Molecular Cell, Vol. 7, 241–248, February, 2001, Copyright ©2001 by Cell Press

Positional Cloning of a Novel Fanconi Anemia Gene, *FANCD2*

Cynthia Timmers,*# Toshiyasu Taniguchi,§# James Hejna,* Carol Reifsteck,* Lora Lucas,* Donald Bruun,* Matthew Thayer,† Barbara Cox,* Susan Olson,* Alan D. D'Andrea,§ Robb Moses,* and Markus Grompe*‡

*Department of Molecular and Medical Genetics
†Vollum Institute
‡Department of Pediatrics
Oregon Health Sciences University
Portland, Oregon 97201
§Dana-Farber Cancer Institute
Harvard Medical School
Boston, Massachusetts 02115

Summary

Fanconi anemia (FA) is a genetic disease with birth defects, bone marrow failure, and cancer susceptibility. To date, genes for five of the seven known complementation groups have been cloned. Complementation group D is heterogeneous, consisting of two distinct genes, *FANCD1* and *FANCD2*. Here we report the positional cloning of *FANCD2*. The gene consists of 44 exons, encodes a novel 1451 amino acid nuclear protein, and has two protein isoforms. Similar to other FA proteins, the FANCD2 protein has no known functional domains, but unlike other known FA genes, *FANCD2* is highly conserved in *A. thaliana*, *C. elegans*, and *Drosophila*. Retroviral transduction of the cloned *FANCD2* cDNA into FA-D2 cells resulted in functional complementation of MMC sensitivity.

Introduction

Fanconi anemia (FA) is an autosomal recessive disorder characterized by progressive bone marrow failure, cancer predisposition, and multiple developmental defects (Fanconi, 1967; Alter, 1993). Cells from FA patients display a characteristic hypersensitivity to agents that produce interstrand DNA cross-links, such as mitomycin C (MMC) or diepoxybutane (DEB). Therefore, the function(s) of the FA proteins pertain to multiple important cellular and organismal processes. FA is genetically heterogeneous, and to date at least seven complementation groups (FANCA–G) have been identified by cell fusion techniques (Joenje et al. 1997, 2000). This observation has resulted in the hypothesis that the FA genes define a multicomponent pathway involved in cellular responses to DNA cross-links (Garcia-Higuera et al., 1999). Five of the FA genes (*FANCA*, *C*, *E*, *F*, and *G*) have been cloned (Strathdee et al., 1992b; The Fanconi Anaemia/Breast Cancer Consortium, 1996; Lo Ten Foe et al., 1996; de Winter et al., 1998, 2000a, 2000b), and the FANCA, C, and G proteins have been shown to form a molecular complex with primarily nuclear localization (Kupfer et al., 1997; Garcia-Higuera et al., 1999; Waisfisz et al., 1999a). FANCC also localizes to the cytoplasm, indicating a potential role in that compartment (Youssoufian, 1994, 1996). No functional domains are apparent in the protein sequences of the FA genes cloned to date, and the biochemical function(s) of the FA pathway remains unknown. Interestingly, database searches reveal no strong homologs of the FANCA, C, E, F, and G proteins in nonvertebrate species. FANCF has weak homology of unknown significance to an *E. coli* RNA binding protein (de Winter et al., 2000b). The formation of the nuclear complex among the FANCA, FANCC, and FANCG proteins is abnormal in all complementation groups studied except in cell lines from the rare group D (Yamashita et al., 1998; Garcia-Higuera et al., 1999; Garcia-Higuera and D'Andrea, 1999; Waisfisz et al., 1999a). Therefore, FANCD may define a function of the FA pathway either upstream, downstream, or independent of the action of the multiprotein FA complex. Here, we report the positional cloning of a gene mutated in the FA group D cell line PD20. In contrast to other previously cloned FA genes, *FANCD2* has highly conserved homologs in lower eukaryotes, indicating the possible conservation of the FA pathway in lower organisms.

Results

Nomenclature

PD20 is an FA cell line that has been assigned to complementation group D because lymphoblasts from this patient failed to complement HSC62 (Strathdee et al., 1992a), the reference cell line for group D (Jakobs et al., 1996). In previous publications, we therefore referred to the gene mutated in PD20 as *FANCD* (Whitney et al., 1995; Jakobs et al., 1996, 1997; Hejna et al., 2000). However, new results described in this paper suggest that the gene mutated in HSC62 (*FANCD1*) and the gene mutated in PD20 (*FANCD2*) are different.

Mutation Analysis of Positional cDNA Candidates

We previously reported the localization of the gene mutated in PD20 to chromosome 3p using microcell-mediated chromosome transfer into FA cell line PD20 (Whitney et al., 1995). Detailed analysis of five microcell hybrids that contained small overlapping deletions encompassing the locus narrowed the candidate region for the *FANCD2* gene to ~200 kb (Hejna et al., 2000). Three candidate ESTs were localized in or near this *FANCD2* critical region (Hejna et al., 2000). Using 5′ and 3′ RACE to obtain full-length cDNAs, the genes were sequenced and the expression pattern of each was analyzed by Northern blot. EST SGC34603 had ubiquitous and low level expression of a 5 kb and 7 kb mRNA similar to previously cloned FA genes (Figure 1) (Strathdee et al., 1992b; The Fanconi Anaemia/Breast Cancer Cosortium, 1996; Lo Ten Foe et al., 1996; de Winter et al., 1998, 2000b). Open reading frames were found for TIGR-A004X28, AA609512, and SGC34603 and were 234, 531,

‖To whom correspondence should be addressed (e-mail: grompem@ohsu.edu).
#These authors contributed equally to this work.

