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Mutations of the Fanconi Anemia Group A Gene (*FAA*) in Italian Patients

Maria Savino,[1] Leonarda Ianzano,[1] Pierluigi Strippoli,[2] Ugo Ramenghi,[3] Araxy Arslanian,[4] Gian Paolo Bagnara,[2] Hans Joenje,[5] Leopoldo Zelante,[1] and Anna Savoia[1]

[1]Servizio di Genetica Medica, IRCCS–Ospedale CSS, San Giovanni Rotondo, Foggia, Italy; [2]Istituto di Embriologia e Istologia, Università di Bologna, Bologna; [3]Ematologia, Dipartimento di Scienze Pediatriche e dell'Adolescenza, Università di Torino, Turin; [4]Centro Regionale di Genetica Umana, E. O. Galliera, Genoa; and [5]Department of Human Genetics, Free University, Amsterdam

Summary

Fanconi anemia (FA) is an autosomal recessive disease characterized by progressive pancytopenia, congenital malformations, and predisposition to acute myeloid leukemia. At least five complementation groups (FA-A–FA-E) have been identified. The relative prevalence of FA-A has been estimated at an average of ~65% but may widely vary according to ethnic background. In Italy, 11 of 12 patients analyzed by cell-fusion studies were assigned to group FA-A, suggesting an unusually high relative prevalence of this FA subtype in patients of Italian ancestry. We have screened the 43 exons of the *FAA* gene and their flanking intronic sequences in 38 Italian FA patients, using RNA-SSCP. Ten different mutations were detected: three nonsense and one missense substitutions, four putative splice mutations, an insertion, and a duplication. Most of the mutations are expected to cause a premature termination of the FAA protein at various sites throughout the molecule. Four protein variants were also found, three of which were polymorphisms. The missense mutation D1359Y, not found in chromosomes from healthy unrelated individuals, was responsible for a local alteration of hydrophobicity in the FAA protein, and it was likely to be pathogenic. Thus, the mutations so far encountered in the *FAA* gene are essentially all different. Since screening based on the analysis of single exons by genomic DNA amplification apparently detects only a minority of the mutations, methods designed to detect alterations in the genomic structure of the gene or in the FAA polypeptide may be helpful in the identification of *FAA* mutations.

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Address for correspondence and reprints: Dr. Anna Savoia, Servizio di Genetica Medica IRCCS–Ospedale CSS, I-71013 San Giovanni Rotondo, Italy. E-mail: genetcss@fg.nettuno.it

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Introduction

Fanconi anemia (FA) is an autosomal recessive disease characterized by progressive bone-marrow failure, various birth defects, and a markedly increased incidence of malignancies (Butturini et al. 1994). Cells cultured from FA patients exhibit an increased level of spontaneous chromosomal aberrations and are hypersensitive to the clastogenic effect of cross-linking agents, such as mitomycin C and diepoxybutane (DEB). The latter feature is used to confirm the FA diagnosis (Auerbach 1993).

FA is genetically heterogeneous: at least five complementation groups (FA-A–FA-E) have been identified (Strathdee et al. 1992*a;* Joenje et al. 1995), each group presumably corresponding to a separate disease gene (*FAA–FAE*) (Buchwald 1995). The relative prevalence of the FA-A subtype has been estimated to be 60%–66%, as determined by complementation analysis and homozygosity mapping (Buchwald 1995; Gschwend et al. 1996). The relative prevalence of FA-A is unusually high in Italy, since 11 of 12 Italian patients analyzed by complementation studies were type FA-A (Savoia et al. 1996).

FAC, which maps to chromosome 9q22.3, was the first gene to be cloned (Strathdee et al. 1992*a;* 1992*b*) whereas *FAA* has only recently been identified (FAB Consortium 1996; Lo Ten Foe et al. 1996*a*). The *FAA* gene has an open reading frame of 4,365 bp that encodes a protein of 1,455 amino acids. It contains 43 exons and spans ~80 kb of genomic DNA on chromosome 16q24.3 (Ianzano et al. 1997), between microsatellites D16S3026 and D16S303 (Pronk et al. 1995; FAB Consortium 1996). *FAA* and *FAC* have no significant homology to any known proteins or to each other and may therefore represent component elements of a novel pathway(s) involved in the prevention of DNA damage.

To date, eight different mutations of the *FAA* gene have been identified, by use of patient RNAs, by SSCP and the protein-truncation test. Five were deletions of one or more exons, which are probably genomic deletions. Another was a TTGG deletion in exon 13, and two were substitutions at different residues in the donor

splice site of intron 7, leading to the utilization of a downstream cryptic splice signal. All, except for the in-frame deletion of exons 16 and 17, caused a shift of the open reading frame and a premature truncation of the FAA protein (FAB Consortium 1996; Lo Ten Foe et al. 1996*a*).

