Molecular Genetic Analysis of the Genetically Heterogeneous Disease Fanconi Anemia

by

Michael Whitney

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Abstract

Fanconi anemia (FA) is a genetically heterogeneous, autosomal recessive disease, characterized by pancytopenia, skin hyperpigmentation, predisposition to leukemia, and defects of the thumb and radial ray. On the cellular level FA cells are hypersensitive to DNA crosslinking agents such as mitomycin C (MMC) and diepoxybutane (DEB). FA cells also exhibit chromosomal instability as characterized by increased chromosomal aberrations both, spontaneous and DNA damage induced. Although the biochemical defect in FA is unknown, it has been proposed to be a defect in DNA repair due to FA cells hypersensitivity to DNA damaging agents. The FA group C gene (FA(C)), which shows no significant sequence homology to any known gene, is the only gene of the five known complementation groups that has been cloned.

In order to determine the fraction of FA caused by FA(C) mutations, we used reverse transcription PCR and chemical mismatch cleavage to examine the FA(C) cDNA in FA patients. It was determined that approximately 20% of FA patients had mutations in the FA(C) gene. Interestingly, a common mutation (IVS4 +4A->T) in the FA(C) gene was identified which accounts for a majority (~83%) of FA cases in Ashkenazi-Jewish individuals. Allele specific oligonucleotide hybridization was then used to experimentally determine the frequency of this mutation in the Jewish population at 2/314. This indicates a carrier frequency of 0.64% with 95%

confidence limits between 0.08-2.3%. This information is currently being used to genetically council FA families of this ethnic group.

Towards the creation of an animal model for FA we have used embryonic stem cell technology to create a disruption of the murine homologue of the FA(C) gene. The creation of this model will aid in the elucidation of the function of FA(C), and permit the testing of novel therapies for the disease. Mice homozygously deleted for exon 9 were generated and phenotypically characterized. Homozygous mutants are viable and exhibit no apparent developmental deformities. Complete blood counts in 6 week old mutants show no evidence of pancytopenia. However, Fac deficient female mice show a marked reduction in fertility. In addition, primary skin fibroblasts from exon 9 deleted mice show increased chromosomal breakage in response to MMC and DEB similar to that seen in human FA patients. These deficits indicate that a functional mutation of the FA(C) gene has been achieved by the exon 9 deletion. Experiments regarding the hematopoietic function of the mutant mice are currently ongoing.

We additionally have used microcell mediated chromosome transfer to map the FA complementation group D gene (FA(D)). Human chromosomes were transferred into an FA(D) immortalized fibroblast cell line (PD-20) to generate microcell hybrid cell lines. PD-20 microcell hybrids which contain a normal chromosome 3p, show functional complementation of the multiple cellular phenotypes associated with FA. Therefore, the FA(D) complementing gene maps

to chromosome 3p. Exclusion mapping was then used in 3 FA(D) families to further map the FA(D) gene to between the microsatellite markers D3S1307 and D3S1619. This corresponds to the chromosomal region between 3p22 and 3p26. This mapping of the FA(D) gene represents a significant and necessary step towards the positional cloning of the FA(D) gene.

Introduction

I. Clinical Characteristics of Fanconi Anemia

Fanconi anemia (FA) is a rare, genetically heterogeneous, autosomal recessive disease. The clinical description of FA was first recorded in 1927 by the Swiss pediatrician Guido Fanconi [1]. The clinical features of FA include developmental defects such as short stature, thumb and radial abnormalities [2], kidney and renal malformations, skin hyperpigmentation, microcephaly, congenital heart defects, hypogenitalism, and mental retardation [3, 4]. The severity and prevalence of these developmental deformities is highly variable, with one or more being present in about 50% of patients [5]. An invariable clinical feature of FA is early onset progressive pancytopenia or aplastic anemia [6, 7]. This pancytopenia represents a bone marrow defect, which results in a lack of FA patients ability to produce sufficient amounts of all peripheral blood lineage's. The onset of the deficiency, in each of the lineage's, including white and red blood cells, as well as platelets, is not usually simultaneous. These blood abnormalities usually present clinically as susceptibility to infection, anemia, or clotting abnormalities. The average age of onset for pancytopenia is 7 years with most FA cases being diagnosed between ages 3 and 7 [8]. There are, however, examples of patients who have been diagnosed well outside these ranges, especially if developmental deformities are not present. Another clinical complication associated with FA is myelodysplastic syndrome,

or acute myeloid leukemia which affects about 52% of FA patients by age 40 [8]. There is currently no clear understanding of the biological defects in FA, nor how these defects lead to the variety of abnormalities seen in FA patients.

If untreated the progressive pancytopenia associated with FA is usually fatal. The only curative therapy available for FA is bone marrow transplantation [9, 10, 11]. Therefore, bone marrow transplantation is the preferred treatment for FA, especially if a related, HLA matched donor is available [12]. This treatment can provide a long term cure for the pancytopenia while having no effect on developmental deformities. The risk of leukemia and other cancers can still be increased after transplantation, especially if leukemia was present prior to transplant. Bone marrow transplantation is usually not recommended when acute myeloid leukemia is present because the lower doses of bone marrow preparative agents which must be used in FA patients can sometimes be insufficient to kill all the leukemic cells [13, 14]. Additional treatments for FA include blood transfusions [15], androgen therapy [16, 17] and cytokine therapies [18-20]. Gene therapy directed approaches could also prove useful as a treatment for FA as the genes defective in FA patients are identified and gene targeting to hematopoietic stem cells becomes possible [21-23].

II. Cellular Characteristics of Fanconi Anemia

On the cellular level FA cells are hypersensitive to DNA crosslinking agents such as mitomycin C (MMC), Diepoxybutane(DEB), and cis-diamminedichloroplatinum II(cis-DDP) [24]. Although there is

a large amount of literature reported on the nature of repair mechanisms for crosslink damage, the defect in FA cells, and the mechanism of action of these clastogens on FA cells is not well understood. Interestingly, FA cells do not show increased sensitivity to other DNA damaging agents such as ultraviolet light, X-rays, or monofunctional DNA alkylating agents such as methyl methane sulfonate (MMS) or ethyl methane sulfonate (EMS). It is because of this specific hypersensitivity of FA cells to DNA crosslinkers that the defect in FA has been proposed to be a deficit in the ability to repair this type of DNA damage.