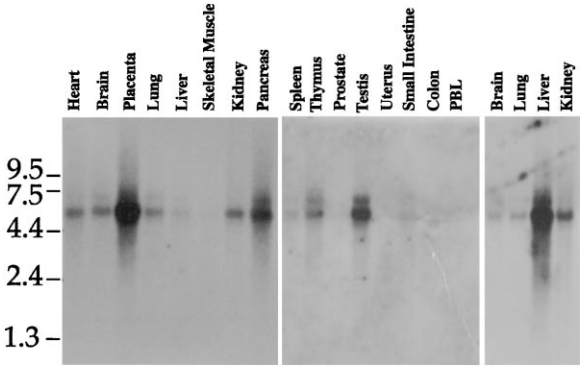

Figure 1. Northern Blot of *FANCD2*

Blots were probed with a full-length FANCD2 cDNA and exposed for 24 hr. The four lanes on the far right are from human fetal tissues, and the others are from human adults. The size markers are given in kbp at the left.

and 4413 bp in length, respectively. All three were analyzed for mutations in PD20 cells by sequencing cloned RT–PCR products. Whereas no sequence changes were detected in TIGR-A004X28 and AA609512, five sequence changes were found in SGC34603 (Table 1). Next, we determined the structure of the SGC34603 gene by using cDNA sequencing primers on BAC 177N7 from the critical region. Forty-four exons were discovered, with the start codon localized in exon 2. The open reading frame predicts a protein of 1451 amino acids (Figure 2). Based on the genomic sequence information, PCR primer pairs were designed, the exons containing putative mutations were amplified, and allele-specific assays were developed to screen the PD20 family as well as 568 control chromosomes. Three of the alleles were common polymorphisms; however, two changes were not found in the controls and, thus, represented potential mutations (Table 1). The first was a maternally inherited A→G change at nt 376. In addition to changing an amino acid (S126G), this alteration was associated with missplicing and insertion of 13 bp from intron 5 into the mRNA. Forty-three of forty-three (100%) independently cloned RT–PCR products with the maternal mutation contained this insertion (data not shown), whereas only 3% (1/31) of control cDNA clones displayed missplced mRNA. The 13 bp insertion generated a frameshift and predicts a severely truncated protein only 180 amino acids in length. The second alteration was a paternally inherited missense change at position 1236 (R1236H). The segregation of the mutations in the PD20 core family is depicted in Figure 3. These findings suggested that SGC34603 is the *FANCD2* gene.

Table 1. *FANCD2* Sequence Alterations

	Mutations	
PD20	nt376a→g	S126G/splice
	nt3707g→a	R1236H
VU008	nt904c→t	R302W
	nt958c→t	Q320X
PD733		deletion of exon 17
	Polymorphisms	
	nt1122a→g	V374V
	nt1440t→c[a]	H480H
	nt1509c→t[b]	N503N
	nt2141c→t[a,b]	L714P
	nt2259t→c	D753D
	nt4098t→g[a,b]	L1366L
	nt4453g→a[b]	3′UTR

[a] PD20 is heterozygous.
[b] VU008 is heterozygous.

The Protein Encoded by *FANCD2* Is Absent in PD20

To further confirm the identity of SGC34603 as *FANCD2*, an antibody was raised against the protein, and Western blot analysis was performed (Figure 4). The specificity of the antibody was shown by transient expression of FANCD2 in PD20 cells (Figure 4c). In wild-type cells, this antibody detected two bands (155 and 162 kDa) that we call FANCD2-S and -L (best seen in Figure 4c). FANCD2 protein levels were markedly diminished in all MMC-sensitive cell lines from patient PD20 (Figure 4a, lanes 2 and 4) but were present in all wild-type cell lines and FA cells from other complementation groups. Furthermore, PD20 cells corrected by microcell-mediated transfer of chromosome 3 also made normal amounts of protein (Figure 4a, lane 3).

Functional Complementation of FA-D2 Cells with the FANCD2 cDNA

We next assessed the ability of the cloned *FANCD2* cDNA to complement the MMC sensitivity of FA-D2 cells. The full-length *FANCD2* cDNA was subcloned into the retroviral expression vector, pMMP-puro, as previously described (Pulsipher et al., 1998). Retrovially transduced PD20 cells expressed both isoforms of the FANCD2 protein, FANCD2-S and FANCD2-L (Figure 4c). Transduction of FA-D2 (PD20) cells with pMMP-FANCD2 corrected the MMC and DEB sensitivity of the cells (Table 2, bottom). Furthermore, in an MMC kill curve assay, retrovirally corrected PD20 cells were as resistant to MMC as cells complemented by an intact human chromosome 3 (data not shown). These results further suggest that the cloned *FANCD2* cDNA encodes the FANCD2-S protein, which can be posttranslationally modified to the FANCD2-L isoform.

Analysis of a Phenotypically Reverted PD20 Clone

We next generated additional evidence demonstrating that the sequence variations in PD20 cells were not functionally neutral polymorphisms. Toward this end, we performed a molecular analysis of a revertant lymphoblast clone (PD20-cl.1) from patient PD20 that was no longer sensitive to MMC. Phenotypic reversion and somatic mosaicism are frequent findings in FA and have been associated with intragenic events such as mitotic recombination or compensatory frameshifts (Lo Ten Foe et al., 1997; Waisfisz et al., 1999b). Indeed, ~60% of maternally derived SGC34603 cDNAs had a novel splice variant inserting 36 bp of intron 5 sequence rather than the usually observed 13 bp (Figure 5). The appearance of this in-frame splice variant correlated with a de novo base change at position IVS5+6 from G to A (Figure 5), and restoration of the correct reading frame was confirmed by Western blot analysis (Figure