In order to screen the *FAA* gene for mutations from genomic DNA, intronic sequences were used to set up PCR and to amplify the 43 exons in 38 patients of Italian ancestry. Ten new mutations and three polymorphisms were identified by RNA-SSCP.

Patients and Methods

Patients

Thirty-eight unrelated FA patients, all of Italian ancestry, had been previously diagnosed on the basis of clinical symptoms in combination with the hypersensitivity to DEB, as determined in a standard chromosomal-breakage test (Auerbach 1993). Lymphoblastoid cell lines were established from 15 of these patients. DNA samples was extracted from cell lines or peripheral blood and were designated, respectively, with "VU" and "FA" numbers. Except for VU578, patients designated as "VU" had been previously reported with clinical symptoms and results from a functional complementation analysis, and 11 of them had been assigned to group FA-A (Savoia et al. 1996).

RNA-SSCP and Direct Sequencing

High-molecular-weight DNA was prepared from peripheral blood or lymphoblast cell lines by use of proteinase K (Sambrook et al. 1989). The *FAA*-gene exons containing the coding region and the flanking regions (Ianzano et al. 1997) were amplified by use of a set of oligonucleotides shown in table 1. PCR was performed in 50 μl containing 50 ng of DNA, 15 pmol of each primer, 2.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 0.01% Tween-20, 0.01% gelatin, 0.01% NP40, and 2 units of *Taq* polymerase. Initial denaturation was for 6 min at 85°C, followed by amplification for 30 cycles, each with denaturation for 30 s at 94°C, annealing for 30 s at the temperature indicated in table 1, and extension for 30 s at 72°C. The amplified products were then used for RNA-SSCP screening (Sakar et al. 1992). Transcription was performed with 14 U of T7 RNA polymerase in a final volume of 10 μl containing 1 × buffer TSC (Promega), 5 nmol of each ribonucleoside, 10 U of RNasin, and 0.2 μl of [α-^{35}S]UTP (>1,000 Ci/mmol), and the product was loaded onto a nondenaturing polyacrylamide gel containing 10% glycerol. Direct sequencing was performed either according to the methods recommended by United States Biochemicals or by use of a fluorescence-labeled dideoxy-nucleotide termination method (dye terminator) in an automated DNA sequencer 373A (ABI).

cDNA Synthesis and PCR

Total RNA from lymphoblasts was prepared by use of guanidinium thiocyanate and by layering the cell homogenate on a cushion of a dense solution of CsCl (Chirgwin et al. 1979). RNA was transcribed by use of 2.5 μM random hexamer, 5 × buffer (BRL kit), 10 M DTT, 500 μM dNTP, 1 U of RNase inhibitor/μl, and 10 U of reverse transcriptase (RT)/μl, for 1 h at 37°C. PCR for the detection of altered splicing was performed as above, with the following two pairs of primers: 597F (5′-C CAT CCC GAC ATG CAT GCT G-3′) (exon 7) and 1011R (5′-AG ATG CTT TCA GCA CAG GGC-3′) (exon 11–12), for the skipping of exon 9, and 2609F (5′-TC CTC ATG TTC AGA TTG TTC-3′) (exon 28) and 2974R (5′-G GAA ATC CAT CAG TGC GTT G-3′) (exon 30), for the alternative cleavage of exon 28. PCR products were purified, subcloned into the pCR™ vector (Invitrogen), and sequenced by dye-primer cycle–sequencing method in an automated DNA sequencer 373A (ABI).

Results

Mutation analysis of the *FAA* gene was performed in 38 FA Italian patients. All patients were diagnosed on the basis of clinical symptoms, such as bone-marrow failure, congenital malformations, and hypersensitivity to DEB, as determined in a standard chromosomal-breakage test (Auerbach 1998). Eleven of these patients belonged to group FA-A, as determined by complementation analysis (Savoia et al. 1996).

Since complementation analysis is a technically demanding and time-consuming procedure, a linkage analysis might be performed to exclude unclassified families from complementation subtypes FA-A and FA-C , by use of markers localized at chromosomes 16q24.2 and 9q22.3, respectively. Of the 27 unclassified families, 11 were eligible for linkage studies, and results were compatible with linkage on chromosome 16q24.3. Three of these families were also excluded from subtype FA-C and, given the high relative prevalence of FA-A in Italy (Savoia et al. 1996), were likely to be group FA-A (Savoia et al. 1997). The other 16 FA DNAs—8 from singleton families and 8 from FA patients whose parents' DNAs were not available—were also included in this study, since most of these patients were likely to be FA-A.