FA cells exhibit chromosome instability, as determined by their increased rate of chromosomal rearrangements, which include chromatid gaps, breaks, radial exchange figure, and endoreduplication [25]. The rate of these chromosomal rearrangements is dramatically increased when growing FA cells are exposed to DNA crosslinkers. This type of chromosome breakage analysis, upon treatment of cells with MMC or DEB, is currently the most reliable diagnostic test for FA [26]. Chromosome breakage accompanied by pancytopenia and any developmental abnormalities is diagnostic for FA [26]. Other cellular phenotypes for FA include reduced growth rate [27], oxygen sensitivity [28], and a marked delay of the G2 phase of the cell cycle after exposure of cells to DNA crosslinking agents [29, 30]. A recent report suggested that a diagnostic test for cell cycle delay could be as reliable as chromosome breakage for detecting FA [31].

III. Genetic Heterogeneity of Fanconi Anemia

Currently, there are five known complementation groups for FA (denoted group A-E), as determined by somatic cell hybridization experiments [32, 33, 34]. This complementation analysis suggests the presence of at least five FA genes. The multiple complementation groups could alternatively be accounted for by mutations in different functional domains of the same gene. This is the case for the recently identified Ataxia-telengactasia (AT) gene for which mutations in only one gene account for all four complementation groups. However, the recent cloning of the FA group C gene which is mutated in only 15% of FA patients demonstrates that there is genetic heterogeneity in FA. The FA(C) gene, representing Fanconi anemia group C, is the only gene, of the five known complementation groups, that has been cloned [35]. The cloning of the FA(C) gene was achieved by using a cDNA complementation approach. For this approach an EBV (Epstein-Barr) based human cDNA library was transfected into an FA(C) lymphoblast cell line (HSC536) in order to identify cDNAs that could complement the hypersensitivity of the FA(C) cells to DNA crosslinkers. The FA(C) cDNA was identified and shown to correct both the MMC and DEB hypersensitivity of the HSC536 cells. A mutation was subsequently found in the HSC536 cell line which resulted in amino acid 554, 4 amino acids from the C-terminus of the protein, being changed from a leucine residue to a proline residue (L554P). As added support that FA(C) is an FA gene we present the identification of additional FA(C) mutations in other FA patients. The results we present show that mutations in FA(C) account for

approximately 15 percent of FA patients [36, 37]. Somatic cell hybridization experiments have been used to estimate the frequency of the other FA complementation groups including, A, B, D, and, E at 50%, 5%, 15%, and, 15%, respectively [34]. The FA(C) polypeptide has no significant sequence homology to any known protein and has been mapped to 9q22 by in situ hybridization and linkage analysis [38, 39]. Although the cloning of the FA(C) gene was considered a significant advance in FA research it has provided no hint to the functional defect in FA.

IV. FA(C) Protein Analysis

Immunohistochemistry studies have recently been reported with antibodies generated against the human FA(C) protein. These studies have included subcellular distribution analysis of the FA(C) protein as well as attempts to identify proteins that physically interact with the FA(C) protein. Immunofluorescense and subcellular fractionation were used to sublocalize the FA gene product primarily to the cytoplasm [40, 41]. It was therefore proposed that the FA(C) gene may function in intracellular signaling and is not directly involved in DNA repair. However, these experiment do not exclude the possibility that the FA(C) protein has some role in DNA repair. In an additional experiment 3 unknown proteins, of molecular masses 65, 50, and 35 kDa, have been shown to physically interact with the FA(C) protein [42]. This experiment utilized a co-precipitation assay using a chimeric FA(C) molecule which had been fused to the constant portion of the human IgG1 heavy chain. This allowed the precipitation of the chimeric FA(C) protein and any interacting proteins with protein A

agarose beads. If these unknown proteins are shown to physically interact with FA(C) in-vivo they represent good candidate genes for other FA complementation groups. Their identification could therefore prove helpful towards advancing our understanding of the biochemical defect in FA.

V. Murine Homologue of FA(C)

A murine homologue (Fac) of the human FA(C) gene has been identified through low stringency DNA hybridization [43]. The murine homologue is 67% identical and 78% similar to the human protein. The murine Fac gene functionally complements the cellular hypersensitivity of human FA(C) cells to MMC and DEB. The Fac RNA is ubiquitously expressed, as determined by PCR analysis of reverse transcribed mouse total RNA from a variety of tissues including liver, small intestine, submucosal gland, brain, lung, heart, spleen, and kidney. Additionally, RNA in-situ hybridization experiments, with Fac anti-sense probe, show tissue specific expression in the later stages of embryonal development. This developmental specific expression may indicate a role for the Fac gene product in these stages of development, and could explain some of the developmental defects seen in FA patients. The identification of the murine FA(C) homologue also allows for the targeted disruption of the Fac gene in embryonic stem cells to generate a mouse model for FA [44, 45]. The targeted disruption of the mouse Fac gene and preliminary phenotypic analysis of homozygous Fac deficient mice will be presented.

VI. Identification of New FA genes

The strategy of cDNA complementation, which was successful for identifying the FA(C) gene [35], has not yet been successful for the identification of additional FA genes. An alternative approach for identifying new FA genes would be to use linkage analysis to map the genes, followed by positional cloning for their isolation. This approach has lead to the identification of a number of other human disease genes in recent years [46]. However, linkage analysis to obtain positional information is hindered, in FA, by genetic heterogeneity and the lack of a simple assay for determining complementation group. One study, about 5 years ago, reported linkage of the FA(A) gene to chromosome 20 using a number of multiplex FA families, for which the complementation group for most families was unknown [47, 48]. Because of this published linkage data we used microcell mediated chromosome transfer to introduce a normal chromosome 20 into a known FA(A) cell line (GM6914). We then tested for the ability of chromosome 20 to functionally complement these cells. Our results indicated that chromosome 20 could not complement FA(A) cells and we therefore concluded that the FA(A) gene is unlikely to reside on chromosome 20. There are no other published reports mapping the chromosomal location of the other FA genes. An alternative approach for mapping a disease gene like FA, which has a testable cellular phenotype, is to use chromosome transfer followed by testing for phenotypic complementation. This approach of functional complementation by microcell-mediated chromosome transfer does not require genetically informative families nor does it

depend on having multiple families of the same complementation group. We are currently using this technique to map the chromosomal location of multiple FA genes.