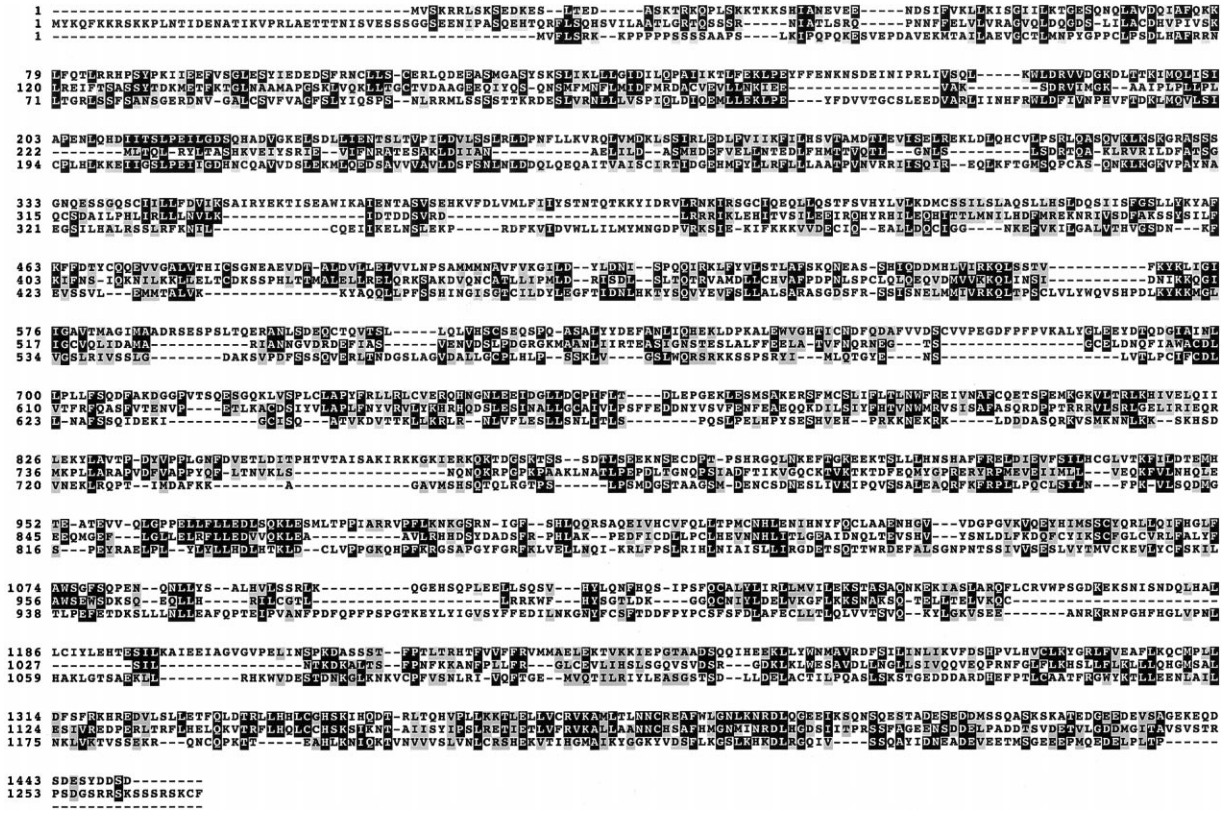

Figure 2. Amino Acid Sequence of Human FANCD2 and Alignment with Fly and Plant Homologs

The human (top), fly (middle), and plant (bottom) FANCD2 protein sequences are shown. Hypothetical proteins from several nonvertebrate eurkaryotes showed highly significant alignment scores using the BEAUTY algorithm (Worley et al., 1995). Black boxes indicate amino acid identity, and gray boxes indicate similarity. The best alignment scores were observed with hypothetical proteins in *Drosophila* ($p = 8.4 \times 10^{-58}$, GenBank accession number AAF55806) and *A. thaliana* ($p = 9.4 \times 10^{-45}$, GenBank accession number B71413).

4a, lane 5). In contrast to all MMC-sensitive fibroblasts and lymphoblasts from patient PD20, PD20-cl.1 produced readily detectable amounts of FANCD2 protein of slightly higher molecular weight than the normal protein.

Analysis of Cell Lines from Other "FANCD" Patients

The antibody was also used to screen additional FA patient cell lines, including the reference cell line for FA group D, HSC 62 (Strathdee et al., 1992a), and two other

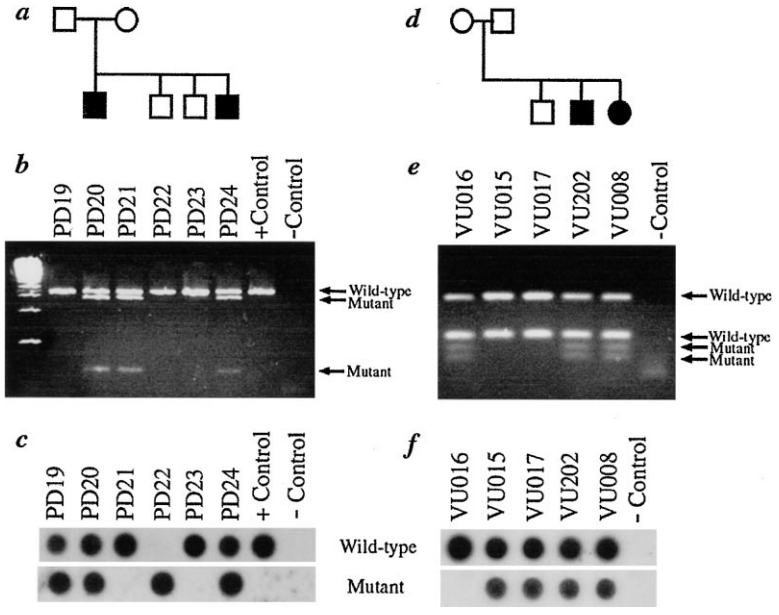

Figure 3. Allele-Specific Assays for Mutation Analysis of Two FANCD2 Families

The family pedigrees (a and d) and (b), (c), (e), and (f) are vertically aligned such that the corresponding mutation analysis is below the individual in question. (a)–(c) depict the PD20 family, and (d)–(f) depict the VU008 family. (b) and (e) show the segregation of the maternal mutations as detected by the creation of a new MspI site (PD20) or DdeI site (VU008). The paternally inherited mutations in both families were detected with allele-specific oligonucleotide hybridization (c and f).