Each exon of the *FAA* gene was amplified from genomic DNA and was analyzed by RNA-SSCP. With this approach, several instances of sequence variations in the

Table 1

Primers Used to Amplify *FAA* Exons

Exon	Primer Sequence[a] (5′→3′) Forward	Reverse	Annealing Temperature (°C)	Product Size[b] (bp)
1	T7-gccgcagccaataggaaggc	gatcggggaaccggcgaaaccg	60	241
2	T7-tttcgaaccgacttctctcc	gaactcccgggctcaggcgac	60	303
3	ggagcttgaatttttttaaatgg	T7-gtcacacaaacatcccatag	56	315
4	gtgctgcatcttaaaataaggc	T7-aacgggcaggtttcctcatc	56	300
5	gcaaagatgaggaaacctgc	T7-ctgttgcctccatccagatc	56	304
6	ggcatggttaagaacctaag	T7-aaacccgtctgattctggg	56	374
7	tgatgggatttagttgagcc	T7-tgagttttacccaagaaccc	58	221
8	T7-tgaagtggatggtctgtgcc	acacttggaataaggacggct	56	241
9	T7-attctcttgtgtgatgcagg	tacctcaaatggaaaggcag	56	219
10	tgaagtggatctgttgtctga	T7-taatttggcagacacctccc	58	241
11	T7-tttcctgacccctctttctg	agcgttaccaaggcaacaagg	56	239
12	cccacaacttttttgatctctg	T7-agacatccctgaacctctcg	56	267
13	gtcatctgtcgcacattggtt	T7-tacacacactcttgaccac	58	267
14	ggaatacttgatcacccagc	T7-aaagctgacagcaaggttgc	58	361
15	T7-tctctccacacaggacactg	ttggggaggccaaggcagtc	60	289
16	agcactgtggatgttggaag	T7-agtttctgctgggacaggtg	60	257
17	tcccagcagaaactgctttg	T7-gaggcaagaccagacatgag	57	254
18	acaaggaagcacaggttgag	T7-acatactgcaggcatcagag	56	275
19–20	tccactctctgaaacaccgg	T7-acgattcttcgcattgtcag	56	375
21	caggctcatactgtacacag	T7-agcacaacagacactcaag	58	304
22	ttgagtgtctttccatctgg	T7-agccacagagctccaaccac	58	239
23	tctctaggccgcagctgtat	T7-ctaatgcctctgcctaatgg	56	291
24	T7-caaagcagaattgcgatgtc	cggagacgagctcatgagtcc	56	290
25	ggactgaatggctagtactg	T7-acgaattgagaagtagcagc	58	229
26	T7-attcgtcttcagtgccctgg	atcaaacgagcatgtgtcact	58	350
27	ccatccagttcggaatgcact	T7-agctgcgtaaacctgaaacg	58	286
28	tgctcagccactcacagtg	T7-agacagtcggcacacac	60	412
29	ggaactctcagctgcaatttc	T7-ccagggtagctcttttcaac	56	261
30	T7-ctgtgtgtcccttactatgg	tgtccctccagagaacccta	53	285
31	T7-aggagaactgggaacttcag	cacgcggcttaaatgaagtg	58	224
32	ccgactggctgggcagtgag	T7-ctaggaccgtcatgagatgct	58	322
33	cttgggaaggagcagagtgt	T7-ttcacacggtcagtacacgc	58	285
34	agcagccactctgcatgttg	T7-ggtttgtgaggacccacaac	58	265
35	tcctgtgagttccttcactc	T7-catccccaaaacagaacacc	58	240
36	ctgtagtggcctgtaggagca	T7-agcaagccagggtgtttagg	58	322
37	aggacttggtttctatggcg	T7-ctgattgaaaccaagcttgc	58	285
38	T7-gaatgacagcacaggtaatt	gctcctgagctagtctggaa	58	242
39	agcaccaggtcggccattct	T7-tgcaagatgcctctg	56	233
40	ccagggtacacgtcccgacc	T7-cagacaacccttcccatctg	58	284
41	T7-gtgctacctttccagcagtc	ccatagtctgcatgctgt	56	325
42	acagcatgcagactatgg	T7-ggcagctgtcaattctcatg	60	251
43	T7-ctgtggtctgaggcctagtg	cactaaagcagtcgaggaga	60	300

[a] T7 = T7 tail (TAA TAC GAC TCA CTA TAG GG).
[b] Includes 20-bp T7 tail.

FAA gene were detected: three nonsense mutations, four alterations in splice sites, a duplication, an insertion, and four missense substitutions (tables 2 and 3).

