.

quencing, to be deletions of exons 15-20 and 16-17, respectively. Heterozygosity for deletions in this region was confirmed by quantitative PCR (see below). In patient EUFA854, a reduction in fragment 5 was a deletion of exons 29-31, again confirmed by quantitative PCR.

A high degree of alternative splicing of the *FANCA* gene was also detected. Exon 14 was commonly spliced out from fragment 3, and exon 30 from fragment 5. Both of these splicing events were seen in RNA prepared from lymphoblastoid control cell lines. An example of the shorter fragment produced in patients and controls, as a result of splicing out of exon 14, is shown in the electrophoretogram from the FCCM analysis in figure 1. Various other alternative splicing events were seen

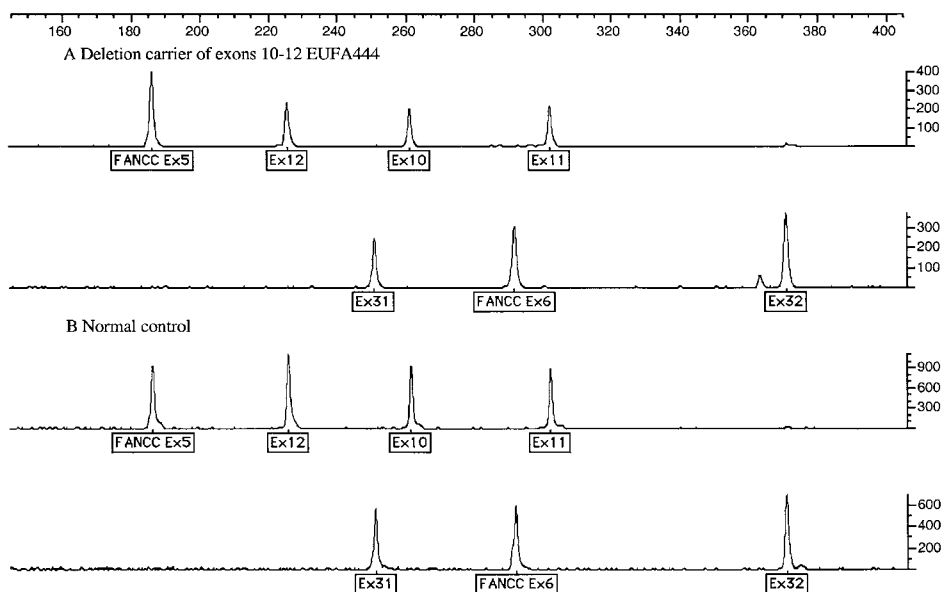


Figure 2 Electrophoretograms showing a deletion carrier of exons 10-12, detected with *FANCA* gene dosage multiplex 1

that, on sequencing, either produced mixed unreadable sequence or, by the detection of a mutation elsewhere in the gene, were excluded as the pathogenic mutation. For example, a transcript lacking exons 21-26 was seen in patient BD255, but this deletion was also present in controls, and both mutant alleles were found in other RT-PCR fragments from this patient.

FCCM Analysis

A total of 18 mutant alleles were detected by screening full-length transcripts from the six overlapping fragments of the *FANCA* coding region by FCCM, and these are listed in table 4. An example of the electrophoretic profiles produced by this analysis is shown in figure 1. Since PCR products are internally labeled by incorporation of fluorescent dUTP, the sizes of the two cleavage products accurately define the position of the mutation, which is then confirmed by sequencing of genomic DNA from the region. All 18 mutant alleles were detected by modification with hydroxylamine, which modifies only mismatched C bases. However, since both the probe and

target were fluorescently labeled, and since biotinylated forward and reverse primers were used for each fragment, mutations such as A→G are detected as a mismatched C on the reverse strand. Although modification was also carried out with osmium tetroxide, no further mutations were found. The use of hydroxylamine alone has been shown to detect ≤95% of all mutations (Rowley et al. 1995; Ellis et al. 1998). The procedure also detected numerous coding-sequence polymorphisms, including 1143G→T, 1235C→T, 1290G→A, 1501G→A, 1927C→G, 2151G→T, 2426G→A, and 4152G→C. These have been excluded as pathogenic mutations in other studies (Levrán et al. 1997; Savino et al. 1997; Wijker et al. 1999; Fanconi Anemia Mutation Database) and have served as useful positive controls for the chemical cleavage.