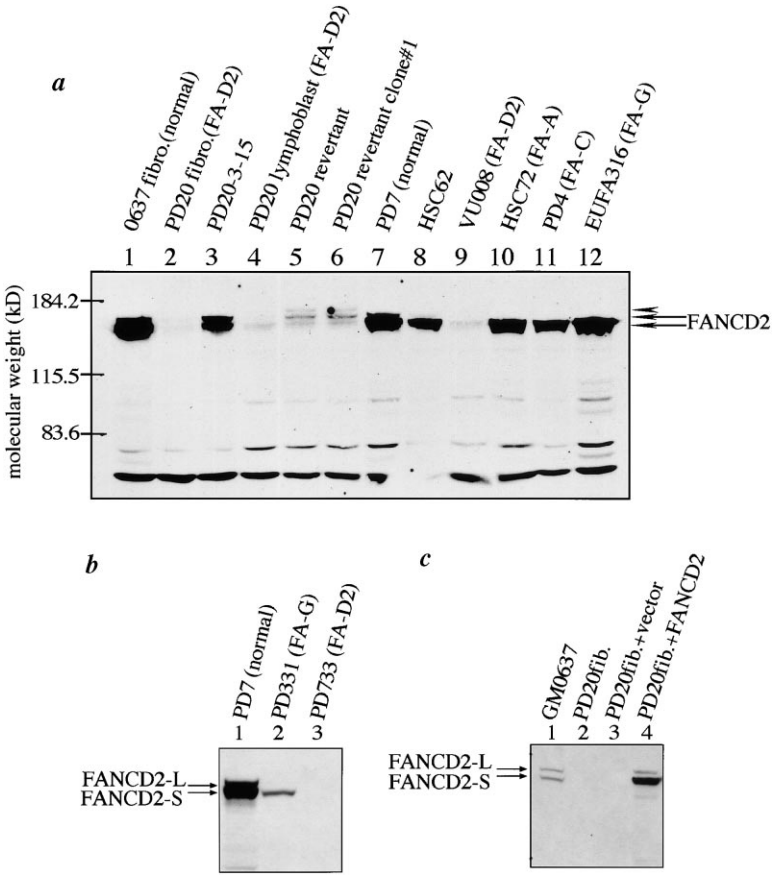

Figure 4. Western Blot Analysis of the FANCD2 Protein in Human Fanconi Anemia Cell Lines

Whole-cell lysates were generated from the indicated fibroblast and lymphoblast lines. Protein lysates (70 μg) were probed directly by immunoblotting with the anti-FANCD2 antiserum. The FANCD2 proteins (155 kDa and 162 kDa) are indicated by arrows. Other bands in the immunoblot are nonspecific.

(a) Cell lines tested included wild-type cells (lanes 1 and 7), PD20 fibroblasts (lane 2), PD20 lymphoblasts (lane 4), revertant MMC-resistant PD20 lymphoblasts (lanes 5 and 6), and chromosome 3p–complemented PD20 fibroblasts (lane 3). Several other FA group D cell lines were analyzed, including HSC62 (lane 8) and VU008 (lane 9). FA-A cells were HSC72 (lane 10), FA-C cells were PD4 (lane 11), and FA-G cells were EUFA316 (lane 12).

(b) Identification of a third FANCD2 patient. FANCD2 protein was readily detectable in wild-type and FA group G cells but not PD733 cells.

(c) Specificity of the antibody. PD20i cells transiently transfected with a FANCD2 expression vector displayed both isoforms of the FANCD2 protein (lane 4) in contrast to empty vector controls (lane 3) and untransfected PD20i cells (lane 2). In wild-type cells, the endogenous FANCD2 protein (two isoforms) was also immunoreactive with the antibody (lane 1).

cell lines identified as group D by the European Fanconi Anemia Registry (EUFAR). VU008 did not express the FANCD2 protein (Figure 4a, lane 9) and was found to be a compound heterozygote with a missense and nonsense mutation, both in exon 12. Neither mutation was found on 370 control chromosomes (Table 1; Figure 3). The missense mutation appears to destabilize the FANCD2 protein, as there is no detectable FANCD2 pro-

Table 2. Chromosome Breakage Analysis of Hybrids and Retrovirally Transduced Cells

Cell Line/Hybrids	DEB (ng/ml)	MMC (ng/ml)	Percentage of Cells with Radials	Phenotype
PD20i	300		58	S
PD24p	300		na[a]	S
VU423p	300		na[a]	S
PD319i	300		52	S
PD20i/VU423p	300		6	R
PD20i/PD24p	300		30	S
PD20i/PD319i	300		0	R
PD20i		40	48	S
VU423i		40	78	S
PD20i/VU423i		40	10	R
VU423i + chr. 3, clone 1		40	74	S
VU423i + chr. 3, clone 2		40	68	S
VU423i + chr. 3, clone 3		40	88	S
PD20i + empty vector	0	0	2	
		40	24	S
	200		62	S
PD20i + FANCD2 vector	0	0	0	
		40	2	R
	200		10	R

Groups of experiments are separated by line spaces. S, cross-linker sensitive; R, cross-linker resistant; i, immortal fibroblast line; p, primary fibroblasts.

[a]Cell viability at this concentration was too low to score for radial formation, indicating the exquisite sensitivity of primary fibroblasts to interstrand DNA cross-links.

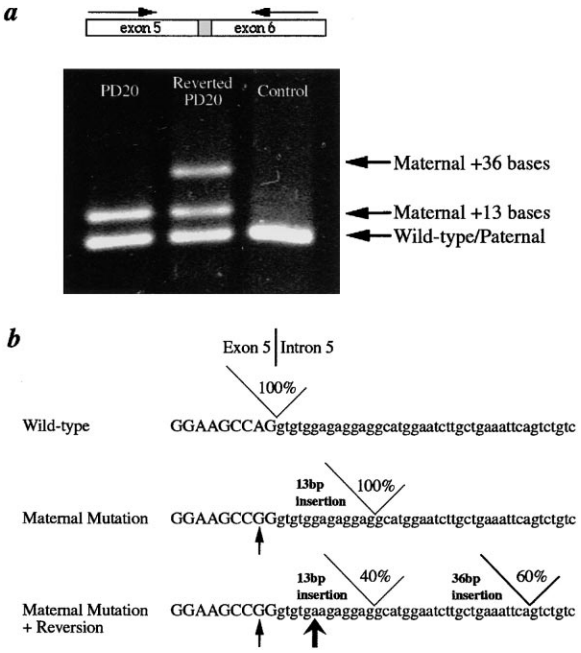

Figure 5. Molecular Basis for the Reversion of PD20 Lymphoblasts

(a) PCR primers to exons 5 and 6 were used to amplify cDNA. Control samples (right lane) yielded a single band of 114 bp, whereas PD20 cDNA (left lane) showed two bands, the larger reflecting the insertion of 13 bp of intronic sequence into the maternal allele. Reverted, MMC-resistant lymphoblasts (middle lane) from PD20 revealed a third, in-frame splice variant of 114 + 36 bp.

