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Reverse mosaicism in Fanconi anemia: natural gene therapy via molecular self-correction

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Abstract. Fanconi anemia (FA) is a genetically and phenotypically heterogenous autosomal recessive disease associated with chromosomal instability and hypersensitivity to DNA crosslinkers. Prognosis is poor due to progressive bone marrow failure and increased risk of neoplasia, but revertant mosaicism may improve survival. Mechanisms of reversion include back mutation, intragenic crossover, gene conversion and compensating deletions/insertions. We describe the types of reversions found in five mosaic FA patients who are compound heterozygotes for single base mutations in FANCA or FANCC. Intragenic crossover could be shown as the mechanism of self-correction in the FANCC patient. Restoration to wildtype via back mutation or gene conversion of either the paternal or maternal allele was observed in the FANCA patients. The sequence environments of these mutations/reversions were indicative of high mutability, and selective advantage of bone marrow precursor cells carrying a completely restored FANCA allele might explain the surprisingly uniform pattern of these reversions. We also describe a first example of in vitro phenotypic reversion via the emergence of a compensating missense mutation 15 amino acids downstream of the constitutional mutation, which explains the reversion to MMC resistance of the respective lymphoblastoid cell line. With one exception, our mosaic patients showed improvement of their hematological status during a three- to six-year observation period, indicating a proliferative advantage of the reverted cell lineages. In patients with Fanconi anemia, genetic instability due to defective caretaker genes sharply increases the risk of neoplasia, but at the same time increases the chance for revertant mosaicism leading to improved bone marrow function.

The genome of long-lived, warm-blooded species such as ours suffers continuous damage from a variety of exogenous and endogenous sources. In order to counteract this threat, a number of gene families have evolved that recognize DNA damage and initiate its repair and/or elimination. Some of these "guardian" functions are carried out by genes that are responsible for the maintenance of our somatic genome. Mutations in such caretaker genes lead to genetic instability in somatic cells and mutator phenotypes, whose most detrimental manifestations are neoplasia (Cahill et al., 2001) and premature aging (de Boer et al., 2002).

During the past few years it has been recognized that genetic instability may not only be detrimental but potentially beneficial for some of the affected patients. This is because genetic instability appears to increase the opportunity for correction of the constitutional genetic error in a proportion of body cells by reversion and/or compensation of the original mutation. For example, in the case of compound heterozygous Bloom syndrome and Fanconi anemia (FA) patients, revertant mosaicism has been described in peripheral blood cells reflecting the side by side existence of defective and self-corrected cell lineages (Ellis et al., 1995 and 1996; Lo Ten Foe et al., 1997). In situa-

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tions where self-correction confers a proliferative advantage, the progeny of the self-corrected precursor cell can be expected to gradually replace the defective cell population (Hirschhorn et al., 1996; Stephan et al., 1996). Somatic cell mosaicism as the result of self-correction has important clinical implications. In the case of Fanconi anemia, clonal expansion of a corrected bone marrow stem or early progenitor cell may lead to complete reversal of the disease phenotype in the periphery such that the definitive laboratory diagnosis must employ other than peripheral blood cells (Joenje et al., 1998). With regard to gene therapy, the observed in vivo growth advantage of self-corrected cells is encouraging, and emergence of mosaicism may in fact mitigate and improve the natural course of the disease in at least some of the FA patients (Lo Ten Foe et al., 1997). In this positive context, it may be justified to speak of "natural gene therapy" (Youssoufian, 1996). A negative aspect concerns bone marrow transplantation where the co-existence of blood cells with different sensitivities towards cytotoxic agents complicates the conditioning regimen. Finally, the phenomenon of somatic cell mosaicism may contribute to the pronounced clinical heterogeneity encountered among patients with Fanconi anemia.

We here document somatic cell mosaicism due to molecular reversion of one of the mutant alleles in five FA patients. We define the underlying constitutional mutations in different patient tissues, and test their functional significance by site-directed mutagenesis in combination with complementation analysis using retroviral vectors. In addition to previously reported mechanisms of reversion of cellular phenotypes (such as back mutation, intragenic crossover, gene conversion, and compensating deletions/insertions; Lo Ten Foe et al., 1997; Waisfisz et al., 1999; Gregory et al., 2001) we describe a compensating missense mutation downstream of the original base change as an in vitro mechanism of phenotypic reversion that explains the MMC resistance of the lymphoblastoid cell line in one of our patients. Four of our five mosaic patients showed gradual improvement of blood counts during a three to six year observation period, indicating the clinical relevance of revertant mosaicism.

Materials and methods

Cell culture, cell cycle studies, and complementation testing

The five patients we describe were referred to our laboratory in order to confirm or rule out the clinical suspicion of Fanconi anemia. Peripheral blood and skin biopsy samples were obtained with informed consent. Mononuclear blood cells isolated by Ficoll separation were used for direct transfections, for 72-hour PHA lymphocyte cultures, and for the establishment of EBV-transformed lymphoid cell lines (LCL). LCL were maintained in RPMI medium (Gibco) with 16 % fetal bovine serum. Fibroblast cultures were established using standard cell culture procedures and MEM medium supplemented with glutamine and 16 % fetal bovine serum. All cell cultures were kept in high humidity incubators equipped with CO_2 and O_2 sensors in an atmosphere of 5 % (v/v) CO_2 and 5 % (v/v) O_2 by replacing air with nitrogen. Chromosome breakage studies following exposure to various concentrations of mitomycin C were performed according to standard protocols (Schroeder-Kurth et al., 1989). Cell cycle studies employed bivariate BrdU/Hoechst flowcytometry as previously described (Poot et al., 1994; Seyschab et al., 1995). Assignment of patient cells to a given FA complementation group was achieved via complementation analysis using retroviral vectors containing inserts of the full-length cDNAs of FANCA or FANCC (Hanenberg et al., 2002; Lobitz et al., manuscript in preparation).

Mutation analysis

Genomic DNA extracted from second to fourth passage primary fibroblast cultures was used for SSCP prescreening. SSCP analysis was performed on native acrylamide gels (Amersham Pharmacia, Germany) at 14 °C. Fragments showing an aberrant SSCP pattern were sequenced using the ABI 310 sequencer to identify the mutations. The FANCA gene was amplified in 41 fragments containing all 43 exons and the FANCC gene was amplified in 14 fragments containing all 14 exons of the gene, using primers flanking the exon/intron boundaries. All detected mutations were confirmed at the cDNA level. In patients where only a single mutation had been detected by SSCP screening we performed RT-PCR in order to rule out large heterozygous deletions. Aberrant bands in 1 % agarose gels of cDNA fragments were sequenced directly.