Nonsense Mutations

Three C→T transitions at positions 790, 2005, and 2314 led to the substitution of a glutamine with a stop codon and, therefore, to a premature truncation of the FAA protein in exons 8, 22, and 25, respectively, in three patients.

The only child of a consanguineous marriage (VU388) was homozygous for the mutation 790C→T (Q264X). The second patient, FA38, was a compound heterozygote for a paternally transmitted mutation, 2005C→T (Q669X). The transition 2314C→T (Q772X), homozygous in patient VU268, created a restriction site for *Mae*I; restriction analysis of the family members showed that both parents carried the substitution (fig. 1). The results were consistent with haplotype data, since the affected sib presented autozygosity at six microsatellite

Table 2

Mutations of the *FAA* Gene

Patient	Complementation Group[a]	DNA[b]	RNA/Protein	Protein	Segregation[c]
VU388[d]	FA-A	790C→T (homozygous)	Q264X	8	P and M
VU330	FA-A	IVS9+3delA (homozygous)	793del34–frameshift	9	P and M
VU262	FA-A	IVS10+1G→T (heterozygous)	ND	10	ND
FA25[d]	Non–FA-C	IVS14+1G→C (homozygous)	ND	14	M
FA38	ND	2005C→T (heterozygous)	Q669X	22	P
VU268[d]	FA-A	2314C→T (homozygous)	Q772X	25	P and M
VU578/FA16	Non–FA-C	IVS28+83C→G (homozygous)	926ins28aa+Stop	28	P
FA19	ND	2831dup2812-2830 (heterozygous)	Frameshift	29	P
VU393	Non–FA-C	3559insG (homozygous)	Frameshift	36	P and M
FA37	ND	3559insG (heterozygous)	Frameshift	36	ND
FA35	ND	4075G→T (heterozygous)	D1359Y	41	ND

[a] Patients were classified as FA-A by complementation analysis (Savoia et al. 1996) and as non–FA-C by linkage exclusion on chromosome 9q22.3 (Savoia et al. 1997); ND = not determined by either complementation or linkage analysis (i.e., singleton families or parents' DNA not available).

[b] The A of the ATG of the initiator Met codon is reported as nucleotide +1.

[c] P = paternal transmission of mutation; M = maternal transmission of mutation; and ND = not determined.

[d] Patient had homozygous microsatellites surrounding the *FAA* locus.

loci, from D16S413 to the telomere of chromosome 16q24.3 (Savoia et al. 1997).

Splice-Site Mutations

Four putative splice-site mutations were identified in the donor splicing site of introns 9, 10, 14, and 28. In order to verify the alterations at the RNA level, the cDNA, when available, was amplified and sequenced.

Patient VU330 was found to be homozygous for an A deletion (IVS9+delA3) in the donor splice site of exon 9. The prediction of the donor splice sites was calculated by the scores for both the wild-type TTgtaagt signal and the mutated TTgtagtt signal, which were 79 and 54, respectively (Shapiro and Senapathy 1987; Ianzano et al. 1997). To determine whether the deletion was responsible for a defective cleavage of intron 9, RT-PCR was performed with primers in exons 7 and 11. A product of reduced size, corresponding to a 34-bp deletion of exon 9, was detected in the patient (fig. 2). The faint major band detected in all samples corresponded to the insertion of an alternatively spliced exon (exon 10a; L. Ianzano, unpublished data). The absence of exon 9 created a shift in the open reading frame and a stop codon 19 amino acids downstream. Sequence analysis revealed the mutation to be present in both parents but not in the unaffected sister. The patient was, however, heterozygous for all the informative microsatellite markers at 16q24.3, suggesting that either the mutation occurred independently on two different chromosomes or that several recombination events had rendered the founder haplotype unidentifiable.

Patient VU262 was heteroallelic for a G→T transversion at the donor site of intron 10 (IVS10+1G→T). The mutation was not inherited by the mother, who was not a carrier (the father's DNA was not available), and it was transmitted to the affected brother as well. The mutation was expected to destroy the splice site and effectuate either the skipping of exon 9 or the recognition of

Table 3

Polymorphisms in the *FAA* Gene

Polymorphism[a]	Restriction Site Abolished	Change in Protein Sequence	Exon	Frequency of Novel Allele
796A/G	*Mae*III	T/A266	9	.29
1501G/A	*Msp*I	G/S501	16	.40
2426G/A	*Bst*NI	G/D809	26	.30

[a] The A of the ATG of the initiator Met codon (FAB Consortium 1996) is reported as nucleotide +1.

