

## Evidence for at Least Eight Fanconi Anemia Genes

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### Summary

Fanconi anemia (FA) is an autosomal recessive chromosomal breakage disorder with diverse clinical symptoms including progressive bone marrow failure and increased cancer risk. FA cells are hypersensitive to crosslinking agents, which has been exploited to assess genetic heterogeneity through complementation analysis. Five complementation groups (FA-A through FA-E) have so far been distinguished among the first 20 FA patients analyzed. Complementation groups in FA are likely to represent distinct disease genes, two of which (*FAC* and *FAA*) have been cloned. Following the identification of the first FA-E patient, additional patients were identified whose cell lines complemented groups A–D. To assess their possible assignment to the E group, we introduced selection markers into the original FA-E cell line and analyzed fusion hybrids with three cell lines classified as non-ABCD. All hybrids were complemented for crosslinker sensitivity, indicating nonidentity with group E. We then marked the three non-ABCDE cell lines and examined all possible hybrid combinations for complementation, which indicated that each individual cell line represented a separate complementation group. These results thus define three new groups, FA-F, FA-G, and FA-H, providing evidence for a minimum of eight distinct FA genes.

### Introduction

Fanconi anemia (FA) is a rare autosomal recessive disease with variable clinical symptoms including skeletal and renal abnormalities, microcephaly, and skin pigmentation abnormalities. Patients suffer from a progressive bone marrow failure (panmyelopathy) and increased cancer risk, predominantly acute myeloid

leukemia (Liu et al. 1994; Buchwald et al. 1997). FA cells are chromosomally unstable and exhibit a marked hypersensitivity to crosslinking agents such as diepoxybutane, mitomycin C, and cisplatin, a feature that is used to assess the FA diagnosis. The crosslinker hypersensitive phenotype has been exploited to assess genetic heterogeneity through complementation analysis. Using an Epstein-Barr virus (EBV)-immortalized lymphoblastoid FA cell line (HSC72) provided with ouabain resistance and hypoxanthine guanine phosphoribosyl transferase (HPRT) deficiency as selection markers, Duckworth-Rysiecky et al. (1985) carried out cell fusions with unmarked lymphoblast lines from six unrelated FA patients, resulting in proliferating hybrids that survived in selective medium. Three hybrids continued to show crosslinker hypersensitivity and were thus assigned to complementation group A, defined by their functional identity with the fusion partner HSC72. The remaining three hybrids had a normal crosslinker sensitivity and were thus assigned to (a) group(s) different from A (then termed “B”) (Duckworth-Rysiecki et al. 1985). Subsequent cell-fusion studies using genetically marked derivatives of the three non-A cell lines revealed that each cell line represented a separate complementation group, termed “B,” “C,” and “D,” thus totaling four groups (Strathdee et al. 1992a). Among 13 additional patients subsequently analyzed one patient’s cell line complemented groups A–D and thus represented a fifth group, FA-E (Joenje et al. 1995). Complementation groups in FA are likely to represent distinct disease genes (Buchwald 1995), since the genes for two groups (*FAC* and *FAA*) have been cloned and mapped to chromosomes 9q3.22 and 16q24.3, respectively (Strathdee et al. 1992a, 1992b; Pronk et al. 1995; Lo Ten Foe et al. 1996; Fanconi Anaemia/Breast Cancer Consortium 1996), while *FAD* has been mapped to chromosome 3p22-26 (Whitney et al. 1995).

Following the identification of the first FA-E patient, additional patients were identified whose cell lines complemented groups A–D (Joenje 1996). To assess their possible assignment to group E, we extended the complementation analysis by introducing suitable selection markers into the original FA-E cell line and three cell

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lines classified as non-ABCD. The results indicate that each of these three cell lines represents a new complementation group, implying the existence of at least eight complementation groups in FA.

## Patients, Material, and Methods

### *Patients and Controls*

EBV-immortalized cell lines were established from blood samples, obtained—after informed consent— from ethnically and clinically unselected and unrelated FA patients. The FA diagnosis was based on clinical symptoms in combination with hypersensitivity to crosslinking agents, as determined in a standard chromosomal breakage test. Ancestries of the patients whose cell lines were studied were Turkish (EUFA130), Dutch (EUFA121), and German (EUFA143 and EUFA173). The cell line EUFA12 was from a healthy control (Lo Ten Foe et al. 1996).

### *Cell Culture*

B lymphoblastoid cell lines were obtained by immortalization with EBV following standard procedures. Culture medium was RPMI1640 supplemented with 1 mM glutamine (Gibco BRL), 1 mM sodium pyruvate, and 10% FCS (Gibco BRL). Selection media contained in addition the antibiotic G418 (400 µg/ml Geneticin; Gibco BRL), hypoxanthine/aminopterin/thymidine (HAT) solution (Gibco BRL), or hygromycin (200 µg/ml; Boehringer Mannheim) as indicated.