Site-directed mutagenesis

In order to confirm the pathogenic status of novel amino acid exchanges they were analyzed by transducing the FANCA wildtype containing the mutations L324R and R951Q as well as the combination of R951Q and Q966A into the pSL1180/FANCA vector (Amersham Biosciences, Freiburg, Germany; modified by introducing wildtype FANCA). Sequences of the phosphorylated mutagenic primers were designed according to the manufacturer's instructions (QuikChange Site-Directed Mutagenesis Kit, Stratagene) and were as follows:

L324R/for 5′-TCAGTCATACCCGGACTCAGATACTC-3′,
L324R/rev 5′-GAGTATCTGAGTCCGGGTATGACTGA-3′,
R951Q/for 5′-CAGATACTGAACAGCAGGACTTCCAC-3′,
R951Q/rev 5′-GTGGAAGTCCTGCTGTTCAGTATCTG-3′,
Q966A/for 5′-CTTTCTCCCTGCGTCCTCGGCTTC-3′ and
Q966A/rev 5′-GAAGCCGAGGACGCAGGGAGAAAG-3′

(modified nucleotides are underlined). The reaction was performed in 50-µl aliquots containing 20 ng of plasmid DNA, 125 ng of each mutagenesis primer, 1.5 mM $MgCl_2$, 1× reaction buffer, 200 nM dNTPs and 2.5 Units *PfuTurbo* DNA Polymerase (Stratagene). An initial denaturation for 30 s at 95 °C was followed by amplification for twelve cycles, each with denaturation for 30 s at 95 °C, annealing for 60 s at 55 °C and extension for 16 min at 68 °C. After placing the reactions on ice for 2 min, 10 Units *Dpn*I were added and the sample was incubated at 37 °C for 1 h to digest the parental supercoiled dsDNA. The FANCA coding sequences carrying the desired mutations were directionally cloned as 4.5-kb *Not*I/*Not*I DNA fragments into the LFAPEG expression vector and sequenced to show the integrity of the entire coding sequence except for the presence of the targeted mutations. The FA-A null cell lines PIR and SNE (both patients carrying large homozygous deletions in the FANCA gene) were stably transfected with the targeted cDNAs and tested for correction of MMC-hypersensitivity as described below.

Retroviral vectors and transfections

The LFAPEG and LFCPEG plasmids were used for complementation studies as previously described (Hanenberg et al., 2002). LFAPEG was used for site directed mutagenesis testing. This vector is a derivative from MSCV2.1 (Hawley et al., 1994) containing FANCA-WT and EGFP (enhanced green fluorescent protein), where the FANCA cDNA is expressed off the retroviral LTR and the EGFP cDNA is under control of the internal murine PGK promotor from MSCV2.1. In LEG (mock transfection), EGFP was directly expressed off the LTR. For site directed mutagenesis studies, the packing cell line, 293T-derived ecotropic Phoenix cells (Nolan, 1996), was transfected with 10–20 µg of the FANCA plasmid DNA using FuGene6 (Roche, Mannheim, Germany). After two days retrovirus-containing supernatants (SNs) were harvested, filtered with 45 µm and used for transducing NIH/3T3-derived pg13 cells (Miller et al., 1991) three times in the presence of 7.5 µg/ml protamine (Roche). GALV-pseudotyped retroviruses were harvested from pg13 cells as previously described (MacNeill et al., 1999). The cell lines of our patients as well as the PIR and SNE cell lines were cultured on the fibronectin fragment CH-296 as described previously (MacNeill et al., 1991; Hanenberg et al., 1996 and 1997; Pollok et al., 1998; Hanenberg et al., 2002). Cells were grown for at least two days after the last exposure to virus supernatants in order to ensure stable expression of the cDNAs from the integrated proviruses. Treated cells were cultured in triplicate in the presence of increasing concentrations of MMC (Medac, Hamburg, Germany). Six days later, cells were harvested for flowcytometry.