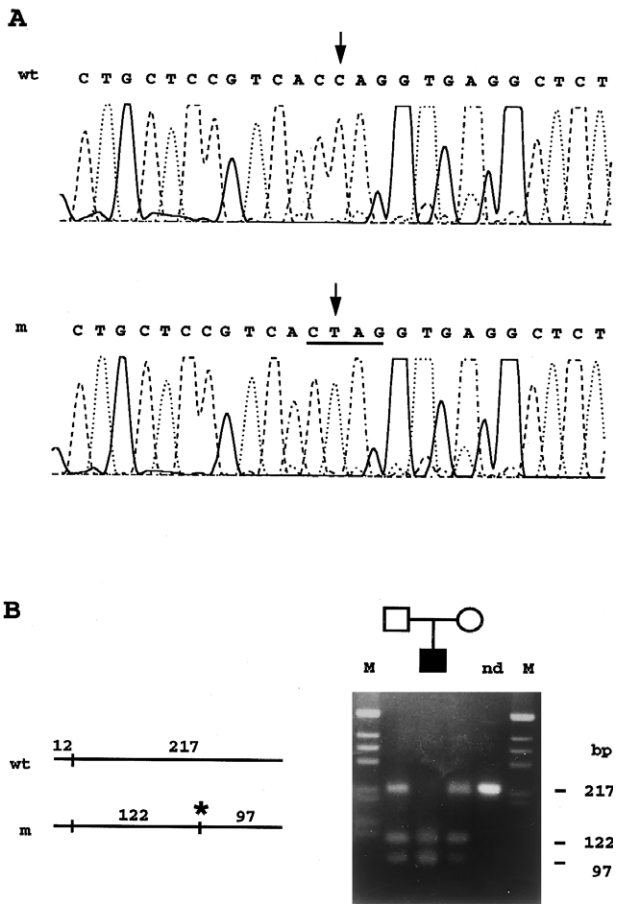

Figure 1 Identification of the 2314C→T mutation. *A,* Nucleotide sequence analysis of the PCR-amplified fragment of exon 24 from a normal control (wt) and patient VU268 (m). The substitution created a restriction site for *Mae*I (*underlined*). *B,* Diagram of the localization of *Mae*I sites in wild-type (wt) and mutated (m) alleles and of digestion of the PCR products from family members. Lane nd, Undigested PCR product. Lanes M, DNA size marker (1-kb ladder).

cryptic splice signals. However, RT-PCR and sequencing analysis did not allow the identification of any altered product. The hypothesis that the mutation may lead to an unstable and undetectable mRNA has not been investigated.

The homozygous transversion IVS14+1G→C in the donor splice site of intron 14 would be expected to determine an alternative splicing event; however, this could not be confirmed, since RNA of FA25 was not available. The patient was homozygous at microsatellites in 16q24.3 (Savoia et al. 1997), and haplotype data were consistent with the segregation of the mutation in the four healthy sisters and the mother. However, since the father's DNA was not available, we cannot exclude a paternal genomic deletion as the second pathogenic FA allele in this family.

In the 5′ region of intron 28, a 60-bp repeat was present twice, at residues 1–60 and 84–133. In patient VU578, a homozygous transversion C→G (IVS28+83C→G) at position 83 of intron 28 created a cryptic donor site AGgtagg with a score of 86, calculated according to the method of Shapiro and Senapathy (1987), higher than the score of 76 for the normal-signal ACgtagg (Ianzano et al. 1997). Since the mutation was only paternally inherited, the second FA allele might be a maternal genomic deletion. We verified the possibility of an alternative cleavage of intron 28 by using RT-PCR with primers in exons 28 and 30 (fig. 3). In the normal control, two bands were observed, the shorter one (366 bp) being the product expected and the longer one (484 bp) corresponding to the insertion of an alternatively spliced new exon (exon 29a; L. Ianzano, unpublished results). In VU578 we detected three products, 366, 449, and 567 bp in size. Sequence analysis showed that the intermediate band represented the mutated cDNA due to the insertion, between exons 28 and 29, of the first 83 bp of intron 28; the major band contained the additional exon 29a (fig. 3). The alternative splicing of intron 28 led to the translation of 28 new amino acids and a stop at codon 955 in the *FAA* gene. The apparent ratio of the wild-type (366 bp) and the mutated (449 bp) alleles in VU578 was ~1:10, suggesting that the splicing machinery recognizes both the normal and the cryptic donor site, but with a different efficiency.