### *Introduction of Selection Markers*

The reference FA-E cell line EUFA130 was provided with two selection markers generating the universal fusion partner EUFA130NT. Resistance to the antibiotic G418 (N) was generated by transfection with pSV2neo, and thioguanine resistance (T) was induced by HPRT mutagenesis as described by Joenje et al. (1995). The HPRT mutation confers sensitivity to growth in medium containing HAT; HAT blocks de novo synthesis of purines and kills HPRT<sup>-</sup> cells, which are unable to use the salvage pathway of purine synthesis. HAT sensitivity is a recessive trait, whereas G418 resistance is dominant, so that fusion hybrids generated from EUFA130NT with unmarked lymphoblasts could be selected by growth in medium containing both G418 and HAT (Joenje et al. 1995). From the cell lines EUFA130, EUFA121, EUFA143, and EUFA173, G418-resistant (N) and hygromycin-resistant (H) derivatives were constructed by transfection with pSV2Neo and pSV2hph, respectively, as described by Strathdee et al. (1992a). Hybrids between N and H fusion partners were selected in medium containing both G418 (400 µg/ml) and hygromycin (200 µg/ml).

### *Complementation Analysis*

Polyethylene glycol-induced cell fusion was carried out as described by Joenje et al. (1995). In brief,  $2 \times 10^7$  lymphoblasts from each fusion partner were mixed, pelleted, and resuspended in serum-free (SF) medium and divided into four equal portions, for four parallel fusion experiments. After centrifugation, cell fusion was induced by gently resuspending the cell pellets in 1 ml of a solution containing 50% polyethylene glycol (type 1000; Merck) in SF medium. After 1 min, 8 ml of SF medium were added, and after 30 min the cells were washed by centrifugation and resuspension in complete medium. After 48 h at 37°C, selection of hybrids was started by adding medium containing HAT plus G418 (400 µg/ml) (fusions with EUFA130NT) or G418 (400 µg/ml) plus hygromycin (200 µg/ml) (fusions with the N and H partners). Successful fusion experiments typically showed outgrowth of hybrids after 2–5 wk of culturing in selective medium.

### *Growth Inhibition Assay*

Crosslinker sensitivity of cell lines and hybrids was assessed in a growth inhibition test (Ishida and Buchwald 1982; Joenje et al. 1995); cultures were initiated at  $5 \times 10^4$ /ml in multiple 5-ml aliquots containing various concentrations of mytomycin C (0–100 nM) and allowed to grow for 3–10 d until the cultures not exposed to MMC had undergone at least three population doublings; all parallel cultures were then counted with a Coulter counter. Hybrids were grown in the continuous presence of hybrid-selective medium to which the various concentrations of MMC were added.

### *Transfection*

Lymphoblasts (EUFA130, -121, -143, and -173) were electroporated with pDR2-based expression plasmids containing the coding sequence of *FAA* or *FAC* as described by Kruyt et al. (1996). Cells surviving hygromycin selection were tested for MMC-induced growth inhibition to assess complementation of crosslinker hypersensitivity (Ishida and Buchwald 1992; Joenje et al. 1995). All transfections were successful, except for EUFA121, which in the case of the *FAA*-expressing plasmid failed to survive hygromycin selection following nine independent transfections.

### *Ploidy of Fusion Hybrids*

Tetraploidy of the selected hybrid cell populations was checked by counting chromosomes in 20 randomly selected metaphases. Of the 61 viable hybrids, 52 had an average chromosome number between 88 and 93 per cell; 4 had 83, 84, 84, and 87 per cell, respectively. Only three hybrids had less than an average of 80 chromosomes per cell, that is, 71, 74, and 79. Two hybrids

(both EUFA121H × EUFA121N) had only 46 chromosomes per cell and were excluded from the analysis.

*Authentication of Hybrids and Transfected Cell Lines*

To exclude inadvertent mix-ups of cell lines during cell culturing, hybrids and transfected cell lines were authenticated by DNA fingerprinting, by use of the VNTR minisatellite marker D1S80 in addition to the microsatellite markers D4S391, D9S175, D10S561, and D17S579, and comparison of the alleles found with those present in the original cell lines.