(b) Schematic representation of splicing at the FANCD exon 5/intron 5 boundary. In wild-type cDNA, 100% of splice events occur at the proper exon/intron boundary, whereas the maternal A→G mutation (indicated by arrow) leads to aberrant splicing, also in 100%. In the reverted cells, all cDNAs with the maternal mutation also had a second sequence change (fat arrow) and showed a mixed splicing pattern with insertion of either 13 bp (40% of mRNA) or 36 bp (60% of mRNA).

tein in lysates from VU008 cells. A third patient, PD733, also lacked FANCD2 protein (Figure 4b, lane 3) and failed to complement PD20 in whole-cell fusions (data not shown). RT–PCR showed the absence of exon 17 causing an internal deletion of the protein (Table 1). The genomic mutations in this patient have not yet been found. The correlation of the mutations with the absence of FANCD2 protein in cell lysates derived from these patients substantiates the identity of *FANCD2* as an FA gene.

In contrast, readily detectable amounts of both isoforms of the FANCD2 protein were found in HSC 62 (Figure 4a, lane 8) and VU423 (data not shown). cDNA and genomic DNA from both cell lines were extensively analyzed for mutations and none were found. In addition, a whole-cell fusion between VU423 and PD20 fibroblasts showed complementation of the chromosome breakage phenotype (Table 2, top). Microcell-mediated chromosome transfer of an intact human chromosome 3 into VU423 cells also did not complement their chromosomal breakage phenotype (Table 2, middle). Taken together, these data suggest that FA group D is genetically heterogeneous, that the gene(s) defective in HSC 62 and VU423 is distinct from *FANCD2*, and that it is not located on chromosome 3.

FANCD2 Homologs

FANCD2 has an open reading frame of 4353 bp encoding a 1451 amino acid protein with a predicted molecular weight of 166 kDa. Database searches revealed no known functional domains or motifs, with the exception of a HMG-like domain at the carboxyl terminus (Bachvarov and Moss, 1991). Unlike previously cloned FA proteins, hypothetical proteins from several nonvertebrate eurkaryotes showed highly significant alignment scores with hypothetical proteins in *Drosophila*, *A. thaliana*, and *C. elegans*. The *Drosophila* homolog has 28% amino acid identity and 50% similarity to FANCD2 (Figure 2). The FANCD2 homologs also have no currently known function or domains, and no functional studies have been carried out in the respective species. No proteins similar to FANCD2 were found in *E. coli* or *S. cerevisiae*. The functional significance of the HMG-like domain is uncertain, as the most highly conserved FANCD2 homologs do not contain a similar domain.

Discussion

Although Fanconi anemia is a rare disease, the pleiotropic effects of FA gene deficiency indicate the importance of their wild-type function for diverse cellular processes including genome stability, apoptosis, cell cycle control, and resistance to DNA cross-links (D'Andrea and Grompe, 1997). At the organismal level, FA proteins are involved in maintenance of hematopoietic and gonadal stem cells as well as the normal embryonic development of many different structures, including the skeleton and urogenital systems (Alter, 1993; Whitney et al., 1996). However, despite the interest of many laboratories in this disorder and the cloning of several FA genes, little progress has been made in the understanding of the biochemical function(s) of the pathway. Similar to other FA genes that have been cloned, the protein sequence of the novel gene identified here does not shed light onto the function of the pathway.

FANCD2 has some unusual properties that may provide some functional insights. First, FANCD2 has highly conserved homologs in organisms that are readily amenable to genetic studies, such as the identification of suppressors. Because no homologs of FANCA, C, E, and G have been identified in nonvertebrates, such experiments have not been possible with the FA genes described to date. In light of the functional connection between BRCA1 and FANCD2 (Garcia-Higuera et al., 2001 [this issue of *Molecular Cell*]), it is noteworthy that BRCA1 does not appear to have a highly conserved homolog in fruit flies. Perhaps the *Drosophila* FANCD2 homolog will be useful in the genetic dissection of this DNA damage–response pathway. Second, as shown in the accompagning paper, FANCD2 colocalizes and interacts with the breast cancer protein, BRCA1 (Garcia-Higuera et al., 2001), suggesting a connection between the pathway and BRCA1-mediated DNA repair.

Our studies also show that Fanconi anemia group D is genetically heterogenous, consisting of at least two genes, *FANCD1* and *FANCD2*. This conclusion is based on the absence of mutations in some group D patients, full complementation between different group D cell lines in whole-cell fusions, and the failure of chromo-

some 3 to correct these cells. Thus, the number of confirmed complementation groups has once again increased from seven to eight (Joenje et al., 2000). It is of interest to note that the original reference cell line for FANCD, HSC62, and the cell lines that have shown mutations in FANCD2 (PD20, VU202 [the sibling of VU008]) have some common properties that distinguish them from other FA complementation groups. In these cells, the nuclear complex between FANCA, G, and C forms normally, whereas the complex does not form in groups A, B, C, E, F, and G (Yamashita et al., 1998; Garcia-Higuera et al., 1999; Garcia-Higuera and D'Andrea, 1999; Waisfisz et al., 1999a). Indeed, work described in the accompanying paper by Garcia-Higuera et al. shows that the FANCA, B, C, E, F, and G proteins are required for posttranslational modification of FANCD2 in response to DNA damage and cell cycle status (Garcia-Higuera et al., 2001). In contrast, cells from group D1 patients are the only FA cells that display normal modification of FANCD2, suggesting that FANCD1 acts in parallel with or downstream from FANCD2.

Experimental Procedures

Northern Hybridizations

Human adult and fetal multitissue mRNA blots were purchased from Clontech (Palo Alto, CA). Blots were probed with ^{32}P-labeled DNA from EST clone SGC34603. Standard hybridization and washing conditions were used. Equal loading was confirmed by rehybridizing the blot with an actin cDNA probe (data not shown).

Mutation Analysis

Total cellular RNA was reverse transcribed using a commercial kit (GIBCO–BRL). The 5′ end section of *FANCD2* was amplified from the resulting patient and control cDNA with a nested PCR protocol. The first round was performed with primers MG471 5′-AATC GAAAACTACGGGCG-3′ and MG457 5′-GAGAACACATGAATGAA CGC-3′. The PCR product from this round was diluted 1:50 for a subsequent round using primers MG492 5′-GGCGACGGCTTCTCG GAAGTAATTTAAG-3′ and MG472 5′-AGCGGCAGGAGGTTTATG-3′. The PCR conditions were as follows: 94°C for 3 min, 25 cycles of 94°C for 45 s, 50°C for 45 s, 72°C for 3 min, and 5 min of 72°C at the end. The 3′ portion of the gene was amplified as described above but with primers MG474 5′-TGGCGGCAGACAGAAGTG-3′ and MG475 5′-TGGCGGCAGACAGAAGTG-3′. The second round of PCR was performed with MG491 5′-AGAGAGCCAACCTGAGCG ATG-3′ and MG476 5′-GTGCCAGACTCTGGTGGG-3′. The PCR products were gel purified, cloned into the pT-Adv vector (Clontech), and sequenced using internal primers.