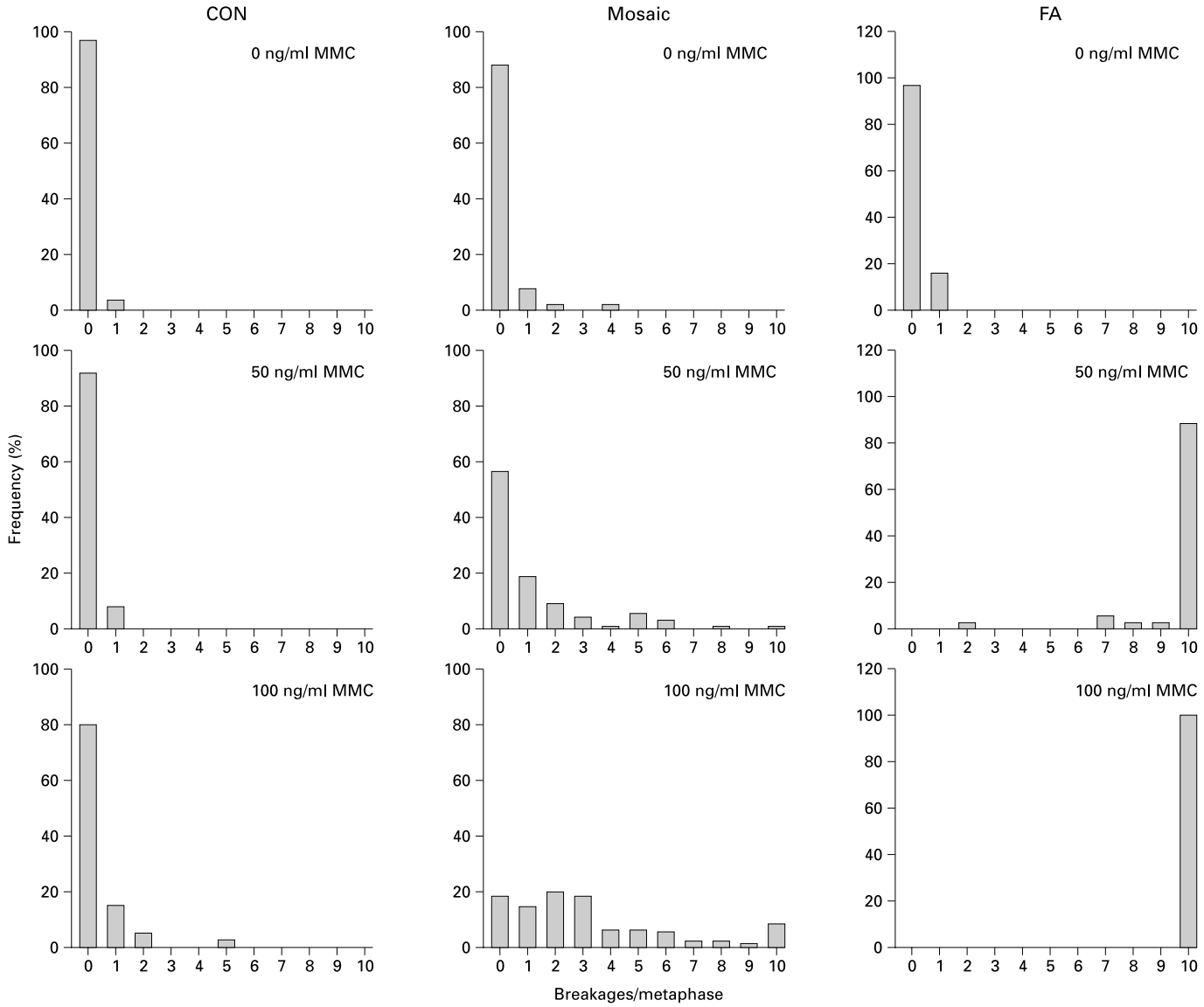

Fig. 1 . Chromosome breakage analysis of 72-hour peripheral blood mononuclear cells in control (left panel), mosaic (center panel) and previously confirmed (right panel) FA blood donors without and after exposure to 50 and 100 ng/ml mitomycin C (MMC). The number of breaks per cell (horizontal axis) was analyzed in 100 metaphases each. Bars represent percentages of metaphases with zero to more than ten breaks per cell. The center panel shows results of the initial chromosome breakage analysis in patient STT with evidence for a bimodal distribution of breakage rates after exposure to MMC.

Flowcytometry

Flowcytometry was carried out on a BD-LSR Instrument (Becton Dickinson, Heidelberg, Germany) equipped with Argon (emission at 488 nm) and Helium-Cadmium (emission at 325 nm) lasers. 30 min prior to cell harvest, the DNA dye Hoechst 33342 was added to the cultures at a final concentration of 8 µg/ml. After harvest by trypsinization, cells were stained for 10 min with propidium iodide (PI) (Sigma, Heidelberg, Germany) at a final concentration of 6 µg/ml. Using the PI vs Hoechst setting, live and dead cells were first separated, followed by the EGFP vs. Hoechst setting, allowing discrimination between successfully transfected (green fluorescence) and non-transfected cells. DNA content distributions showing G_1 and G_2 phase peaks were determined for EGFP positive cells.

Results

When 72-hour cultures of peripheral blood mononuclear cells from healthy donors are exposed to increasing concentrations of MMC, there is only a minimal increase of metaphases that show more than three breakage events per cell (Fig. 1, leftmost columns). In contrast, metaphases from FA patients show more than ten breaks per cell after exposure to MMC (Fig. 1, rightmost columns). In our five patients, chromosome breakage studies of 72-hour mononuclear blood cell cultures yielded various degrees of bimodal breakage distributions after exposure to 50 and 100 ng/ml MMC, indicating the simultaneous presence of MMC-sensitive and MMC-resistant cells (Fig. 1, mid-

dle columns, patient STT). Bivariate BrdUrd/Hoechst flowcytometry of 72-hour peripheral blood mononuclear cell cultures of our five patients revealed cell cycle distributions and G_2 phase accumulations intermediate (Fig. 2b) between controls (Fig. 2a) and previously confirmed FA patients (Fig. 2c). At the time of their first diagnostic evaluation, the cell cycle test placed all five of our patient blood samples within the lower end of the Fanconi anemia cohort (Fig. 2d). However, during the course of a 3–6 years observation period, their cell cycle patterns gradually merged with those of the healthy controls (data not shown).

The bimodal distribution of chromosomal breakage rates and the results of the cell cycle analysis therefore suggested the presence of a mixture of MMC-sensitive and MMC-resistant mononuclear blood cells, and a mixture of cells with a disturbed and a normal cell cycle pattern. EBV-transformed lymphoid cell lines of the five patients were uniformly MMC-resistant. In contrast, the patients' fibroblast cultures were uniformly MMC-sensitive as evidenced by elevated chromosome breakage (with unimodal breakage distributions), G_2 phase blockage, and lack of cell growth following five-day exposures to 3 ng/ml MMC (data not shown). Altogether, these findings indicated an FA-positive cellular phenotype in the patients' fibroblasts, whereas the patients' mononuclear blood cells consisted of variable mixtures of cells with FA and control cell phenotypes. In order to clarify the cellular genotype-phenotype correlations in our patients, complementation and mutation studies were performed with DNA isolated from their fibroblasts, and the results were compared to DNA isolated from their primary peripheral blood mononuclear cells and EBV-transformed lymphoid cell lines.