Microcell mediated chromosome transfer can be used to move chromosomes from one cell line to another [49, 50]. One usually uses a chromosome donor cell line which contains a known chromosome which has been marked with a dominant selectable marker. Alternatively, donor cells can be randomly marked and their chromosomes transferred into a recipient cell [51]. Microcells, which are represented by as few as one chromosome surrounded by a nuclear and cytoplasmic membrane, are prepared by micronucleating donor cells with colcemid and cytochalasin B, followed by the physical isolation of these micronuclei by centrifugation. Microcells are then fused into recipient cells using polyethylene glycol (PEG) and hybrid clones are selected with the appropriate selective agent. A microcell hybrid is therefore a recipient cell which has taken up a marked chromosome from the donor cell line. Unmarked chromosomes will also be transferred but they will eventually be lost as they are not selected for.

We have used microcell mediated chromosome transfer as the first step towards the positional cloning of a new FA gene. Our variation of this technique utilizes functional complementation to identify the chromosome bearing the FA(D) gene. Similar approaches have previously been used successfully for mapping other disease genes, including the DNA repair associated disorder Bloom's syndrome [52]. For our experiments microcell hybrids were generated by transferring individual neomycin resistance marked human

chromosomes, from a mouse A9 cell, into an immortalized FA(D) fibroblast cell line PD-20. This PD-20 immortalized fibroblast cell line was generated from an FA skin sample by the Oregon Health Sciences University Fanconi Anemia Cell Repository [53]. It was placed into complementation group D by the following criteria: 1) Mutations were not found by chemical-cleavage in the FA(C) gene [36], 2) PD-20 cross-complements a known group A immortalized fibroblast (GM6914), upon whole cell fusions [53], 3) Lymphoblasts derived from the PD-20 patient do not cross-complement a known FA(D) lymphoblast cell line [34]. Upon generation of PD-20 microcell hybrids, they were assayed for phenotypic correction and thereby the FA(D) complementing chromosome was identified.

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A common mutation in the FA(C) gene causes Fanconi anemia in Ashkenazi-Jewish individuals

Michael A. Whitney[1], Hiroshi Saito[1], Petra M. Jakobs[1], Rachel A. Gibson[2], Robb E. Moses[1], and Markus Grompe*[1]

Department of Molecular and Medical Genetics[1], Oregon Health Sciences University, 3181 Sam Jackson Park Road, L 103, Portland, Oregon 97201 Division of Medical and Molecular Genetics[2], UMDS Guy's Hospital, 8th floor Guy's Tower, London SE1 9RT, United Kingdom

* To whom correspondence should be addressed

Summary

Fanconi anemia is an autosomal recessive disease for which five known complementation groups exist. Recently, the gene defective in complementation group C (FA(C)) has been cloned. In order to determine the fraction of Fanconi anemia caused by FA(C) mutations, we used reverse transcription PCR and chemical mismatch cleavage (CMC) to examine the FA(C) cDNA in 17 FA cell lines. 4/17 patients (23.5%) had mutations in this gene. Two Ashkenazi Jewish individuals were homozygous for an identical splice mutation. Three additional Jewish patients bearing this allele were found upon screening 21 more families. We conclude that a common mutation in FA(C) accounts for the majority of Fanconi anemia in Ashkenazi-Jewish families.

Introduction

Fanconi anemia (FA) is an autosomal recessive disorder characterized by pancytopenia, defects of the thumb and radius, hyperpigmentation, short stature and susceptibility to cancer [1]. Fanconi anemia cells are hypersensitive to DNA cross-linking agents such as mitomycin C or diepoxybutane and the basic defect in FA has been proposed to be a defect in repair of DNA cross-links [2]. Cells treated with DNA-cross linkers display chromosomal aberrations at a high frequency including chromatid breaks, gaps and endoreduplication [3].

Somatic cell hybridization experiments [4] and genetic linkage studies[5] have demonstrated the existence of at least 5 complementation groups, termed A, B, C and D. Recently the gene defective in group C was cloned by transfection of a cDNA expression library and complementation of the cellular mitomycin C hypersensitivity [6]. The FA(C) gene has a coding region of 1677 bp and is expressed ubiquitously. The function of the FA(C) protein has not yet been determined.

The proportion of FA cases due to mutations in FA(C) is currently not known. Mutations were found in 3 out of 10 FA cell lines in the original cloning report [6]. To determine the incidence and nature of FA(C) mutations among randomly ascertained FA patients, reverse transcription PCR (RT-PCR) and chemical mismatch cleavage (CMC) were utilized to scan the entire FA(C) coding region for mutations [7]. CMC has high specificity and sensitivity in mutation analysis [8] and,

therefore, is reliable in detecting as well as excluding the presence of mutations.

Many workers in the field are utilizing transformed cell lines available through publicly accessible repositories (ATCC, Camden) for complementation and biochemical studies. The complementation groups to which these cells belong are not known. We here report the use of mutation analysis on 17 such cell lines to categorize them into a group containing changes in the FA(C) cDNA and a group which does not.

In Ashkenazi Jewish patients we found that a single splice mutation in the FA(C) gene accounts for the majority of Fanconi anemia. The implications of this finding for heterozygote detection and future therapy are discussed.

Results

RT-PCR and CMC

Fibroblast and lymphoblast cell lines from 17 patients were analyzed. The FA(C) cDNA was amplified in 2 overlapping PCR products of ~1200 bp each in all patients (see figure 1B). Agarose gel electrophoresis showed the presence of two shortened 5' PCR products in each of 2 patients: GM 449 and GM 4510 (data not shown). This result was reproduced in several separate RT-PCR amplifications and therefore was thought to represent a possible splicing error or deletion in these cell lines. All other PCR products were of normal length.