Quantitative Fluorescent Multiplex PCR

Three separate quantitative multiplex PCR assays were established that collectively screened for heterozygous deletions in 11 exons across the *FANCA* gene.

Table 4

Mutations Detected in the *FANCA* Gene of FA Patients

Patient	Mutation 1	Effect	Exon(s)	Method of Detection	Mutation 2	Effect	Exon(s)	Method of Detection
EUFA353	154C→T ^a	R52X	2	FCCM	*5' UTR-522*del	Deletion	*5' UTR-5*	Dosage
BD237	*5' UTR-522*del	Deletion	*5' UTR-5*	Dosage	1115-1118del ^d	Frameshift	13	FCCM
EUFA1030	*427-522*del	Deletion	*5*	Dosage	987-990del ^b	Frameshift	11	Dosage
EUFA444	*5' UTR-1900*del	Deletion	*5' UTR-21*	Dosage	1115-1118del ^d	Frameshift	13	FCCM
EUFA750	*5' UTR-3066del	Deletion	*5' UTR-31	Dosage	3788-3790del ^{a,b}	1263delF	38	FCCM
BD812	*5' UTR-3066del	Deletion	*5' UTR-31	Dosage	Not detected			
EUFA651	523-1359del	Deletion	6-14	Dosage	Not detected			
EUFA123	IVIS6-2A→G ^a	Del13AA	I6	RT-PCR	Not detected			
EUFA186	597-1826del	Deletion	7-20	Dosage	Not detected			
BD220	862G→T	E288X	10	FCCM	Homozygote			
BD255	862G→T	E288X	10	FCCM	4015delC	Frameshift	41	FCCM
EUFA056	1007-3066del	Deletion	12-31	Dosage	1827-2778del	Deletion	21-28	Dosage/exon PCR
EUFA763	1191-1194del ^a	Frameshift	13	FCCM	3971C→T	P1324L	40	FCCM
EUFA755	1360-1626del	Deletion	15-17	Dosage/RT-PCR	1471-1826del	Deletion	16-20	Dosage/RT-PCR
EUFA006	1360-1826del ^c	Deletion	15-20	RT-PCR/Dosage	3788-3790del ^{a,b}	1263delF	38	FCCM
EUFA779	IVS15-1G→T	Splicing	16	RT-PCR	Not detected			
EUFA417	1471-1626del ^{d,e}	Deletion	16-17	RT-PCR/Dosage	3788-3790del ^{a,b}	1263delF	38	FCCM
EUFA137	IVS16+3A→C	Exon skip	i16	RT-PCR	Not detected			
EUFA057	1771C→T ^b	R591X	19	FCCM	Homozygote			
EUFA134	1792G→A ^a	D598N	20	FCCM	1627-1900del	Deletion	18-21	Dosage
EUFA854	2779-3066del	Deletion	29-31	Dosage/RT-PCR	Not detected			
EUFA583	*2982-4365del	Deletion	*31-43	Dosage	Not detected			
BD32	3329A→C	H1110P	33	FCCM	Homozygote			
EUFA413	3403-3405del	1135delF	34	FCCM	Not detected			
BD460	3629-3630insT	Frameshift	37	FCCM	Homozygote			
EUFA097	3786C→G	F1262L	38	FCCM	4080G→C	M1360I	41	FCCM

NOTE.—An asterisk (*) denotes that the deletion endpoint is undefined.

^a Previously described by Wijker et al. (1999).

^b Previously described by Levrán et al. (1997).

^c Previously described by Lo Ten Foe et al. (1996).