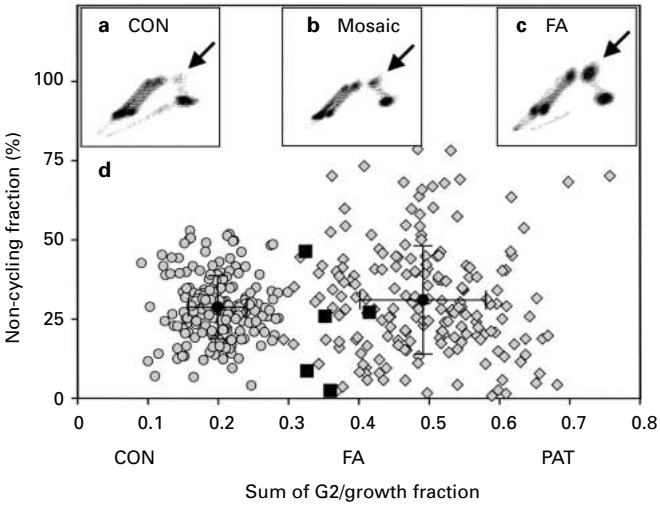

Fig. 2. (**a–c**) 72-hour cell cycle distributions of PHA-stimulated peripheral blood monunuclear cells analyzed via two-parameter flowcytometry (BrdU/Hoechst technique, 27). First cell cycle G_2 phase compartments are denoted by arrows. Compared to controls (**a**), mosaic (**b**) and previously confirmed (**c**) FA patients show increasing accumulations of cells in the G_2 phase and decreasing cell cycle progression. (**d**) Sum of G_2 phases over growth fraction (horizontal axis) versus percentage of G_0/G_1 phase cells (vertical axis) were assessed via flowcytometry and plotted for controls (solid circles), the five mosaic patients at their initial presentation (solid squares) and previously confirmed FA patients (solid diamonds). Our five mosaic patients display positions within, but near the leftmost margin of the FA patient cluster. Mosaicism has not been excluded for other FA patients with similar low X-axis positions.

Patient 1, **URD**, was assigned to complementation group A by showing elimination of G_2 phase blockage after transfection of his fibroblasts with the FANCA vector (Fig. 3a). Mutation analysis (SSCP and sequencing) of the patients' fibroblast cultures revealed compound heterozygosity for two FANCA nonsense mutations (856C→T, Q286X in exon 10 and 3976C→T, Q1326X in exon 40; Table 1). Sequencing of the corresponding exons in DNA prepared from the patient's MMC-resistant lymphoblastoid cell line showed that the exon 10 nonsense mutation had reverted to wildtype (Fig. 3b). The same reversion was found in DNA extracted from the patient's peripheral blood mononuclear cells. As a consequence of the reversion, the patient's blood cells have acquired a heterozygous state which normalizes their chromosome breakage rates and cell cycle patterns, and which explains the MMC resistance of the patient's LCL. These changes were accompanied in vivo (beginning at age 11) by improvements of the patients' haematological parameters (Fig. 4).

Patient 2, **STT**, was assigned to complementation group A by retroviral transfection of his fibroblast culture. Mutation analysis (SSCP and sequencing) revealed a single nonsense mutation, 862G→T, E288X, in exon 10 of FANCA, which has been described before (Morgan et al., 1999). Sequencing of the complete genomic DNA failed to detect a second mutation. On the basis of cDNA analysis a defective promoter region is unlikely because of the presence of a second allele. Rather, the distribution of heterozygous polymorphisms limited to the 5′ and 3′ ends of the gene suggests the presence of a large intragenic deletion, including exon 10, as the second mutation (data not shown). Sequencing of the patient's MMC-resistant lymphoblastoid cell line and of his native peripheral blood mononuclear cells showed that the nonsense mutation in exon 10 had reverted to wildtype, both in the patient's T- and B-cells (data not shown).

Clinically, the patient suffered from anemia and thrombocytopenia right after birth. His erythrocyte and leukocyte counts improved gradually during his first year of life, whereas his thrombocyte counts started to improve between ages 3 and 6. Currently, at age 12, thrombocytes are 112 Gpt/l, hemoglobin is 6.9 mmol/l, erythrocytes are 3,500 Gpt/l and leukocytes 7.4 Gpt/l.

Patient 3, **MRB**, was assigned to complementation group A by normalization of his cell cycle pattern after transfection of his fibroblasts with the FANCA vector (Fig. 5a). Mutation analysis of the patient's fibroblast DNA revealed two not previously reported mutations, one splice-site and one missense mutation. The paternal splice-site mutation IVS9-1G→T destroys the conserved acceptor splice-site in front of exon 10. An alternative AG dinucleotide dowstream is used, which is located in exon 10 and results in a 32 nt deletion at the mRNA level, leading to a frameshift and a truncated protein nine amino acids downstream. The maternal missense mutation (971T→G, L324R, exon 11) affects a conserved residue in the murine *Fanca* sequence, suggesting its functional significance. In order to provide definitive proof of its pathogenicity, the

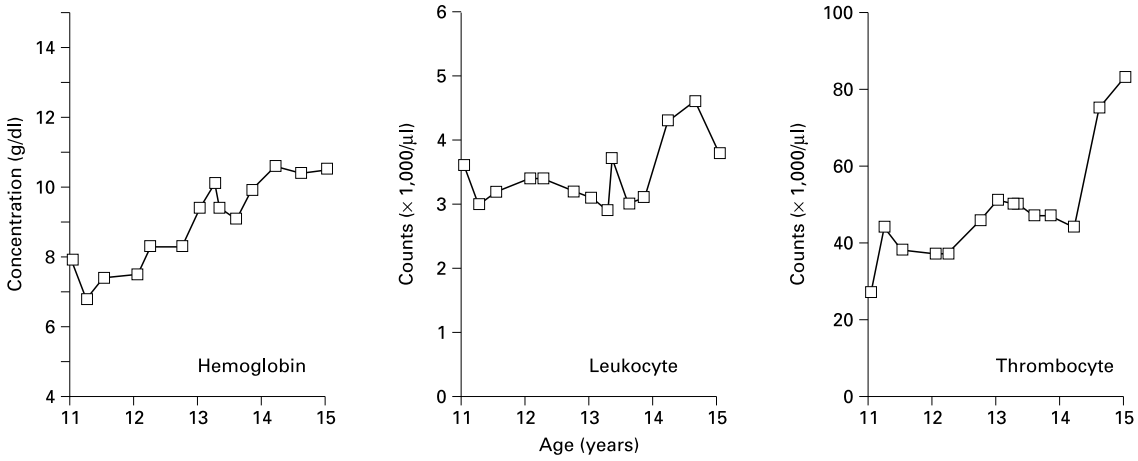

Fig. 3. Complementation and mutation analysis in patient URD. (**a**) Elimination of G_2 phase blockage of virus-transfected fibroblasts after MMC treatment in patient URD shown by flowcytometry. After gating on EGFP-positive cells, mock-transfected fibroblasts (EGFP only) display massive G_2 phase accumulation, whereas the G_2 phase peak of fibroblasts transfected with the vector LFAPEG containing the wild type FANCA plus EGFP cDNAs has returned to control levels. URD fibroblasts transfected with FANCC or FANCG cDNA containing viruses (LFCPEG and LFGPEG) behave like mock-transfected fibroblasts (data not shown). (**b**) URD fibroblasts carry the heterozygous mutation 856C→T, Q256X, leading to a premature stop codon, whereas the lymphoblastoid cell line derived from patient URD displays apparent homozygosity for the wild type codon CAG (glutamine). The same reversion was found in the patients' native peripheral blood mononuclear cells (data not shown).