Duplication and Insertion

A duplication and a single base insertion were detected in one patient and in two patients, respectively. Patient FA19 carried a paternally inherited heterozygous duplication of 19 bp, GA AAT TCA ACC TGA AGC TG (residues 3012–2830), in exon 29, causing a shift of the open reading frame and a stop codon four amino acids downstream. An insertion of a single G at residue 3559 (3559insG) in exon 36 was detected in two unrelated individuals, VU393 and FA37. This mutation, which leads to a frameshift and a stop codon, was homozygous in the first patient, the only child of a consanguineous marriage, and was heterozygous in the other.

In the absence of functional data, it is difficult to determine conclusively whether an observed variation in sequence represents a pathogenic mutation. However, the majority of sequence alterations described in this paper are predicted to truncate the FAA protein and are thus likely to represent pathogenic mutations. One hundred chromosomes from healthy unrelated individuals were analyzed for the presence of each variation, by different approaches, such as restriction analysis, restriction site–generating PCR (RG-PCR), or RNA-SSCP. None of the previously described mutations was detected in the normal controls (data not shown).

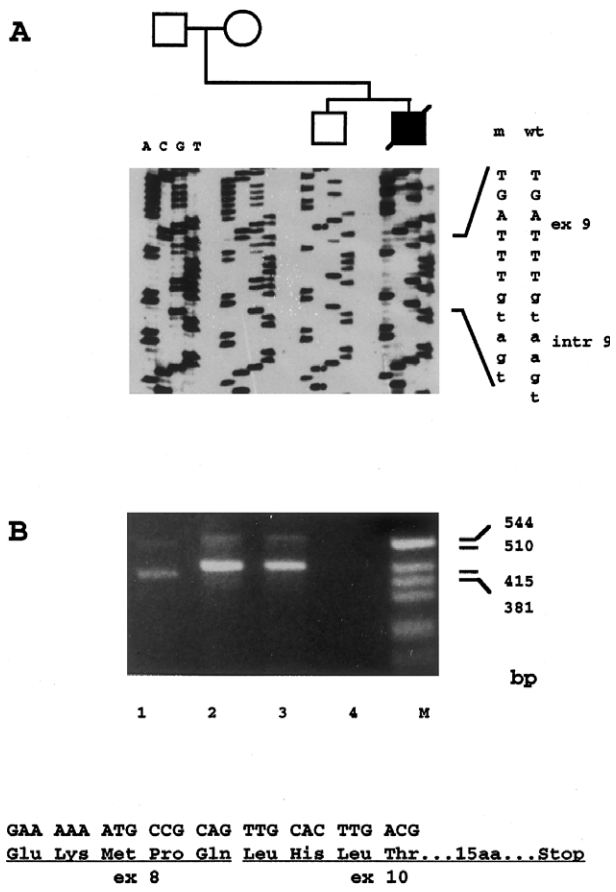

Figure 2 Detection of the IVS9+3delA mutation. *A,* Sequence analysis of the amplified product of exon 9 in all members of the family. *B,* RT-PCR products resulting from use of primers in exons 8 and 11 of patient VU330 (lane 1), different lymphoblastoid cell lines (lanes 2 and 3), and a negative control (lane 4). Lane M, DNA size marker (1-kb ladder).

Missense Substitutions

Some amino acid substitutions were detected. Among these, only one (D1359Y) was not found in non-FA chromosomes, and it involved a change from a charged to a hydrophobic amino acid. A local variation in hydrophobicity, with a possibly deleterious effect on FAA-protein activity was detected by the PC/GENE analysis program. The other amino acid substitutions were more conservative and were not predictive of any significant conformational changes. In fact, T/A266, G/S501, and G/D809 were found to be polymorphisms, as assessed by restriction-enzyme analysis. The respective allele frequencies, estimated for 100 chromosomes from unrelated individuals, are listed in table 3.

Discussion

Knowledge of the mutation spectrum occurring in the *FAA* gene may contribute significantly to pathogenesis studies in FA and may help to design mutation-screening strategies. The identification of pathogenic mutations in FA patients allows for the assignment of patients to complementation group FA-A and thus removes the need for a much more laborious complementation study. In addition, knowledge of the mutations allows for a rapid prenatal diagnosis, as well as for carrier testing in the FA families.

RNA-SSCP screening of all 43 exons of the FAA gene led to the identification of nonsense mutations, alterations in donor sites, an insertion, and a duplication, most of which predict a premature truncation of the FAA protein. Two mutations affecting the intronic sequences led to splicing aberrations. In one family, a nucleotide deletion in the donor splice site caused the skipping of the respective exon. In another patient, an intronic substitution generated a cryptic splice site and an alternative cleavage of the transcript, although a small amount of the wild-type messenger was produced. It was found that IVS10+1G→T did not alter the mRNA in two siblings. However, an instability of the RNA carrying this mutation cannot be excluded.