^d Previously described by Fanconi Anaemia/Breast Cancer Consortium (1996).

^e Previously described by Levrán et al. (1998).

Exons were selected on the basis of robust amplification in multiplex PCR and by noting which exons were most frequently deleted as the work proceeded, and they included 5, 10–12, 17, 21, 27, 31, 32, 35, and 43. In these assays, the peak area of each exon is measured against all other exons in the multiplex, including two exons from a different locus, to produce a series of dosage quotients (for details, see Methods subsection). A typical electrophoretogram and a typical statistical profile of this analysis are shown in figure 2 and table 3, respectively. Genomic DNA from the 26 cell lines was used to screen for intragenic deletions by use of these three multiplex PCR reactions. A total of 17 large deletions were detected, which removed 1–31 exons of the *FANCA* gene (see table 4 and fig. 3). The multiplex assay also detects microdeletions within an exon, by means of size discrimination; in this way, patient EUFA1030 was found to be heterozygous for a 4-bp deletion in exon 11. The deletion data in figure 3 indicate that all deletions detected in the FA-A patients included one or more of the following five exons: 5, 11, 17, 21, and 31. A further multiplex was therefore designed that includes these five exons and exon 1 of the Myelin protein zero gene as an external control (see Methods subsection and table 2). This one multiplex, instead of the three other assays, can now be used for *FANCA* deletion screening.

RT-PCR analysis and polymorphic marker typing were used to map the approximate endpoints of the deletions. In six patients the most 5' exon deleted was exon 5 (table 4). RT-PCR assays were therefore designed to amplify from exon 1 of the gene to a downstream exon known, from the dosage assay, to be present. These assays did not produce smaller transcripts, which suggested that the deletion endpoints extended upstream of the primer site in exon 1. A PCR was then carried out on genomic DNA, with a primer upstream of the *FANCA* 5' UTR and a primer 3' to the deletion, but no products were detected, again suggesting that the deletion extended beyond the promoter. The extent of these

deletions was further investigated by typing the microsatellite polymorphic marker D16S3407, which is located 16 kb upstream of the 5' UTR of the gene (Whitmore et al. 1998), in these patients and, if available, in parental samples. Since five of the six patients typed were homozygous for the marker (which has a heterozygosity of .77), the deletions may extend even farther upstream of the *FANCA* gene. This was confirmed in two of these five patients (EUFA353 and BD812), since parental DNA was available and informative with this marker, and both patients showed lack of inheritance of the parental allele that was linked to the deletion. The endpoints of deletions in EUFA cell lines 134, 186, 417, 651, 755, and 854 were defined by nested RT-PCR with primers outside the exons found to be deleted by the dosage assays followed by sequencing of the products (see table 4). The precise breakpoints of the exon 16–17 deletion in EUFA417 are different from the two deletions of these exons reported by Levrant et al. (1998), since primers for those breakpoints did not generate the expected PCR products. In EUFA056, the endpoints of one deletion could be deduced as exon 21–28 since exons 21 and 28 were homozygously deleted and exons 20 and 29 were present; the other mutant allele in this patient is a deletion of exons 12–31, which was precisely defined in the dosage assay and overlaps the exon 21–28 deletion. The endpoint of the deletion in EUFA583 could not be defined, since the deletion extended beyond the 3' UTR of the gene.

The analysis of *FANCA* deletion endpoints in this series of patients suggests at least four common breakpoints; there were four breaks in intron 31, four in intron 20, and up to five in the region 5' to the gene (table 4). In view of the involvement of *Alu*-repeat elements in the generation of three published *FANCA* deletions (Centra et al. 1998; Levrant et al. 1998), we searched the complete genomic sequence of the *FANCA* gene (AC005565, AC005567, and AC005360) for *Alu* repeats and compared the number of repeats with the number of known