Table 1. Patient codes, birthdates and constitutional mutations as determined from fibroblast DNA

Patient code	Year of Birth	Complementation group	Mutations	Reference	Mechanism of reversion
URD	1987	A	856C>T, Q256X 3976C>T, Q1326X	Wijker et al., 1999	Back mutation to WT
STT	1992	A	862G>T, E288X not detected	Morgan et al., 1999	Back mutation to WT
MRB	1994	A	IVS9-1G>T 971T>G, L324R		Back mutation to WT
RNT	1991	C	67delG IVS11-2A>G	Strathdee et al., 1992	Intragenic cross-over
EUFA173	1974	A	Del exons 17-31	Joenje et al., 2000	
			2852G>A, R951Q 2897A>C, E966A	Joenje et al., 2000	Back mutation to WT Compensatory missense mutation

substitution was introduced into pSL1180/FANCA by site directed mutagenesis and the altered FANCA coding sequence was directionally cloned as a 4.5-kb *Not*I/*Not*I DNA fragment into the LFAPEG expression vector. After packaging this plasmid into the retroviral vector, the FANCA –/– cell lines SNE and PIR were stably transfected with the targeted FANCA cDNA and tested for correction of their MMC-hypersensitivity. As shown in Fig. 5b, transfection with the targeted FANCA vector failed to abrogate the G_2 phase cell cycle blockage of FANCA null cells, just like mock transfection with the EGFP-only vector (LEG) or transfection with the FANCC containing vector (LFCPEG). In contrast, transfection of null cells with the LFAPEG vector carrying the wildtype FANCA gene restored a normal cell cycle pattern. Altogether, these experiments prove that the amino acid change L324R is a pathogenic mutation. Sequencing of the patient's MMC-resistant EBV-transformed cell line and of his native peripheral blood mononuclear cells revealed that the mutation 971G→T had reverted to wildtype (Fig. 5c), explaining the reversion of the patient's blood cells to functional heterozygosity.

Within an observation period of four years, the patient's thrombocyte counts increased from 38 to 139 Gpt/l, total leukocytes from 4.1 to 5.6 Gpt/l, Granulocytes from 1.2 to 3.5 Gpt/l and hemoglobin from 7.4 to 8.1 mmol/l. This suggests that the reversion event took place in a very early progenitor of all these cell lineages.

Patient 4, **RNT**, was assigned to complementation group C by transfection of his cultured fibroblasts with the FANCC vector. His genomic DNA (from fibroblasts) had the known founder mutation 67delG (previously published as 322delG, Strath-

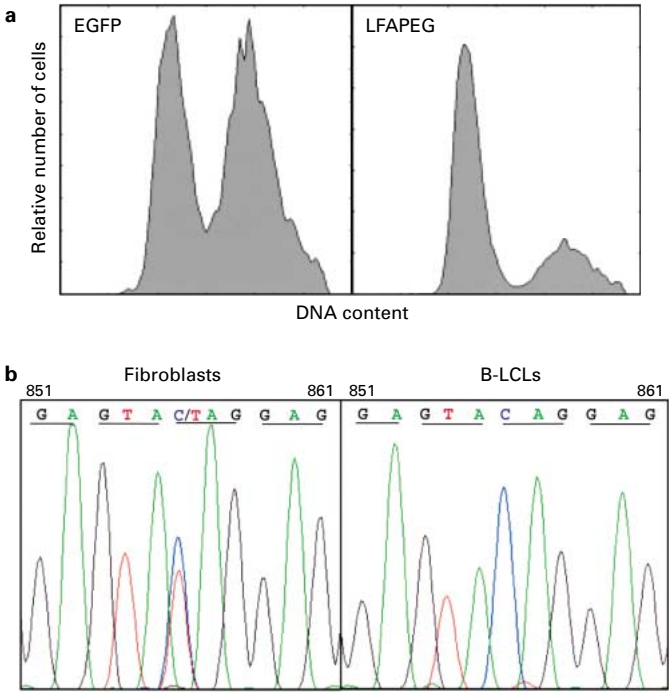

Fig. 4. Time course of haematological parameters in patient URD.