**Results and Discussion**

To be able to select for heterokaryotic hybrids resulting from fusions with the reference FA-E cell line EUFA130 we generated a universal fusion partner, EUFA130NT, by genetic marking with both neomycin resistance (N, conferring resistance to the antibiotic G418) and HPRT deficiency (T, conferring resistance to 6-thioguanine and sensitivity to medium containing HAT). After fusion with any unmarked cell line, selec-

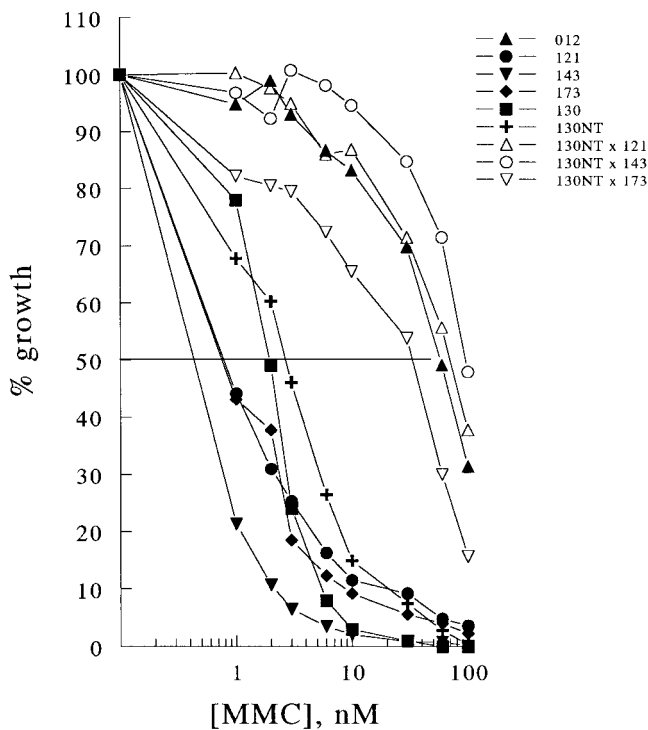
tive outgrowth of hybrid cell lines was achieved by culturing in medium containing both G418 and HAT. EUFA130NT cells were fused with the three non-ABCD cell lines EUFA121, EUFA143, and EUFA173, and selected hybrids were analyzed for complementation by testing their sensitivity to MMC-induced growth inhibition. As shown in figure 1, all three hybrid cell lines were complemented for crosslinker sensitivity ( $IC_{50}$  values 30–100 nM vs. <1–3 nM for the single cell lines). These results indicated that patients EUFA121, EUFA143, and EUFA173 did not belong to group E.

To assess possible heterogeneity within these three non-ABCDE patients, we prepared two drug-resistant derivatives from each cell line, N and H, by transfection with the plasmids pSV2neo and pSV2hph, respectively, thus allowing hybrids to be selected in medium containing both the antibiotic G418 and hygromycin as described by Strathdee et al. (1992a). Similarly marked derivatives of the FA-E cell line EUFA130 were included in this study.

Table 1 summarizes the results from all the fusion experiments from which viable hybrids were generated, involving 16—including all reciprocal—combinations. As expected, all hybrids combining two differently marked derivatives of the same cell line (homokaryons) continued to show crosslinker sensitivity, indicating that complementation was not an artifactual consequence of the cell fusion and selection procedure. In contrast, all heterokaryotic hybrids were complemented, that is, exhibiting  $IC_{50}$  values of  $\geq 30$  nM. Some hybrids (e.g., EUFA143N × EUFA130H) were even more resistant to MMC than was the average wild-type or complemented hybrid cell line; we have no explanation for this phenomenon. Overall, the data indicate that each of the four cell lines analyzed belongs to a distinct complementation group.

Transfection experiments further substantiated this result. As expected, non-ABCD cells successfully transfected with expression plasmids containing cDNAs for *FAC* and *FAA*—known to correct the defect in FA-C and FA-A cell lines, respectively (Strathdee et al. 1992b; Lo Ten Foe et al. 1996)—failed to be corrected for crosslinker hypersensitivity (results not shown; for details, see Patients, Material, and Methods).

Our results thus indicate the existence of three new complementation groups in FA: FA-F, FA-G, and FA-H, represented by patients EUFA121, EUFA143, and EUFA173, respectively, adding up to a total of eight complementation groups (table 2). Unlike groups A–D, to which multiple patients have been assigned (Joenje et al. 1995, Joenje 1996), groups E–H are represented by single patients. However, since ongoing complementation studies have identified 12 additional patients as being non-ABCD (Joenje 1996; F. di Summa, A. B. Oostra, M. A. Roomans, H. Joenje, unpublished data), it seems



**Figure 1** Complementation of MMC sensitivity in hybrids prepared from EUFA130NT (FA-E) cells with EUFA121, EUFA143, and EUFA173. Growth inhibition was assessed as described in Patients, Material, and Methods. Curves are shown for EUFA130 (■), EUFA130NT (+), EUFA121 (●), EUFA143 (▼), EUFA173 (◆), and the hybrids EUFA130NT × EUFA121 (△), EUFA130NT × EUFA143 (○), and EUFA130NT × EUFA173 (▽). EUFA12 (▲) is from a healthy control. All hybrids of EUFA130 with EUFA121, -143, and -173 are complemented, indicating nonidentity with the E group.