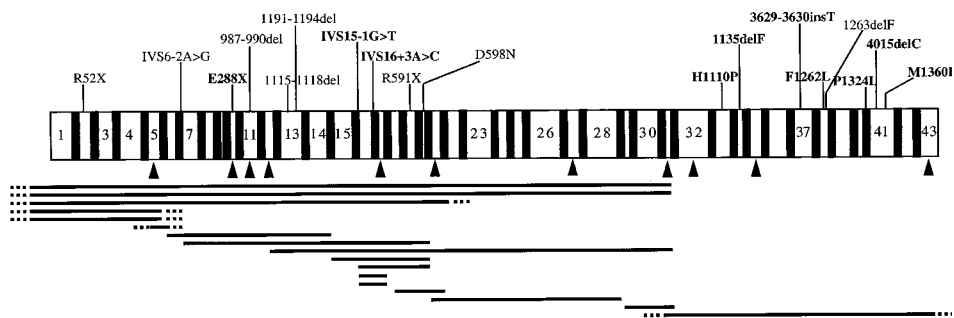


Figure 3 Mutations detected in the *FANCA* gene in patients with FA. The figure is a schematic representation of the 43 exons of the *FANCA* gene. Novel mutations are shown in boldface. Large deletions are indicated by horizontal lines, which are dotted where endpoints are undefined. ▲ = Exons included in dosage multiplex assays.

deletion breakpoints that we have detected in this and a previous study (Wijker et al. 1999). The results of this analysis (fig. 4) show that the most common breakpoints all occur in genomic regions that contain four or more *Alu* repeats and that none of the breakpoints occur in introns with fewer than two repeats. The presence of a deletion breakpoint correlated strongly with the presence of an *Alu* repeat ($P < .001$, Fisher's exact test), and linear regression analysis showed a highly significant correlation between the number of *Alu* repeats and the number of breakpoints detected ($P = 2.8 \times 10^{-5}$).

FANCA Mutation Spectrum

A total of 33 different mutations were identified in this study, 23 of which were novel (fig. 3). This very heterogeneous spectrum included three splice-site mutations, two in-frame deletions of single-amino acid codons, three nonsense mutations, five missense mutations, five microdeletions or insertions that caused frameshifts, and 15 different large intragenic deletions (table 4 and fig. 3). The large intragenic deletions accounted for 17 (40%) of the 43 mutant alleles, making them by far the most common class of mutation in the *FANCA* gene. No single mutation was common, but E288X was found in two unrelated British patients, and 1263delF, which was originally described by Levrant et al. (1997), was found in two French patients and one German patient. The pathogenic status of the five missense mutations was verified initially by confirming their cosegregation with

the disease phenotype in the family and their absence in 100 ethnically matched control chromosomes. D598N is a nonconservative substitution that alters an amino acid residue that is conserved in the mouse *Fanca* gene (H. van de Vrugt, personal communication). H1110P is a nonconservative change that is not conserved in the mouse but has been shown to be nonfunctional in a cellular complementation assay (Kupfer et al. 1999). The other three (F1262L, P1324L, and M1360I) are conservative changes, but the altered residues are all conserved in the mouse. The sensitivity of the two-step mutation-screening assay can be deduced from the fact that at least one mutation was detected in all 26 FA-A cell lines tested, and 43 (83%) of the possible 52 mutant alleles were found.

Discussion

The modest detection rates reported from mutation screening of the *FANCA* gene, coupled with several reports of intragenic deletions (Centra et al. 1998; Levrant et al. 1998; Wijker et al. 1999), led us to investigate whether these findings were connected. We therefore devised a mutation-screening protocol that would detect both small mutations, such as point mutations and microdeletions or insertions, and gross deletions that were present in the heterozygous state. The protocol was used to screen RNA and DNA from 26 cell lines that had been selected solely on the basis of their having been shown, by complementation analysis, to be FA group A.