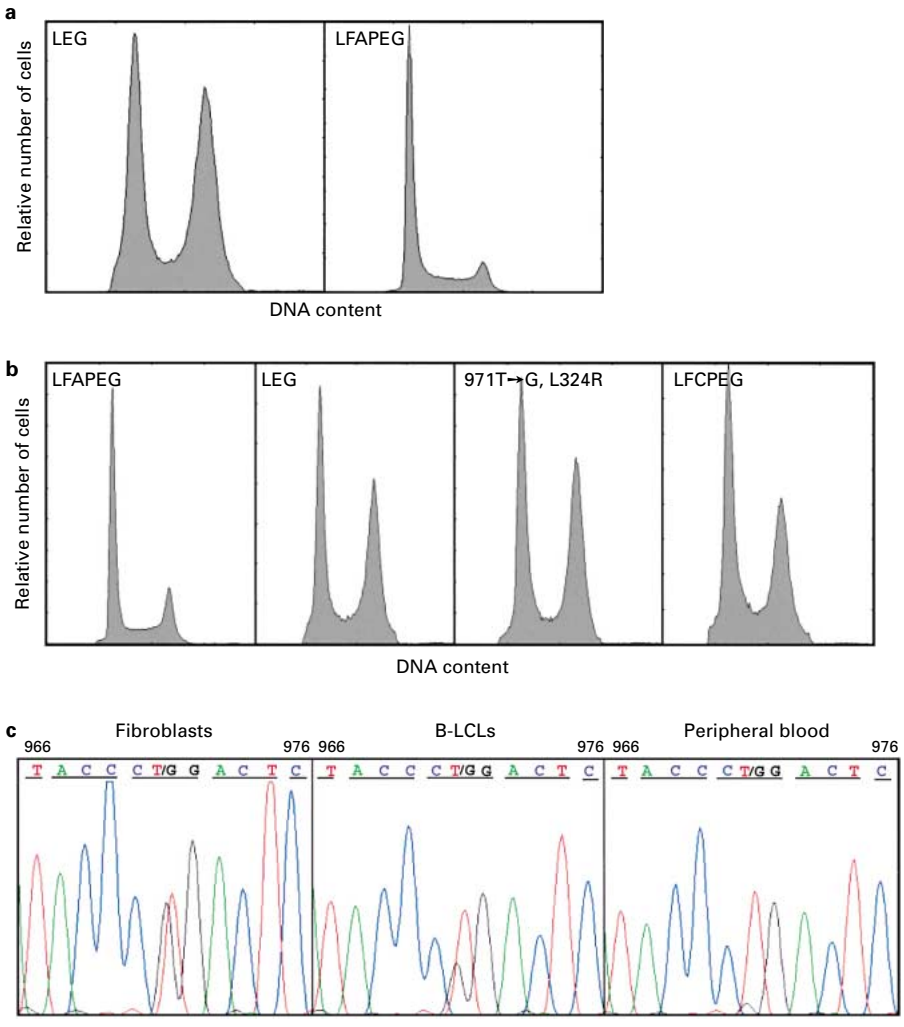

Fig. 5. Complementation and mutation analysis in patient MRB. (**a**) Mock-transfected fibroblasts (LEG) show G_2 phase accumulations typical for FA cells, whereas G_2 phase accumulations of fibroblasts treated with the complementing vector containing FANCA cDNA (LFAPEG) have returned to control levels. MRB fibroblasts transfected with FANCC- or FANCG-containing viruses (LFCPEG and LFGPEG) behave like mock-transfected fibroblasts (data not shown). (**b**) Testing for pathogenicity of the maternal missense mutation 971T→G, L324R, of patient MRB, using the FANCA –/– lymphoblastoid cell line SNE. The respective cell cycle distributions (from left to right) show complementation of SNE after transfection with the FANCA vector (LFAPEG), lack of complementation in mock-transfected cells (LEG), lack of complementation after transfection with LFAPEG vector containing the 971T→G mutation (which proves the pathogenicity of the mutation), and lack of complementation with the FANCC (LFCPEG)-containing vector. (**c**) Sequencing analysis of the reversion of patient MRB. His fibroblasts carry the heterozygous missense mutation 971T→G, L324R, whereas the mutated maternal allele decreases in the lymphoblastoid cell line and in native peripheral blood cells, confirming the patient's incomplete mosaic status. In the patient's fibroblasts, the proportion of wild type (T) and mutated nucleotides (G) amounts to 1:1. This ratio increases to 2:1 in the lymphoblastoid cell line which was established in the year 1998, but two years later the patient's native peripheral blood cells exhibit a wild type to mutant ratio of 10:1, indicating a selective growth advantage of the reverted cell population.

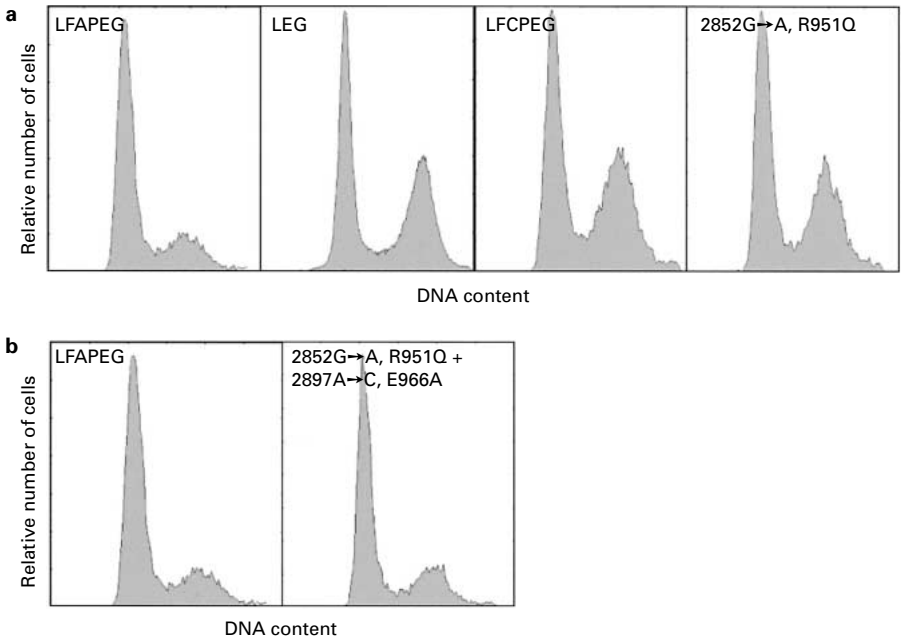

Fig. 6. Complementation assays in patient EUFA173. (**a**) Cell cycle distributions (from left to right) indicating successful complementation of EUFA173 fibroblasts by the FANCA vector (LFAPEG), lack of complementation in mock-transfected fibroblasts (LEG), lack of complementation after transfection with the FANCC vector (LFCPEG) and lack of complementation after transfection with the FANCA vector containing the maternal missense 2852G→A mutation (which proves its pathogenicity). (**b**) Cell cycle distributions (from left to right) showing successful complementation of the PIR cell line (FANCA –/– cells) after transfection with the FANCA vector (LFAPEG) and after transfection with the FANCA vector containing both the 2852G→A, R951Q, and the 2897A→C, E966A, mutations. The latter result indicates the compensatory nature of the in vitro 2897A→C mutation.

dee et al., 1992) in addition to a not previously reported splice-site mutation IVS11-2A→G, which destroys the conserved acceptor splice site in front of exon 12 and leads to skipping of exon 12. In contrast to the previous patients, both mutations were still present in the patient's MMC-resistant EBV-transformed cell line indicating a mechanism other than back-mutation for the self-correction of his blood cells. There were no detectable sequence variations in the vicinity of the two mutations, but their location at opposite ends of the gene suggested the possibility of self-correction via intragenic crossover. In order to separate the two different alleles from patient RNT, his full-length FANCC cDNA was amplified using *Pfu-Turbo* DNA Polymerase (Stratagene) and primers containing a tail with a *Not*I-binding site. The full-length fragment was cloned as a *Not*I/*Not*I DNA fragment into the pSL1180 vector and transfected into TOP TEN electrocompetent cells (Gibco). Exons 1 and 12 of the resulting 26 colonies were sequenced in order to prove intragenic crossover by the detection of both wildtype alleles and alleles carrying both mutations. Eight colonies carried a single mutation, 15 colonies showed wildtype sequences, whereas three colonies included the 67delG mutation as well as a lack of exon 12, confirming intragenic crossover as the mechanism of reversion in patient RNT. The presence of colonies with only one of the two mutations suggests that mosaicism had been incomplete at the time when the cell line was established. Due to the patient's sudden death from cerebral hemorrhage, no primary blood cells were available for further studies. Since there was no improvement of his haematological parameters during the observation period of three years prior to his death, the somatic recombination event giving rise to mosaicism may have been limited to a common precursor of his T- and B-cell lineages.