**Table 1**

**Complementation Analysis of Four Non-ABCD FA Lymphoblastoid Cell Lines**

	EUFA130H (5.6 ± 1.5; N = 5)	EUFA121H (1.0 ± .6; N = 4)	EUFA143H (1.6 ± .9; N = 3)	EUFA173H (2.5 ± 1.5; N = 6)
EUFA130N (2.4 ± 1.0; N = 7)	3.6 ± 1.1 (4/24)	61.3 ± 8.5 (4/4)	83.8 ± 25 (4/4)	73.0 ± 19.9 (4/4)
EUFA121N (1.3 ± .7; N = 4)	40.0 ± 0 (1/24)	2.5 ± 0 (3/12)	90.0 ± 14.1 (4/4)	30.0 ± 5.0 (3/4)
EUFA143N (2.4 ± 1.2; N = 5)	193.0 ± 67.5 (4/4)	78.0 ± 9.6 (4/4)	3.7 ± 1.4 (3/4)	38.8 ± 4.8 (4/4)
EUFA173N (1.7 ± 1.4; N = 4)	67.0 ± 6.7 (5/15)	57.5 ± 11.9 (4/4)	95.0 ± 17.3 (4/4)	3.8 ± .3 (4/4)

NOTE.—Cell lines, marked with G418-resistance (N) or hygromycin-resistance (H), were fused, and, following selection in medium containing G418 plus hygromycin, hybrid cell lines were assayed for MMC sensitivity (see Methods). EUFA130 is the reference group E cell line. Numbers indicate average IC<sub>50</sub> values for multiple independently obtained hybrids, ± SD (cf. fig. 1). The numbers of successful fusions per total attempted fusions are indicated in parentheses. Values of IC<sub>50</sub> for the parental cell lines, determined in parallel with the relevant hybrids, are indicated within parentheses. Value of IC<sub>50</sub> for the healthy control cell line EUFA12 was 54 ± 22 (N = 17); multiple healthy controls were 47 ± 12 nM, range 32–70 nM (N = 9).

likely that at least some of these patients are to be assigned to groups E–H, while others may represent still new complementation groups. Further cell-fusion studies will be necessary to resolve this question.

With the implication of eight separate disease genes FA has an even higher degree of locus heterogeneity than xeroderma pigmentosum, with seven complementation groups (Hoeijmakers and Bootsma 1994). This heterogeneity complicates the molecular diagnosis of FA. The fact that certain complementation groups prevail in specific populations (FA-C in Ashkenazi Jews [Whitney et al. 1993; Verlander et al. 1994], FA-A in South African Afrikaans-speaking people [Pronk et al. 1995] and Italians [Savoia et al. 1996]) helps to set priorities for mutation screens. On the other hand, all eight complementation groups have been found in a sample of 21 FA patients recruited from a relatively small geographic area (Germany and the Netherlands [Joenje 1996]), which suggests that mutation detection may remain cumbersome for a sizable proportion of FA patients worldwide.

Given the similar phenotype of FA patients and the specific features that characterize FA cells, it is likely

that FA genes function in a molecular pathway of some complexity. This pathway is likely to be novel, since the FA gene products identified to date (FAA and FAC) have no homologies to any known proteins (D’Andrea 1996). At the clinical level, mutations in FA genes are associated with developmental abnormalities (typically affecting the skeleton and kidneys), bone marrow failure, and increased cancer risk (Buchwald et al. 1997). At the cellular level, chromosomal instability, spontaneous cell-cycle arrest, and hypersensitivity to cross-linking agents are typically associated with the FA phenotype. In addition, recent work has indicated that FA cells are more prone to apoptosis than are non-FA cells (Kruyt et al. 1996; Cumming et al. 1996; Whitney et al. 1996). The relationship between these diverse cellular functions is unknown, but the FA genes must play an integrating role. Unlike in xeroderma pigmentosum, where the implicated genes are known to be interconnected through their role in transcription and DNA repair/replication (Hoeijmakers and Bootsma 1994), the molecular pathology of FA is still elusive and continues to be a challenge for geneticists and molecular biologists (D’Andrea 1996). Whether the functional defect in FA involves FA gene products forming a multi-protein complex, a reaction sequence, or a combination of these remains to be elucidated. Identification of the remaining six or more FA genes should facilitate our understanding of this disease.

**Table 2**

**Cell Lines Representing Eight FA Complementation Groups**

Group	Cell Line	Reference
FA-A	HSC72	Duckworth-Rysiecki et al. (1985)
FA-B	HSC230	Strathdee et al. (1992a)
FA-C	HSC536	Strathdee et al. (1992a)
FA-D	HSC62	Strathdee et al. (1992a)
FA-E	EUFA130	Joenje et al. (1995)
FA-F	EUFA121	Present study
FA-G	EUFA143	Present study
FA-H	EUFA173	Present study

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