Consistent with linkage and haplotype analysis in Italian pedigrees, which revealed a high frequency of compound heterozygotes and a wide variety of haplotypes (Savoia et al. 1997), the results reported in this paper suggest that different mutations characterize the Italian FA alleles, whereas none of these seems to occur at a high frequency. The degree of mutation homogeneity was expected to be high in the genetic isolate of the Brenta basin (Savoia et al. 1997). Unexpectedly, even though patients VU330, VU268, and VU388 shared the same haplotype, they were found to carry different mutations, suggesting that different mutational events have occurred on the same chromosome. Except for VU330, who had two distinct haplotypes, the homoallelic pa-

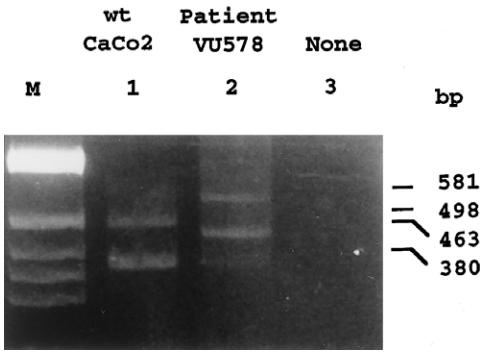

Figure 3 Identification of the alternative splicing of intron 28, resulting from the IVS28+83C→G mutation in patient VU578; results shown are for RT-PCR using primers in exons 28 and 30 of the CaCo2 cell line (lane 1), VU578 (lane 2), and a negative control (lane 3). Lane M, DNA size marker (1-kb ladder).

tients were either from a consanguineous marriage or autozygous at the loci surrounding the *FAA* locus. The other seven patients were compound heterozygotes; however, in none of these has the second FA allele been characterized.

Surprisingly, screening of all 43 exons of the *FAA* gene has led to the identification of only 10 mutations, in 11 of 38 patients. This low number could be attributed to several causes. First, FA is a genetically heterogeneous disease (Strathdee et al. 1992*a;* Joenje et al. 1995), and so some of the patients may in fact not belong to group FA-A. However, since 11 patients were classified as FA-A by complementation analysis but only four (VU388, VU330, VU262, and VU268) have been characterized for one or two mutations, the nonassignment to FA-A group does not seem to be the reason for the low mutation yield. Second, a subset of FA alleles in certified FA-A patients might have escaped the screening process, either because of the limitations of the technical procedure or because of their localization in either introns or the promoter region. Finally, evidence for genomic deletions has been reported previously (FAB Consortium 1996). For example, in patient VU337, exons 18–21 were deleted in the RNA, and in situ hybridization indicated that one FA allele is a large genomic deletion of the entire *FAA* gene (data not shown). It might be that some factors, such as an intrinsic instability in the *FAA* region, might lead to genomic deletions or other structural rearrangements of the gene. Therefore, to increase the efficiency of mutation screens, a different strategy may be useful, such as Southern blotting, FISH analysis, and methods designed to detect alterations at the protein level (FAA antibodies or the protein-truncation test).

In the other cloned FA gene, *FAC,* a total of 10 pathogenic mutations have been identified (Strathdee et al. 1992*b;* Gibson et al. 1993, 1996; Murer-Orlando et al. 1993; Whitney et al. 1993; Verlander et al. 1994; Lo Ten Foe et al. 1996*b,* and in press). Except for two amino acid substitutions, the other sequence variations would predict, as in *FAA,* premature terminations of the FAC protein. However, a wider spectrum of *FAC* mutations has to be expected, since only single mutations were identified in several heterozygous patients. Indeed, since a large number of the FA patients were unclassified, the rate of mutations in the *FAC* gene may be underestimated, since some alterations may have escaped the screening procedures.

The FAA mutations described so far are widely distributed over the *FAA* gene, and all but one were found only in single patients (FAB Consortium 1996; Lo Ten Foe et al. 1996*a*). This situation hampers a detailed genotype-phenotype correlation study, which would otherwise be helpful in the identification of possible associations with a mild or severe form of the disease. A further difficulty is due to a wide clinical heterogeneity in FA, not only among patients from different families of the same complementation group A but also among patients within one family (Savoia et al. 1996). However, patients VU388, VU330, VU268, and VU393, homozygous for a nonsense or frameshift mutation, have a relatively severe phenotype, with multiple congenital malformations and severe pancytopenia (Savoia et al. 1996). Continued screening for mutations may localize critical domain or functional residues of the FAA protein and, in this way, may help to provide a better understanding of the molecular pathogenesis in FA patients.