Allele-Specific Assays

Allele-specific assays were performed in the PD20 family and 290 control samples (=580 chromosomes) The PD20 family is of mixed Northern European descent, and VU008 is a Dutch family (Whitney et al., 1995). Control DNA samples were from unrelated individuals in CEPH families (n = 95), samples from unrelated North American families with either ectodermal dysplasia (n = 95) or Fanconi anemia (n = 94). The maternal nt376a→g mutation in the PD20 family created a novel MspI restriction site. For genomic DNA, the assay involved amplifying genomic DNA using the primers MG792 5′-AGGAGA CACCCTTCCTATCC-3′ located in exon 4 and MG803 5′- GAAGTTG GCAAAACAGACTG-3′, which is in intron 5. The size of the PCR product was 340 bp, yielding two fragments of 283 bp and 57 bp upon MspI digestion if the mutation was present. For analysis of the reverted cDNA clones, PCR was performed using primers MG924 5′-TGTCTTGTGAGCGTCTGCAGG-3′ and MG753 5′-AGGTTTTGA TAATGGCAGGC-3′. The paternal exon 37 mutation (R1236H) in PD20 and exon 12 missense mutation (R302W) in VU008 were tested by allele-specific oligonucleotide (ASO) hybridization (Wu et al., 1989). For the exon 12 assay, genomic DNA was amplified with primers MG979 5′-ACTGGACTGTGCCTACCCACTATG-3′ and MG984 5′-CCTGTGTGAGGATGAGCTCT-3′. Primers MG818 5′-AGA GGTAGGGAAGGAAGCTAC-3′ and MG813 5′-CCAAAGTCCACTT CTTGAAG-3′ were used for exon 37. Wild-type (5′-TTCTCCCGA AGCTCAG-3′ for R302W and 5′-TTTCTTCCGTGTGATGA-3′ for R1236H) and mutant (5′-TTCTCCCAAAGCTGAG-3′ for R302W and 5′-TTTCTTCCATGTGATGA-3′ for R1236H) oligonucleotides were end-labeled with [γ-^{32}P]ATP and hybridized to dot-blotted target PCR products as previously described (Wu et al., 1989). The VU008 nonsense mutation (Q320X) in exon 12 created a novel DdeI site. The wild-type PCR product digests into a 117 and 71 bp product, whereas the mutant allele yields three fragments of 56, 61, and 71 bp in length. PCR in all of the above assays was performed with 50 ng of genomic DNA for 37 cycles of 94°C for 25 s, 50°C for 25 s, and 72°C for 35 s.

Generation of an Anti-FANCD2 Antiserum

A rabbit polyclonal antiserum against FANCD2 was generated using a GST-FANCD2 (N-terminal) fusion protein as an antigen source. A 5′ fragment was amplified by polymerase chain reaction (PCR) from the full-length *FANCD2* cDNA with the primers DF4 EcoRI (5′-AGCC TCgaattcGTTTCCAAAAGAAGACTGTCA-3′) and DR816 Xh (5′- GGT ATCctcgagTCAAGACGACAACTTATCCATCA-3′). The resulting PCR product of 841 bp encoding the amino-terminal 272 amino acids of the FANCD2 polypeptide was digested with EcoRI/XhoI and subcloned into the EcoRI/XhoI sites of the plasmid pGEX4T-1 (Pharmacia). A GST-FANCD2 (N-terminal) fusion protein of the expected size (54 kDa) was expressed in *E. coli* strain DH5α, purified over glutathione S-Sepharose, and used to immunize a New Zealand White rabbit. An FANCD2-specific immune antiserum was affinity purified over an AminoLink Plus column (Pierce) loaded with GST protein and over an AminoLink Plus column loaded with the GST-FANCD2 (N-terminal) fusion protein.

Immunoblotting

Cells were lysed with 1× sample buffer (50 mM Tris-HCl [pH 6.8], 86 mM 2-mercaptoethanol, 2% sodium dodecyl sulfate [SDS]), boiled for 5 min, and subjected to 7.5% polyacrylamide SDS gel electrophoresis. After electrophoresis, proteins were transferred to a nitrocellulose filter in transfer buffer (Tris 25 mM, glycine 200 mM) at 400 mA at 4°C for 4 hr. The filter was blocked for 1 hr in 5% nonfat milk in TBS (50 mM Tris-HCl [pH 8.0], 150 mM NaCl) and was incubated in primary antibody (1:1000 dilution) in TBS plus 0.1% (v/v) Tween 20 (TBS-T) overnight at 4°C . After extensive washing in TBS-T, the filter was incubated in horseradish peroxidase anti-rabbit Ig secondary antibody (Amersham) at 1:5000 dilution in TBS-T for 1 hr at room temperature. After extensive washing in TBS-T, enzyme-linked chemiluminescence (Amersham) was performed.

Cell Lines and Transfections

PD20i is an immortalized and PD733 a primary FA fibroblast cell line generated by the Oregon Health Sciences Fanconi Anemia Cell Repository (Jakobs et al., 1996). PD20 lymphoblasts were derived from bone marrow samples. VU008 is a lymphoblast and VU423 a fibroblast line generated by the European Fanconi Anemia Registry (EUFAR). VU423i was an immortalized line derived by transfection with SV40 T-antigen (Jakobs et al., 1996) and telomerase (Bodnar et al., 1998). The other FA cell lines have been previously described (Strathdee et al., 1992a; Joenje et al., 1997). Human fibroblasts were cultured in αMEM and 20% fetal calf serum. Transformed lymphoblasts were cultured in RPMI 1640 supplemented with 15% heat-inactivated fetal calf serum.