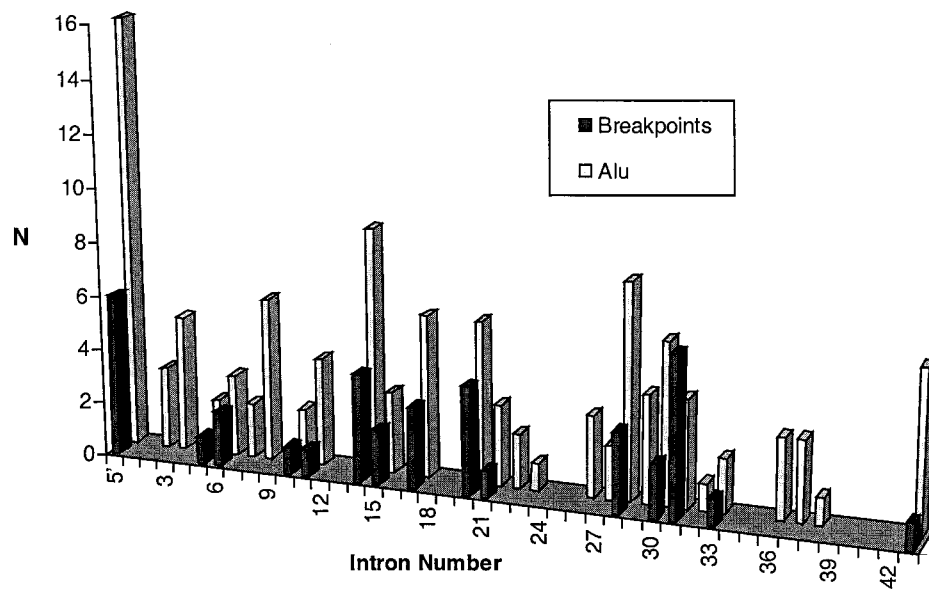


Figure 4 *FANCA* deletion breakpoints and *Alu* repeats. The number of deletion breakpoints includes those defined in this study and in the study by Wijker et al. (1999). The number of *Alu*-repeat elements shown for the 5' end of the gene is for the sequence from the microsatellite D16S3407 to the initiation codon of *FANCA*. The number for the 3' end is for 3 kb of sequence downstream of the stop codon.

The quantitative PCR assay detected 17 deletions of the *FANCA* gene in these cell lines, which accounted for 40% of the mutant alleles observed. This result suggests that gross deletions of one or more exons of the *FANCA* gene are the most common form of mutation in this gene. The endpoints of 10 of the 17 deletions were established by RT-PCR and sequencing (table 4 and fig. 3). In six of the other deletions, genomic PCR with a primer from the *FANCA* 5' UTR showed that the deletion is likely to extend upstream of the gene, and five patients were apparently homozygous for a highly informative microsatellite polymorphism, D16S3407, that lies 16 kb upstream of the initiation codon (Whitmore et al. 1998). This suggests that the deletion extends beyond this marker in at least some of these, as confirmed by family studies with this marker in two cases. In one of these cases, BD812, the deletion extends to exon 31 and must therefore remove ≥ 80 kb of DNA. The remaining deletion extends 3' to the last exon of *FANCA*, exon 43. The presence of several common breakpoints in this series of deletions and the evidence that three fully characterized deletions of *FANCA* appear to result from *Alu*-mediated recombination (Centra et al. 1998; Levrán et al. 1998) led us to compare the breakpoints of all the deletions that we have detected in this and another study (Wijker et al. 1999) with the location of *Alu* repeats in *FANCA* that were obtained from its complete genomic sequence. This analysis (fig. 4) revealed a highly significant correlation between the breakpoints and the presence of multiple repeats and provides strong experimental support for the suggestion that this may be a common method for the generation of *FANCA* deletions (Centra et al. 1998; Levrán et al. 1998). The frequency of these repeats in *FANCA* would then explain the high prevalence of gross deletions in its mutation spectrum. It might also indicate a higher mutation rate for *FANCA* as compared to other FA genes, which would explain why FA-A is by far the most common complementation group in this disorder (Joenje et al. 1997).