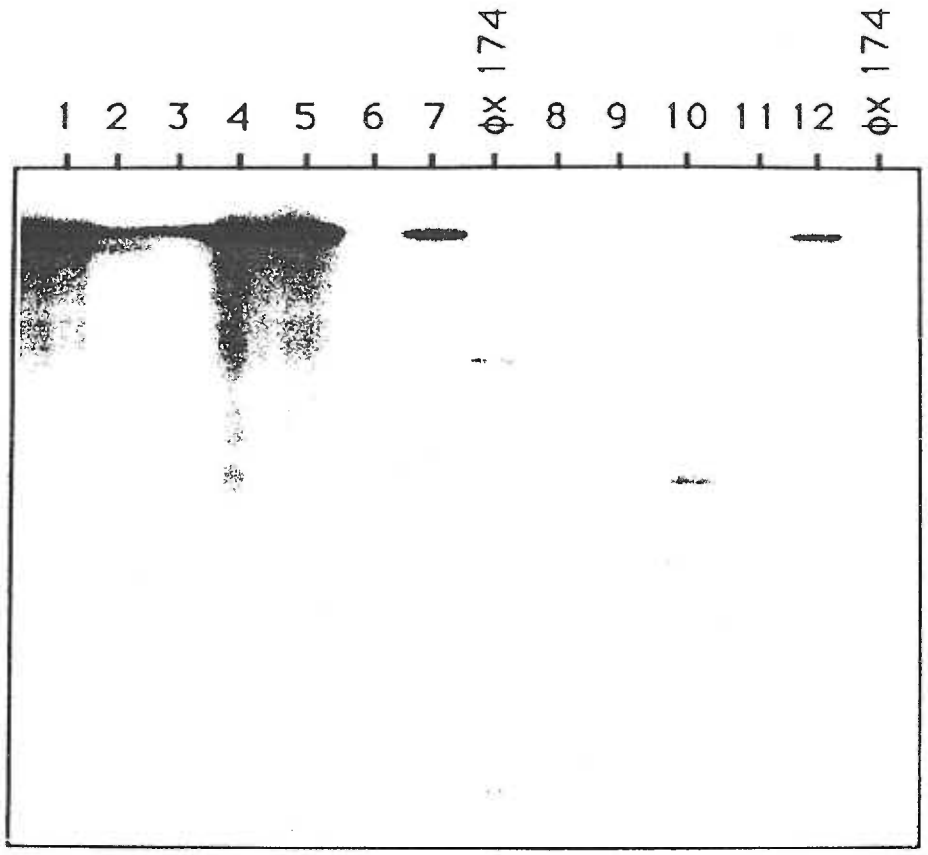

Figure 1A: Chemical mismatch cleavage gel. Lanes 1 and 2 were from PD-4L, with a 1 bp deletion resulting in cleavage with both hydroxylamine (1) and osmium tetroxide (2). Lanes 3-7 were from patients that didn't have FA(C) mutations. Lanes 8 and 10 were from GM 449 with a splice mutation, which leads to a deletion of exon 4. Lanes 9 and 11 were from GM 4510, with the identical alteration. In lanes 8 and 9, the antisense wild-type strand was labeled and cleavage occurred at the 3' end of the deleted exon. In lanes 10 and 11 the sense strand produced 2 cleavage products, indicating partial usage of a cryptic splice donor. Lane 12 contained the cleavage product resulting from a missense mutation in GM 1309 (hydroxylamine cleavage). The size marker was HaeIII cut PhiX174.

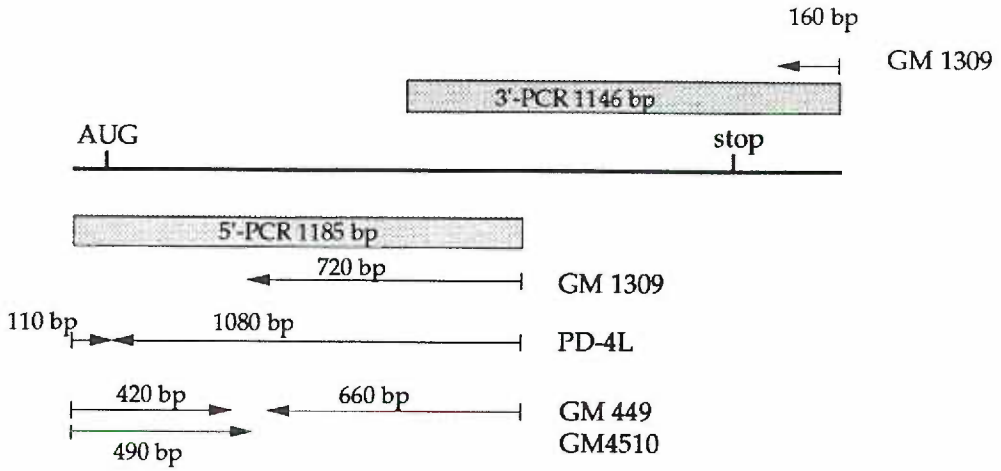

Figure 1B: Schematic representation of CMC results. The FA(C) cDNA and its translation start and stop codons are shown. The 5' and 3' PCR products are depicted as filled bars above and below the cDNA. The cleavage products are represented by arrows beginning at the labeled primer and their approximate length is given above. The designation of the cell line is given next to the arrows.

CMC was used to determine the presence and localization of mutations in all patients. Cleavage products were identified within the 5' PCR product in 4 cell lines: GM 449, GM 1309, GM 4510 and PD-4L. Cleavage results are shown in figure 1A and a schematic representation of the data is given in Figure 1B. GM 1309 yielded a cleavage band of ~720 bp with hydroxylamine and anti-sense probe, indicating a G-mismatch. Both hydroxylamine and osmium tetroxide produced a ~1080 bp band in PD-4L when the anti-sense probe was used. When the sense probe was employed, a 107 bp product was seen with both chemicals. This pattern is consistent with either a

small deletion or an insertion. Osmium tetroxide and hydroxylamine also both resulted in cleavage in GM 449 and GM 4510. The pattern seen in these two cell lines was identical. A single band of ~660 bp was produced by the anti-sense probe. The sense probe resulted in 2 cleavage bands of ~490 and 420 bp respectively. This cleavage pattern was consistent with the presence of 2 deletions, with common 3' and different 5' break-points.

Only one cell line (GM 1309) yielded a cleavage with the 3' PCR product. Cleavage was seen with anti-sense probe and Osmium tetroxide (~160 bp), indicating an A mismatch. The cell lines, their ethnic origin and the result of the mutation analysis are listed in (see table 1).