The last patient in this series is patient **EUFA173**, who had previously been re-assigned from complementation group H to group A by the Amsterdam group (Joenje et al., 2000). We confirmed this re-assignment by showing normalization of the G_2-phase cell cycle blockage after transfection of his primary fibroblasts with the wildtype FANCA vector. Two mutations in the FANCA gene, a large deletion spanning exons 17 to 31 and the missense mutation 2852G→A, R951Q, in exon 29 had been previously described by the group of H. Joenje (Joenje et al., 2000). Even though the maternal missense mutation affects a position that is conserved in the murine gene, its pathogenic status had yet to be proven. The strategy used was the same as described for the L324R substitution in patient MRB. As shown in Fig. 5a, transfection of PIR and SNE with LFAPEG/2852 failed to reduce the G2-phase accumulations of the null mutant cells, confirming the pathogenicity of the R951Q substitution. Sequencing of the patient's MMC-resistant lymphoblastoid cell line revealed the presence of both mutations. However, a second amino acid substitution located 15 amino acids downstream of the first one (2897A→C, E966A, Exon 30) was also present. In order to test the possibility whether this E966A-alteration (not at a conserved residue in the mouse genome) might act as a compensatory mutation, both substitutions were introduced into pSL1180/FANCA and transfected into our null cell lines PIR and SNE. As shown in Fig. 6b, transfection with the double mutant decreased G_2 phase cell cycle blockage in the same way as transfection with the wildtype LFAPEG vector. The restoration of normal cell cycle progression by the presence of both amino acid changes is suggestive of restoration of normal protein function by the second mutation. Much to our surprise, sequencing of the patient's peripheral blood mononuclear cells did not show the second amino acid substitution that had been found in his lymphoblastoid cell line, but rather showed a reversion of the maternal mutation 2852G→A to wildtype. Likewise, a newly established lymphoblastoid cell line from the patient contained only a reversion of 2852G→A.

This proves that the second amino acid change found in the patient's original LCL must have arisen *in vitro* during long-term cultivation. Since no other mutation had been found in the original LCL, the substitution E966A therefore seems to be responsible for the MMC resistance of this cell line, since it obviously compensates the defective function caused by the constitutional mutation. With regard to the reversion event, the G→A mutation creates a new direct repeat, CAGCAG, that could trigger back mutation via a slippage mechanism. There are a number of Alu repeats in the region of exons 27 to 33 of FANCA that are known to increase the likelihood of replication errors (Morgan et al., 1999).

From a clinical point of view it is worth noting that, between the ages 15 and 21, the patient's thrombocyte counts increased from 54 to 142 Gpt/l, his total leukocytes rose from 2.5 to 4.8, his granulocytes from 0.8 to 2.5 Gpt/l, and his hemoglobin from 7.5 to 9.8 mmol/l.

Discussion

Somatic cell mosaicism due to forward somatic mutation is increasingly recognized as explanation for deviations from predicted genotype-phenotype correlations (Gottlieb et al., 2001). In contrast, somatic cell mosaicism due to reverse somatic mutation (revertant mosaicism) is less well-known, but should in principle contribute in the same way to variable expressivity of genetic disorders (Wahn et al., 1998; Jonkman, 1999). In recessive diseases, somatic reversion of one of the two inherited mutations restores heterozygosity in the descendants of the reverted cell. Depending on the mechanism of somatic reversion, the function of the affected cell lineage may be partly or completely restored. Complete restoration of a cellular phenotype to wildtype usually results from mechanisms such as intragenic recombination, gene conversion, or back-mutation (reverse point mutation). Partial restoration of protein function has been observed with so-called compensating or second site mutations. Such second site somatic mutations in *cis* leave the constitutional mutation unchanged but alter the downstream DNA sequence via insertion, deletion or point mutation. As a consequence of the compensating mutation a protein with at least partial function is produced (Waisfisz et al., 1999).

Revertant mosaicism has been described as a rare cause of phenotypic modulation in single instances of X-linked and autosomal recessive diseases (for review see Jonkman, 1999; Wada et al., 2001), but revertant mosaicism appears to be a rather frequent phenomenon in the autosomal recessive genetic instability syndromes. For example, in Bloom syndrome, compound heterozygosity combined with genetic instability sets the stage for somatic reversion of the high SCE-phenotype via intragenic recombination (Ellis et al., 1995 and 1996). In occasional homozygous Bloom syndrome individuals back mutation has been reported as well (Ellis et al., 2001). In FA, intragenic recombination, gene conversion and compensatory second site mutations have been reported in lymphoblastoid cell lines of four FANCC patients, and cytogenetic evidence for mosaicism in five additional unclassified patients (Lo Ten Foe et al., 1997; Waisfisz et al., 1999). A single compound heterozygous FANCA patient has been reported to date who developed mosaicism as a consequence of a putative back mutation (Gregory et al., 2001), and two FANCA patients with homozygous mutations were shown to harbor compensatory mutations in their peripheral blood mononuclear cells (Waisfisz et al., 1999). The case history of patient EUFAR173 demonstrates that such a compensatory change can also arise during the *in vitro* cultivation of lymphoblastoid cells, explaining the conversion of these cells from MMC sensitivity to MMC resistance.

Intragenic crossover could be shown as the likely mechanism of self-correction in our FANCC patient. Intragenic crossover represents a reversion mechanism that requires compound heterozygosity. It has been described as the predominant mechanism of reversion in Bloom syndrome (Ellis et al., 1995 and 1996) and in the lymphoblastoid cell line of a single FANCC patient (Lo Ten Foe et al., 1997), but there are no reports to date of self-correction via intragenic recombination for any of the other known FA genes. The previously published FANCC patient had a single base deletion in exon 1 (67delG) together with a single base insertion in exon 14 (1806insA). Our FANCC patient has the same single base deletion in exon 1 but a different second mutation located in exon 12. In both patients, the intragenic recombination event seems to have been facilitated by the mutually distant locations of the paternal and the maternal mutations.