The two-step mutation-screening protocol that we developed in this study detected at least one mutation in all 26 cell lines screened and 43 (83%) of the expected 52 mutant alleles. This compares well with the results of other published studies. Savino et al. (1997) detected mutations in only 29% of Italian patients with FA, although complementation analysis showed that the prevalence of FA-A in Italy was $>90\%$ (Savoia et al. 1996). Levrán et al. (1997) found mutations in 69% of patients unselected for complementation group, but 35% of these mutations were amino acid substitutions of unknown pathogenic status, and Wijker et al. (1999) detected mutations in 49% of probable FA-A patients. The highest detection rate was reported by Tachibana et al. (1999), who found mutations in 12 of 15 Japanese FA patients by direct sequencing; 22 (73%) of a possible 30 mutant

alleles were detected. None of these studies included quantitative analysis of *FANCA* exon copy number. Our finding that 40% of the mutant alleles detected in 26 FA-A cell lines were heterozygous deletions of multiple exons suggests that mutation-detection rates can be substantially improved by incorporation of a quantitative assay into screening protocols. Since all of the mutations observed in our study involved one or more of exons 5, 11, 17, 21, and 31, we replaced the three multiplex PCRs that we developed initially by a single multiplex that incorporated these five exons and a control exon from an unrelated gene. This could serve as a useful and rapid initial molecular screen for FA in patients of unknown complementation group or of uncertain diagnosis.

The combination of FCCM and dosage analysis failed to detect 9 (17%) of a possible 52 mutant alleles. There are several possible explanations for this. The undetected mutations could be intragenic deletions of exons that were not included in the dosage multiplexes; collectively, the three multiplexes screened only 11 of the 43 exons, and the RT-PCR analysis would not detect all such deletions—for a variety of reasons, including the “noise” created by numerous alternative splicing events. The undetected mutations could also be complex genomic rearrangements, such as duplications or inversions, or they might conceivably be located in regions of the gene that were not screened in our protocol, such as the promoter or 3' UTR. Finally, a small proportion of point mutations are not detected by FCCM if they are located in particular sequence contexts (Rowley et al. 1995; Ellis et al. 1998). Ultimately, no mutation-screening protocol is perfect, and a judgment has to be made as to whether a large amount of additional effort and expense can be justified in order to achieve 100% sensitivity of detection.

The very heterogeneous nature of the mutation spectrum in the *FANCA* gene is exemplified in this study: 33 different mutations were found in the 26 patients, of which 23 were novel mutations. The most interesting mutations, from the perspective of an attempt to describe the functional architecture of the *FANCA* protein, are those which do not cause premature termination of translation, such as the missense mutations and the deletions of single-amino acid codons. Almost all of these are clustered in the carboxy-terminal region of amino acid residues 1110–1360 (table 4), which may reflect the presence of an important functional domain involved in protein-protein interactions or in transport to the nucleus. In this group, only H1110P has, as yet, been confirmed as disrupting the function of the *FANCA* protein (Kupfer et al. 1999). Introduction of the others into the wild-type cDNA is in progress, in order to test them for absence of functional complementation.

Finally, the high sensitivity of the two-step screening procedure described in this article suggests that it may

be usefully applied to mutation detection in other disorders. The utility of the multiplex quantitative assay for detection of deletions in the heterozygous state could be exploited to search for gross deletions in autosomal dominant disorders such as familial breast cancer, since evidence for such deletions in the BRCA1 gene is beginning to emerge (Puget et al. 1999). Also, in view of the general importance of *Alu*-mediated deletions in human genetic disorders (Purandare and Patel 1997) and the increasing availability of the complete genomic sequence of human genes, an initial electronic search for the location and density of such repeats may be a useful prelude to the design of mutation-screening strategies for a specific gene.

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Electronic-Database Information

URLs for data in this article are as follows:

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim>
 Fanconi Anemia Mutation Database, <http://www.rockefeller.edu/fanconi/mutate>

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