Cell line	Cell type	Repository	Ethnicity	FA(C) mutations	Reference
GM 1309	TF	Camden	BL	G139E+nt2045	22
PD-4L	L	OHSU	CA	ΔG322	cat
GM 449	F	Camden	AJ	IVS4 +4 A->T	Ger
GM 4510	L	Camden	AJ	IVS4 +4 A->T	31
PD-77L*	L	OHSU	AJ	IVS4 +4 A->T	cat
PD-25F*	F	OHSU	AJ	IVS4 +4 A->T	cat
GM 8010	L	Camden	WH	none found	cat
GM 368	F	Camden	BL	none found	cat
GM 646	F	Camden	WH	none found	cat
GM 1746	F	Camden	AR	none found	31
GM 2053	F	Camden	AR	none found	31
GM 2061	F	Camden	WH	none found	cat
GM 2361	F	Camden	WH	none found	cat
GM 2362	F	Camden	WH	none found	cat
CRL 1196	F	ATCC	?	none found	cat
HG 261	F	ATCC	CA	none found	cat
PD-9L	L	OHSU	CA	none found	cat
PD-15L	L	OHSU	CA	none found	cat
PD-20L	L	OHSU	CA	none found	cat

Table 1. Chemical cleavage and ASO results. Cell type: F = primary fibroblasts; TF = transformed fibroblasts; L = EBV transformed lymphoblasts. Ethnic background: BL = black; CA = caucasian; AJ = Ashkenazi Jewish; AR = arab; WH = white, with no further specification; ? = unknown. Reference: cat = cell line catalog from the appropriate repository; Ger = Dr. German, personal communication. The two cell lines marked with a star, were not analyzed by CMC, only by ASO hybridization.

DNA sequencing

The PCR products were subcloned and the regions containing sequence alterations defined by CMC were analyzed by DNA sequencing. All base and amino acid positions are given according to Strathdee et al [6]. The sequence change in GM 1309 was found to be a G->A transition in base 671, altering codon 139 from Glycine (GGG) to Glutamic acid (GAG). This allele is designated G139E. In addition, GM 1309 also had a cleavage in the 3' untranslated region. This change resides in nucleotide 2045, altering an A to a C. Since the cDNA regions containing these changes were amplified independently, it could not be determined whether they were localized on the same strand or whether they represent two alleles.

In PD-4L, the deletion of one G at position 322 was found causing a frameshift as well as a truncated message. This mutation is identical to one reported by Strathdee et al. (1992) and was from a patient in the same family.

Both GM 449 and GM 4510 yielded the same aberrant splice products, with no full length wild-type message detectable. Two cDNA deletions were found in both patients, one larger and one smaller, and are shown in figure 2. The large deletion corresponded to a clean 111 bp in-frame deletion (nt 601-711) of FA(C) exon 4 [9]. In contrast, the smaller deletion represented a partial removal of 40 bp (nt 672-711) of this exon and probably resulted from the use of a cryptic splice donor sequence within exon 4 (figure 2). The smaller

deletion resulted in a frame-shift. In keeping with the CMC results, the 3' boundary of the deletions was identical, but the 5' ends were different.

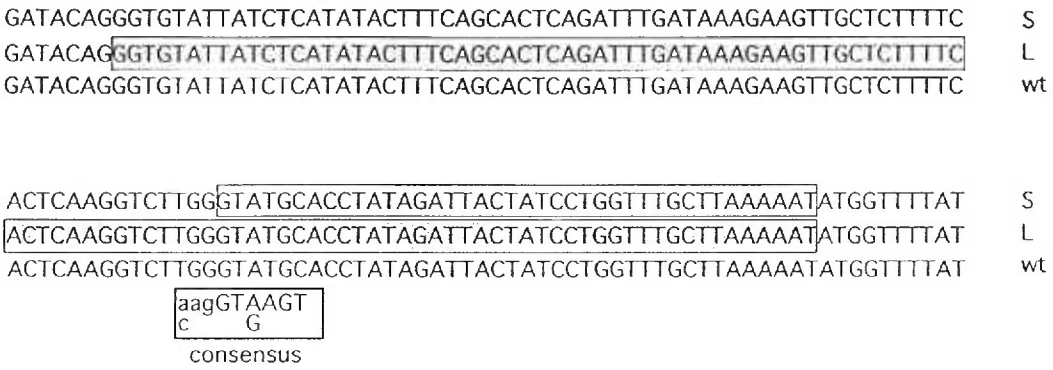

Figure 2: Alignment of wild-type FA(C) cDNA and mutant cDNAs. The smaller exon deletion (S) is shown on top as a clear box, the full exon deletion (L) in the middle as a shaded box. The wild-type sequence (wt) is given below from nt 594-721. The 9 bp consensus splice donor sequence is aligned with the potential cryptic splice donor within exon 4.

Analysis of the splice junctions of exon 4

The deletions seen in GM 449 and GM 4510 were most consistent with a mutation of the splice donor of intron 4. Exon 4 and its surrounding splice junctions were therefore amplified from genomic DNA by PCR and sequenced in both wild-type and splice-defective patients. A single base change was found in the 4th intronic base, changing the sequence from a consensus A to T, the least frequent base at that position [10, 11]. This allele is termed IVS4+4 A->T. Figure 3 shows the mutation along with the wild-type and splice donor consensus sequences. The Shapiro and Senapathy score was 85 in the wild-type and 73 in the mutant splice donor sequences [10]. The putative exonic splice donor used to produce the smaller deletion had a score of 72.

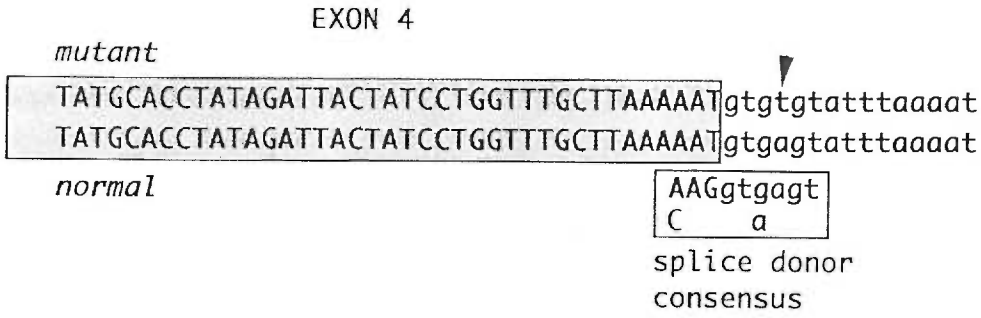

Figure 3: The IVS4 +4 A->T mutation. Location of the mutation responsible for aberrant FA(C) splicing in Ashkenazi-Jewish patients. The arrow indicates the altered base. The top sequence is mutant, the bottom sequence wild-type. Exonic sequences are in uppercase and boxed in gray, intronic sequences in lowercase. A splice donor consensus sequence is given below.