In the remaining four of our five patients the uniformity of the type of reversion is impressive. Each of these four patients carries compound mutations in the FANCA gene, and in each of them one of the two constitutional point mutations has reverted back to the original wildtype sequence. In each of these four cases reversion is complete in the patient's B-cell derived lymphoblastoid cell lines, whereas the findings in peripheral blood mononuclear cells (mostly T-cells) indicate variable degrees of co-existence of mutated and reverted cells. Three of the four reversions took place in the region of exons 10 to 11 of the FANCA gene which is known as a highly mutable region due to the abundance of repetitive elements (Morgan et al., 1999; Wijker et al., 1999). To illustrate this point, Table 2 summarizes the types of DNA sequence motifs surrounding the respective mutation/reversion events, including motifs found within ± 20 bp that are known to play an important role in the breakage and rejoining of DNA (Cooper and Krawcak, 1993; Huff et al., 1995; Levran et al., 1997; Ianzano et al., 1997).

How can the apparent non-randomness of the reversion events, leading in each instance to the precise restoration of the wildtype sequence, be reconciled with the stochastic nature of genetic changes? One obvious explanation concerns the constraints imposed by selection which convey a proliferative advantage on cells with complete rather than partial restoration of protein function. In the case of our patients, hypothetical random base alterations resulting in back mutation would overwhelmingly yield non-conservative amino acid exchanges such that protein function might be impaired. In addition, there might be constraints imposed by DNA structure which would favor the restoration of the original sequence. For example, in our patients STT and URD the respective mutations/reversions are located at the 5′ end of a palindromic sequence whose hairpin structure might be important for protein binding. Back

Table 2. Sequence environments and DNA motifs surrounding the mutation/reversion sites in FANCA (mutations and reversions are shown in bold letters)

Patient code	Exon	Mutation	Reversion	Mutation motifs[a] (+/- 20 bp around the mutation)	Direct repeats (+/- 20 bp)
STT	10	AGGAG**T**AGTCCTCC	A[GGAG**G**AGTCCTCC][b]	2 CTT, TGGA, CAGG(CCTG)	AGGAGG, AGTAGT, TCCTCC
URD	10	**T**A[GGAGGAGTCCTCC][b]	**C**A[GGAGGAGTCCTCC][b,c]	CTT, 2 TGGA, CAGG	AGGAGG, TCCTCC
MRB (UPN127)	11	CCC**G**GA	C**C**C**T**GA[c]	2 CTT	CCC, TTCTTC, ACTCACTC
EUFA173 (Blood cells)	29	C**A**GCAGG[c]	C**G**GCAGG[c]	CAGG, 2 CTT, TGGA	CAGCAG
EUFA173 (Lymphoblastoid cell line)[d]	30	C**A**GCAGG[c]	TTTCTCCCTG**C**GTCC	2 CTT, 2 CAGG, TGGA	TTTCTCCC, GGGGG

[a] CTT = Topoisomerase I cleavage site consensus sequence (Bullock et al., 1975). TGGA = Deletion hot spot consensus sequence, which resembles the putative arrest site for DNA polymerase α (Krawcak and Cooper, 1991). CAGG/CCTG = Mutation hot spot sequence (Huff et al., 1995; Levran et al., 1997).
[b] The sequence in parentheses [] represents a palindrome.
[c] Motifs directly found in the mutation locus are underlined.
[d] In the lymphoblastoid cell line of patient EUFA173, the reversion is located 45 nucleotides downstream of the mutation.

mutation combined with selection therefore might explain the surprisingly uniform pattern of reversion in our FANCA-patients.

Other possibilities of reversion of course are intragenic recombination and gene conversion. Recombination and gene conversion events can be excluded in two of our FANCA patients because their second allele carries a deletion within the homologous sequence. Moreover, recombination and gene conversion events require somatic pairing between homologous chromosomes, a process which is exceedingly rare in somatic cells. Since human chromsome 16 (containing the FANCA locus) harbors large blocks of heterochromatin known to promote somatic pairing (Haaf et al., 1986), the possibility of somatic pairing cannot be ruled out for the non-deletion FANCA patients. Additional studies with intragenic and flanking polymorphic markers will be required to distinguish between back mutation, intragenic recombination or gene conversion as possible mechanism for restoration of the wildtype sequence. Given the postulated involvement of FA genes in recombinational types of DNA repair (Howlett et al., 2002; Thompson and Schild, 2002), it would be surprising if, in patients with defective FA gene functions, recombination or gene conversion rather than back mutation would surface as the chief mechanism of reversion.

The clinical course of FA is highly variable and may be determined in part by complementation group and mutation type (Gillio et al., 1997; Faivre et al., 2000). Whether revertant mosaicism leads to clinical improvement depends on when and where the reversion occurred during evolution of the various bone marrow cell lineages (Lo Ten Foe et al., 1997; Gregory et al., 2001). From a hematological and functional point of view, both T- and B-lymphocytic cell types were reverted in all of our patients. The fact that we observed bimodality of chromosome breakage and intermediate cell cycle patterns at the initial evaluation of these patients probably reflects the relative longevity of subpopulations of peripheral blood lymphocytes in which constitutionally defective cells persist long after somatically self-corrected cells have emerged. In contrast to the patient's normal lymphocyte situation, their erythroid, leukocytic and megakaryocytic cell lineages showed various degrees of impairment. However, the status of these affected cell lineages improved over time in four of our five patients. In only a single patient (RNT, FANCC) the hematological situation did not improve during a three-year observation period, and he died from cerebral haemorrhage at six years of age after his thrombocyte counts had continuously decreased to 11 Gpt/l and his hemoglobin from 7.0 to 3.7 mmol/l. In this patient, the reversion event must have been limited to a progenitor cell of the lymphocytic cell lineages. In our FANCA patients, the reversion must have taken place in a common progenitor of all blood cell lineages, possibly a hematopoietic stem cell. With respect to the prospects of gene therapy, the obvious *in vivo* selective advantage of spontaneously reverted cells is encouraging, even though selection appears to be slow and self-corrected as well as defective cells may co-exist for many years in long-lived cell compartments such as the patient's lymphocytes.

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