Whole-Cell and Microcell Fusions

For the whole-cell fusion experiments, a PD20 cell line (PD20i) resistant to hygromycin B and deleted for the HPRT locus was used (Jakobs et al., 1997). Controls included PD24 (primary fibroblasts from affected sibling of PD20) and PD319i (Jakobs et al., 1997) (immortal fibroblasts from a non-A, C, D, or G FA patient). Cells (2.5×10^5) from each cell line were mixed in a T25 flask and allowed to recover for 24 hr. The cells were washed with serum-free medium and then fused with 50% PEG for 1 min (Mercer and Schlegel, 1979). After removal of the PEG, the cells were washed three times with

serum-free medium and allowed to recover overnight in complete medium without selection. The next day, cells were split 1:10 into selective medium containing 400 μg/ml hygromycin B (Roche Molecular) and 1× HAT. After the selection was complete, hybrids were passaged once and then analyzed as described below. Microcell-mediated transfer of human chromosome 3 into VU423 cells was performed as previously described by us (Whitney et al., 1995).

Retroviral Transduction of FA-D2 Cells and Complementation Analysis

The full-length *FANCD2* cDNA was subcloned into the vector pMMP-puro (Pulsipher et al., 1998). Retroviral supernatants were used to transduce PD20F, and puromycin resistant cells were selected. Cells were analyzed for MMC sensitivity by the crystal violet assay (Naf et al., 1998).

Chromosome Breakage Analysis

Chromosome breakage analysis was performed by the Cytogenetics Core Lab at OHSU (Portland, OR). For the analysis (Cohen et al., 1982), cells were plated into T_{25} flasks, allowed to recover, and then treated with 300 ng/ml of DEB for two days. After treatment, the cells were exposed to colcemid for 3 hr and harvested using 0.075 M KCl and 3:1 methanol:acetic acid. Slides were stained with Wright's stain, and 50–100 metaphases were scored for radials.

Acknowledgments

This work was supported by NHLBI program project grant 1PO1HL48546 (M. G., S. O., and R. E. M.) and R01HL52725 (A. D'A.). We thank H. Joenje (Free University of Amsterdam, The Netherlands) and the European Fanconi Anemia Registry for making cell lines VU008 and VU423 available. We also thank the Fanconi Anemia Research Fund for their support.

Received August 14, 2000; revised December 11, 2000.

References

Alter, B.P. (1993). Fanconi's anaemia and its variability. Br. J. Haematol. *85*, 9–14.

Bachvarov, D., and Moss, T. (1991). The RNA polymerase I transcription factor xUBF contains 5 tandemly repeated HMG homology boxes. Nucleic Acids Res. *19*, 2331–2335.

Bodnar, A.G., Ouellette, M., Frolkis, M., Holt, S.E., Chiu, C.P., Morin, G.B., Harley, C.B., Shay, J.W., Lichtsteiner, S., and Wright, W.E. (1998). Extension of life-span by introduction of telomerase into normal human cells. Science *279*, 349–352.

Cohen, M.M., Simpson, S.J., Honig, G.R., Maurer, H.S., Nicklas, J.W., and Martin, A.O. (1982). The identification of Fanconi anemia genotypes by clastogenic stress. Am. J. Hum. Genet. *34*, 794–810.

D'Andrea, A.D., and Grompe, M. (1997). Molecular biology of Fanconi anemia: implications for diagnosis and therapy. Blood *90*, 1725–1736.

de Winter, J.P., Waisfisz, Q., Rooimans, M.A., van Berkel, C.G., Bosnoyan-Collins, L., Alon, N., Carreau, M., Bender, O., Demuth, I., Schindler, D., et al. (1998). The Fanconi anaemia group G gene FANCG is identical with XRCC9. Nat. Genet. *20*, 281–283.

de Winter, J.P., Leveille, F., van Berkel, C.G., Rooimans, M.A., van Der Weel, L., Steltenpool, J., Demuth, I., Morgan, N.V., Alon, N., Bosnoyan-Collins, L., et al. (2000a). Isolation of a cDNA representing the Fanconi anemia complementation group E gene. Am. J. Hum. Genet. *67*, 1306–1308.

de Winter, J.P., Rooimans, M.A., van Der Weel, L., van Berkel, C.G., Alon, N., Bosnoyan-Collins, L., de Groot, J., Zhi, Y., Waisfisz, Q., Pronk, J.C., et al. (2000b). The Fanconi anaemia gene FANCF encodes a novel protein with homology to ROM. Nat. Genet. *24*, 15–16.

Fanconi, G. (1967). Familial constitutional panmyelocytopathy, Fanconi's anemia (F.A.) I. Clinical aspects. Semin. Hematol. *4*, 233–240.

The Fanconi Anaemia/Breast Cancer Consortium. (1996). Positional cloning of the Fanconi anaemia group A gene. Nat. Genet. *14*, 324–328.

Garcia-Higuera, I., and D'Andrea, A.D. (1999). Regulated binding of the Fanconi anemia proteins, FANCA and FANCC. Blood *93*, 1430–1432.

Garcia-Higuera, I., Kuang, Y., Naf, D., Wasik, J., and D'Andrea, A.D. (1999). Fanconi anemia proteins FANCA, FANCC, and FANCG/XRCC9 interact in a functional nuclear complex. Mol. Cell. Biol. *19*, 4866–4873.

Garcia-Higuera, I., Taniguchi, T., Ganesan, S., Meyn, S., Timmers, C., Hejna, J., Grompe, M., and D'Andrea, A.D. (2001). Interaction of the Fanconi anemia proteins and BRCA1 in a common pathway. Mol. Cell *7*, this issue, 249–262.

Hejna, J.A., Timmers, C.D., Reifsteck, C., Bruun, D.A., Lucas, L.W., Jakobs, P.M., Toth-Fejel, S., Unsworth, N., Clemens, S.L., Garcia, D.K., et al. (2000). Localization of the Fanconi anemia complementation group D gene to a 200-kb region on chromosome 3p25.3. Am. J. Hum. Genet. *66*, 1540–1551.

Jakobs, P.M., Sahaayaruban, P., Saito, H., Reifsteck, C., Olson, S., Joenje, H., Moses, R.E., and Grompe, M. (1996). Immortalization of four new Fanconi anemia fibroblast cell lines by an improved procedure. Somat. Cell. Mol. Genet. *22*, 151–157.