In order to verify that this change did not represent a normal polymorphism, a total of 50 chromosomes from 25 unrelated, normal individuals were tested by allele specific oligonucleotide (ASO) hybridization [12]. The mutant allele was not found in any normal individuals. GM 449 and GM 4510 both were confirmed to be homozygous for the mutation (see figure 4).

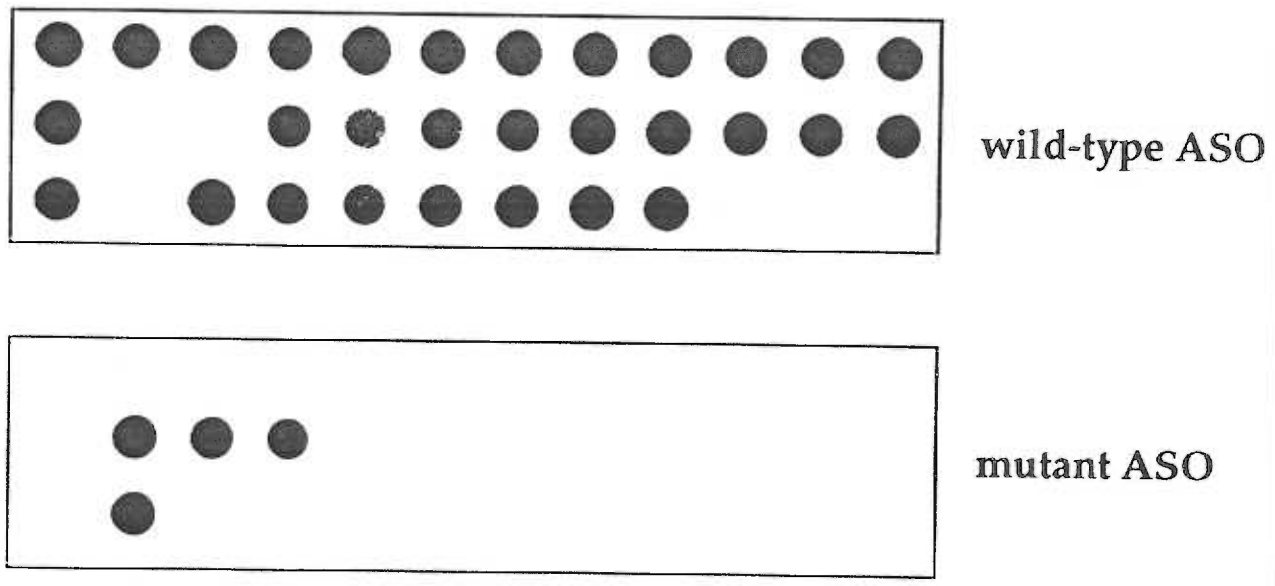

Figure 4: Allele specific oligonucleotide hybridization. The top panel shows hybridization of the wild-type oligonucleotide, the bottom with the mutant oligonucleotide. In 3 positions hybridization occurred only with only the mutant oligonucleotide (homozygous for IVS4 +4 A->T), whereas one heterozygous sample gave signal with both ASOs. The rest of the samples were homozygously normal.

To determine the incidence of this allele among FA families, 21 additional, unrelated Fanconi cell lines were assayed using ASO hybridization. Of these, three additional families were found to have the IVS4 +4 A->T splice donor mutation. PD-L77 and PD-F25, cell lines from affected individuals, were homozygous for this mutation, whereas PD-L92 and PD-L93 were heterozygous. PD-PD-L92 and PD-L93 were derived from the parents of a patient, whose cells were not available. Other family members were also analyzed in the PD-F25 and PD-L77 pedigrees. The parents are heterozygotes in both families. PD-F25 has two unaffected siblings, one a heterozygote, the other homozygously normal.

All individuals bearing the exon 4 splice mutant allele were of Ashkenazi-Jewish descent.

Discussion

Several autosomal recessive disorders are known to cluster in the Ashkenazi-Jewish population. Due to founder effects one or very few mutations account for the majority of cases. Among these "common allele diseases" Tay-Sachs and Gaucher's disease are best recognized [13, 14]. However, no association between Jewish ancestry and Fanconi anemia has been reported to date. Our results show that a single splice mutation in the FA(C) gene accounts for the majority of this disease among Ashkenazi-Jewish people. All affected individuals were homozygous for the alteration and no non-Jewish Fanconi patients or unaffected controls carried this allele. Three families among the 21 studied by ASO hybridization, were of known

Ashkenazi-Jewish descent and in all 3 the exon 4 splice mutation was found. The aberrant cDNA products found in these patients are consistent with splice donor mutations, for which both exon skipping and use of cryptic splice donor have been reported [15, 16]. These findings have important clinical implications. Fanconi anemia is an early onset disease with high mortality and morbidity and families desire prenatal diagnosis. Carrier detection for the FA(C) IVS4+4A->T allele is feasible with the ASO assay described here and could be implemented without difficulty, should the frequency of the mutation be sufficiently high. Additionally, protocols for gene therapy trials for complementation group C have already been submitted [17]. Fanconi anemia patients of Ashkenazi Jewish descent are likely to be candidates for this therapeutic approach.

Although Fanconi anemia is a rare genetic disease, the delineation of the basic defect in this disorder promises to yield insights into mechanisms of mammalian DNA repair, cell cycle regulation and hematopoeitic stem cell biology. The FA genes from other complementation groups are being sought by a variety of approaches, including cDNA complementation and linkage analysis [18-20]. For these studies, knowledge about the complementation group of a cell line or family is important. Also, additional cell lines with known FA(C) mutations are needed for experiments regarding the function of this protein. Three cell lines in the original cloning report had such mutations [21]. One cell line (HSC536N) had a missense change (L553P) and the two others carried the same single bp deletion (Δ322G).

Among the 17 available FA cell lines studied by us, we found only 4 with sequence alterations in the FA(C) cDNA. Because of the high sensitivity of CMC [8], all 13 cell lines, which were negative in the CMC analysis are very likely (>95%) to represent other, non-C complementation groups.