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References

Auerbach AD (1993) Fanconi anemia diagnosis and the diepoxybutane (DEB) test. Exp Hematol 21:731–733

Buchwald M (1995) Complementation groups: one or more per gene? Nat Genet 11:228–230

Butturini A, Gale RP, Verlander PC, Adler-Brecher B, Gillio A, Auerbach AD (1994) Hematologic abnormalities in Fanconi anemia: an international Fanconi anemia study. Blood 84: 1650–1655

Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18:5294–5299

FAB Consortium (1996) Positional cloning of the Fanconi anemia group A gene. Nat Genet 14:324–328

Gibson RA, Hajianpour A, Murer-Orlando M, Buchwald M, Mathew CG (1993) A nonsense mutation and exon skipping in the Fanconi anaemia group C gene. Hum Mol Genet 2: 797–799

Gibson RA, Morgan NV, Goldstein LH, Pearson IC, Kesterton IP, Foot NJ, Jansen S, et al (1996) Novel mutations and polymorphisms in the Fanconi anemia group C gene. Hum Mutat 8:140–148

Gschwend M, Levran O, Kruglyak L, Ranade K, Verlander PC, Shen S, Faure S, et al (1996) A locus for Fanconi anemia on 16q determined by homozygosity mapping. Am J Hum Genet 59:377–384

Ianzano L, d'Apolito M, Centra M, Savino M, Levran O, Auerbach AD, Cleton-Jansen A-M, et al (1997) The genomic organisation of the Fanconi anemia group A (*FAA*) gene. Genomics 41:309–314

Joenje H, Lo Ten Foe JR, Oostra AB, van Berkel CGM, Rooi-

mans MA, Schroeder-Kurth T, Wegner R-D, et al (1995) Classification of Fanconi anemia patients by complementation analysis: evidence for a fifth genetic subtype. Blood 86:2156–2160

Lo Ten Foe JR, Kruyt FAE, Zweekhoest MBM, Pals G, Gibson RA, Mathew CG, Joenje H, et al. Exon 6 skipping in the Fanconi anemia C gene associated with a nonsense/missense mutation (775C→T) in exon 5. Hum Mutat (in press)

Lo Ten Foe JR, Rooimans AA, Bosnoyan-Collins L, Alon N, Wijker M, Parker L, Lightfoot J, et al (1996*a*) Expression cloning of a cDNA for the major Fanconi anemia gene, *FAA*. Nat Genet 14:320–323

Lo Ten Foe JR, Rooimans MA, Joenje H, Arwert F (1996*b*) Novel frameshift mutation (1806insA) in exon 14 of the Fanconi anemia C gene, *FAC*. Hum Mutat 7:264–265

Murer-Orlando M, Llerena JC, Birjandi F, Gibson RA, Mathew CG (1993) *FACC* gene mutations and early prenatal diagnosis of Fanconi's anaemia. Lancet 342:686

Pronk JC, Gibson RA, Savoia A, Wijker M, Morgan NV, Melchionda S, Ford D, et al (1995) Localization of the Fanconi anemia complementation group A gene to chromosome 16q24.3 by linkage analysis and allelic association. Nat Genet 11:338–340

Sakar G, Yoon H-S, Sommer S (1992) Screening for mutations by RNA single strand conformation polymorphism (rRNA): comparison with DNA-SSCP. Nucleic Acids Res 20:871–878

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Savoia A, Piemontese MR, Savino M, Zatterale A, Pronk J, Arwert F, Joenje H, et al (1997) Linkage analysis of Fanconi anemia in Italy and mapping of the complementation group A gene. Hum Genet 99:93–97

Savoia A, Zatterale A, Del Principe D, Joenje H (1996) Fanconi anemia in Italy: high prevalence of complementation group A in two geographic clusters. Hum Genet 97:599–603

Shapiro MB, 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

Strathdee CA, Duncan AMV, Buchwald M (1992*a*) Evidence for at least four Fanconi anemia genes including *FACC* on chromosome 9. Nat Genet 1:196–198

Strathdee CA, Gavish H, Shannon WR, Buchwald M (1992*b*) Cloning of cDNAs for Fanconi's anemia by functional complementation. Nature 356:763–767

Verlander PC, Lin JD, Udono MU, Zhang Q, Gibson RA, Mathew CG, Auerbach AD (1994) Mutation analysis of the Fanconi anemia gene *FACC*. Am J Hum Genet 54:595–601

Whitney MA, Saito H, Jakobs PM, Gibson RA, Moses RE, Grompe M (1993) A common mutation in the *FACC* gene causes Fanconi anaemia in Ashkenazi Jews. Nat Genet 4: 202–205