Jakobs, P.M., Fiddler-Odell, E., Reifsteck, C., Olson, S., Moses, R.E., and Grompe, M. (1997). Complementation group assignments in Fanconi anemia fibroblast cell lines from North America. Somat. Cell. Mol. Genet. *23*, 1–7.

Joenje, H., Oostra, A.B., Wijker, M., di Summa, F.M., van Berkel, C.G., Rooimans, M.A., Ebell, W., van Weel, M., Pronk, J.C., Buchwald, M., and Arwert, F. (1997). Evidence for at least eight Fanconi anemia genes. Am. J. Hum. Genet. *61*, 940–944.

Joenje, H., Levitus, M., Waisfisz, Q., D'Andrea, A., Garcia-Higuera, I., Pearson, T., van Berkel, C.G., Rooimans, M.A., Morgan, N., Mathew, C.G., and Arwert, F. (2000). Complementation analysis in Fanconi anemia: assignment of the reference FA-H patient to group A. Am. J. Hum. Genet. *67*, 759–762.

Kupfer, G.M., Naf, D., Suliman, A., Pulsipher, M., and D'Andrea, A.D. (1997). The Fanconi anaemia proteins, FAA and FAC, interact to form a nuclear complex. Nat. Genet. *17*, 487–490.

Lo Ten Foe, J.R., Rooimans, M.A., Bosnoyan-Collins, L., Alon, N., Wijker, M., Parker, L., Lightfoot, J., Carreau, M., Callen, D.F., Savoia, A., et al. (1996). Expression cloning of a cDNA for the major Fanconi anaemia gene, FAA. Nat. Genet. *14*, 320–323.

Lo Ten Foe, J.R., Kwee, M.L., Rooimans, M.A., Oostra, A.B., Veerman, A.J., van Weel, M., Pauli, R.M., Shahidi, N.T., Dokal, I., Roberts, I., et al. (1997). Somatic mosaicism in Fanconi anemia: molecular basis and clinical significance. Eur. J. Hum. Genet. *5*, 137–148.

Mercer, W.E., and Schlegel, R.A. (1979). Phytohemagglutinin enhancement of cell fusion reduces polyethylene glycol cytotoxicity. Exp. Cell Res. *120*, 417–421.

Naf, D., Kupfer, G.M., Suliman, A., Lambert, K., and D'Andrea, A.D. (1998). Functional activity of the Fanconi anemia protein FAA requires FAC binding and nuclear localization. Mol. Cell. Biol. *18*, 5952–5979.

Pulsipher, M., Kupfer, G.M., Naf, D., Suliman, A., Lee, J.S., Jakobs, P., Grompe, M., Joenje, H., Sieff, C., Guinan, E., et al. (1998). Subtyping analysis of Fanconi anemia by immunoblotting and retroviral gene transfer. Mol. Med. *4*, 468–479.

Strathdee, C.A., Duncan, A.M., and Buchwald, M. (1992a). Evidence for at least four Fanconi anaemia genes including FACC on chromosome 9. Nat. Genet. *1*, 196–198.

Strathdee, C.A., Gavish, H., Shannon, W.R., and Buchwald, M. (1992b). Cloning of cDNAs for Fanconi's anemia by functional complementation. Nature *356*, 763–767.

Waisfisz, Q., de Winter, J.P., Kruyt, F.A., de Groot, J., van der Weel, L., Dijkmans, L.M., Zhi, Y., Arwert, F., Scheper, R.J., Youssoufian, H., et al. (1999a). A physical complex of the Fanconi anemia proteins FANCG/XRCC9 and FANCA. Proc Natl Acad Sci U S A *96*, 10320–10325.

Waisfisz, Q., Morgan, N.V., Savino, M., de Winter, J.P., van Berkel, C.G., Hoatlin, M.E., Ianzano, L., Gibson, R.A., Arwert, F., Savoia, A., et al. (1999b). Spontaneous functional correction of homozygous

Fanconi anaemia alleles reveals novel mechanistic basis for reverse mosaicism. Nat. Genet. *22*, 379–383.

Whitney, M., Thayer, M., Reifsteck, C., Olson, S., Smith, L., Jakobs, P.M., Leach, R., Naylor, S., Joenje, H., and Grompe, M. (1995). Microcell mediated chromosome transfer maps the Fanconi anaemia group D gene to chromosome 3p. Nat. Genet. *11*, 341–343.

Whitney, M.A., Royle, G., Low, M.J., Kelly, M.A., Axthelm, M.K., Reifsteck, C., Olson, S., Braun, R.E., Heinrich, M.C., Rathbun, R.K., et al. (1996). Germ cell defects and hematopoietic hypersensitivity to gamma-interferon in mice with a targeted disruption of the Fanconi anemia C gene. Blood *88*, 49–58.

Worley, K.C., Wiese, B.A., and Smith, R.F. (1995). BEAUTY: an enhanced BLAST-based search tool that integrates multiple biological information resources into sequence similarity search results. Genome Res *5*, 173–184.

Wu, D.Y., Nozari, G., Schold, M., Conner, B.J., and Wallace, R.B. (1989). Direct analysis of single nucleotide variation in human DNA and RNA using in situ dot hybridization. DNA *8*, 135–142.

Yamashita, T., Kupfer, G.M., Naf, D., Suliman, A., Joenje, H., Asano, S., and D'Andrea, A.D. (1998). The Fanconi anemia pathway requires FAA phosphorylation and FAA/FAC nuclear accumulation. Proc. Natl. Acad. Sci. USA *95*, 13085–13090.

Youssoufian, H. (1994). Localization of Fanconi anemia C protein to the cytoplasm of mammalian cells. Proc. Natl. Acad. Sci. USA *91*, 7975–7979.

Youssoufian, H. (1996). Cytoplasmic localization of FAC is essential for the correction of a prerepair defect in Fanconi anemia group C cells. J. Clin. Invest. *97*, 2003–2010.

GenBank Accession Numbers

The GenBank accession numbers for the candidate cDNAs are G26488 for EST TIGR-A004X28, G26135 for the EST SGC34603/FANCD2, and AA609512 for the third EST. The accession numbers for the human *FANCD2* exons/gene structure are AF273222–AF273251 and AF340183 for the FANCD2 cDNA. Accession numbers for the *FANCD2* homologs are AAF55806 (*Drosophila*), CAB10276 and AAD23659 (*A. thaliana*), and CAB63365 (*C. elegans*).