Of the CMC positive cells from non-Jewish patients, GM 1309 is of particular interest [22]. GM 6914, an SV40 transformed FA cell line used by many investigators, was derived from GM 1309, a primary fibroblast cell line, and is thought to represent complementation group A [4, 23]. It is therefore likely that the 2 sequence alterations found in the FA(C) gene of GM 1309 represent polymorphisms and not mutations.

PD-4L bears the Δ322G allele and was derived from a sibling of the patient described by Strathdee et al [6]. Although only one altered allele was found, the mutation found is clearly disruptive for gene function. In compound heterozygotes for autosomal recessive diseases the two mutant alleles often are not found in equal abundance as mRNA and only one allele is detectable by mRNA analysis [24].

Methodology

Cell lines

Cell lines GM 00368, GM 00449, GM 00646 , GM 01309, GM 01746, GM 02053, GM 02061, GM 02361, GM 02362, GM 02363, GM 04510, GM 08010 were obtained from the NIGMS Human Genetic Mutant Cell Repository in Camden, New Jersey. All of these were fibroblast lines except GM 4510 and 8010, which were lymphoblast lines. CRL 1196 and HG261 were fibroblasts cell lines and were obtained from American Type Culture Collection (ATCC) in Rockville, Maryland. All other cell lines were established at the Oregon Health Sciences University FA cell repository and are designated by the initials PD.

Reverse Transcription and PCR

10 μg of total cellular RNA, prepared from cultured lymphoblasts or fibroblasts by the RNAzol method [25], was used for reverse transcription utilizing a BRL RT kit and 0.5 ug of random hexamers as primers. The FA(C) cDNA was amplified [26] from normal and affected cells in 2 overlapping segments, denoted the 5' (1185 bp) and 3' (1146 bp) products. The primers for the 5' amplification were 5'-TTAATGTGTGCCGACCATTTCCTTC-3' and 5'-ATGCTTCAGTGTCTGGAGCCAG-3'. Primers 5'-GTCTGAGAAGGATCGAATGC-3' and 5'-TTATCAAGCTGACGGTCTGG-3' were used for the 3' amplification. PCR reactions were done with Kogan [27] buffer (10% DMSO) and the thermal profile was as follows: 95 °C x 7 min, 40 cycles of 90°C x 30 sec, 53 °C x 30 sec and 72 °C x 3 min 30 sec, 72 °C x 8 min.

Chemical mismatch cleavage

The chemical mismatch cleavage (CMC) analysis was carried out as described by Cotton [7] and Grompe [28]. Briefly, the probe for CMC was prepared by reamplification of a wild-type PCR product using end labeled PCR primers [29]. A sense and an antisense probe were prepared separately by using one radioactivity labeled and one unlabeled PCR primer. Ten ng of gel purified probe and 150 ng of mutant PCR product were mixed, boiled and allowed to reanneal, forming heteroduplexes. Heteroduplex DNA was subjected to either hydroxylamine modification for the detection of C and G mismatches or osmium tetroxide for the identification of T and A changes. Following chemical modification, the reactions were stopped with tRNA and ethanol precipitation. Piperidine cleavage was then carried out for 30 min at 90 °C, again followed by ethanol precipitation. The samples were redissolved in 10 ul formamide loading buffer and electrophoresed in a denaturing polyacrylamide gel followed by autoradiography.

Sequence analysis

PCR products were subcloned into pBluescript (Stratagene) and double-stranded dideoxy sequencing [30] performed on plasmid DNA prepared with the boiling miniprep method [29].

Genomic PCR

Intronic sequences flanking exon 4 of the FA(C) gene were kindly provided Dr. C. Mathew [9]. PCR primers, 5'-GTAGGCATTGTACATAAAAG-3' and 5'-TGGCACATTCAGCATTAAAC-3', capable of amplifying the splice junctions for this exon, were designed and PCR carried out on genomic DNA. DNA from the 2 patients with exon 4 splice mutations as well a normal controls were amplified with this method, subcloned and sequenced.

Allele Specific Oligonucleotide (ASO) Hybridization

Templates for ASO hybridization 12 were generated using PCR primers flanking exon 4 and 200 ng genomic DNA as described above. The PCR products were then denatured in 0.4 M NaOH and spotted onto Hybond N+ (Amersham) using a dot blot apparatus. Mutant (5'-AAAATGTGTGTATTT-3') and normal (5'-AAAATGTGAGTATTT-3') oligo-nucleotides corresponding to the IVS4 +4 A->T change were end labeled with [32P]-ATP and polynucleotide kinase [29]. Hybridization conditions were 6 x SSPE, 0.5% SDS at 30 °C for 3 h, followed by two ten minute washes at 22 °C and 26 °C in 4 x SSPE, 0.5% SDS.

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The Ashkenazi-Jewish Fanconi anemia mutation: Incidence among patients and carrier frequency in the at-risk population

Michael A. Whitney, Petra Jakobs, Michael Kaback, Robb E. Moses and Markus Grompe*

Department of Molecular and Medical Genetics, Oregon Health Sciences University, 3181 Sam Jackson Park Road, L 103, Portland, OR 97201, and Department of Pediatrics and Reproductive Medicine, UCSD School of Medicine, Children's Hospital, San Diego CA 92123

* To whom correspondence should be addressed

Summary

Fanconi anemia (FA) is an autosomal recessive disease for which at least four complementation groups exist. Recently the gene that corrects the defect in group C cells (FA(C)) has been cloned. We have previously identified a common mutation in the FA(C) gene which accounts for a majority of FA cases in Ashkenazi-Jewish individuals. We here describe the use of allele specific oligonucleotide (ASO) hybridization to determine the frequency of this mutation among additional Jewish FA patients and to determine the carrier frequency in the Jewish population. The common IVS4 +4A->T allele was found on 19/23 (83%) Jewish FA chromosomes, indicating that it is indeed responsible for most cases of FA among Ashkenazi Jews. The carrier frequency was 2/314 for Jewish individuals and the mutant allele was not detected in 130 non-Jewish controls.

Introduction

Fanconi anemia is an autosomal recessive disorder characterized by pancytopenia, defects of the thumb and radius, hyperpigmentation, short stature and susceptibility to cancer [1]. Somatic cell hybridization experiments have demonstrated the existence of at least 4 complementation groups, termed A, B, C and D [2, 3]. Recently the gene defective in group C was cloned. [4] and several mutations in FA(C) have been described [4, 5]. We have previously reported a mutation denoted IVS4 +4A->T in FA(C) that appears to account for the majority of FA cases in Ashkenazi-Jewish individuals [6]. Here we report the use of ASO hybridization to determine the frequency of this mutation among additional Jewish FA patients and to measure the carrier frequency in the Jewish population. This study addresses two important questions regarding the FA(C) IVS4 +4 A->T mutation: 1) Do all Jewish FA patients have the IVS4 +4 A->T mutation? 2) How high is the carrier frequency for this mutation within the Jewish population?

Results and Discussion

White blood cell pellets for the carrier testing were obtained from the California Tay-Sachs Disease Prevention program (M.K.). All samples from FA patients were obtained from the Oregon Health Sciences University Fanconi Anemia Cell Repository. The ethnic background of the patients was determined by questionnaire. DNA

was prepared from white blood cell pellets provided by the California Tay-Sachs Disease Prevention program using the Chelex (Biorad) method of DNA preparation [7, 8]. DNA from cells in the OHSU cell repository was isolated using the salting out method of Miller et al. [9]. Genomic DNA from individuals was PCR amplified and the ASO assay for the IVS4 +4 A->T mutation was performed as described previously [6].

In the OHSU cell repository there are registered 11 FA patients for which both parents are Ashkenazi-Jewish and one patient who has a Jewish mother and a non-Jewish father. Nineteen of the 23 (83%) Jewish FA chromosomes analyzed carried the IVS4 +4 A->T allele. The 95% confidence limits for this measurement are 61% - 95% [10]. This splice mutation in FA(C) therefore accounts for the majority of cases of FA in this ethnic group. All Jewish patients that carried this mutation were homozygous except the one patient whose father was not of Jewish descent. Of 39 non-Jewish FA patients screened by ASO none carried this mutation. This demonstrates that this allele is not commonly responsible for FA in non-Jewish individuals. Two Ashkenazi Jewish FA patients did not carry the IVS4 +4 A->T allele at all.

Through collaboration with the California Tay Sachs Prevention program our laboratory screened DNA samples from 315 Jewish and 130 non-Jewish individuals. The results obtained from DNA analysis of some Ashkenazi-Jewish individuals, with no family history of FA, are shown in figure 1. Of the 315 Jewish individuals tested only two were found to be carriers for the IVS4 +4 A->T while no non-Jewish carriers were detected.

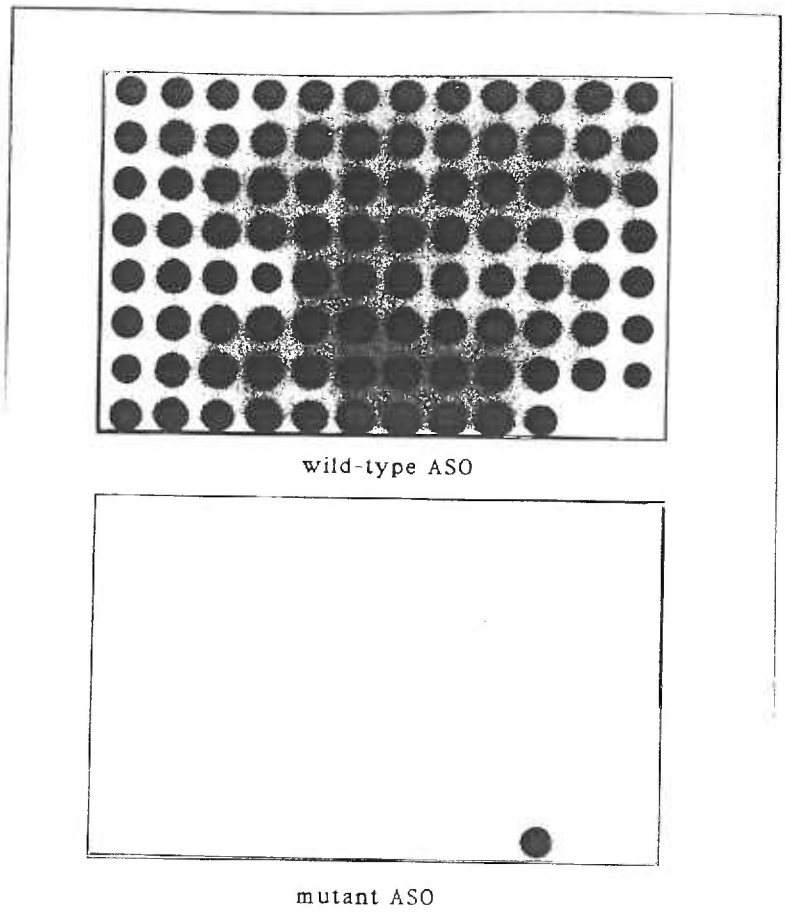

Figure 1. ASO detection of IVS4 +4 A->T carriers. The top panel was probed with wild-type and the bottom panel probed with the mutant ASO. Three samples hybridized to the mutant oligonucleotide. One (bottom row) is a known heterozygous control, whereas the other two positives (second from bottom row) represent carriers detected through random sampling of Jewish individuals.

Because PCR amplification from DNA samples obtained by Chelex-extraction are not always successful we verified the presence of PCR product in each sample by re-hybridizing all blots with the wild-type

oligonucleotide. Successful PCR amplification was achieved in all but one of the white cell pellet samples yielding a final carrier frequency of 2/314 individuals (1/157 = 0.64%; 95% confidence limits 0.08%-2.3%) [10].

The information provided here will prove useful for genetic counseling in FA families of Jewish descent. Due to the high morbidity and mortality of this disease, many families are interested in prenatal and carrier testing. With the availability of an ASO assay, these diagnostic tests can be performed rapidly and cheaply for any interested family.

There are several genetic diseases, such as Gaucher's and Tay-Sachs disease, that are known to cluster in the Jewish population. [11-13]. The carrier frequency for Tay-Sachs disease is high enough and the disease phenotype is sufficiently severe to warrant population based carrier testing in this disorder. While FA is also a debilitating condition with a low life expectancy, we have shown here that the frequency of carriers in the at-risk population is low and thus it appears impractical to perform routine carrier screening in the Ashkenazi Jewish population. However, the carrier frequency of the common IVS4 +4A->T mutation determined here will aid in the genetic counseling of Jewish families with FA. If a known carrier for FA complementation group C marries a spouse of Ashkenazi Jewish descent, the risk of having a child affected by FA is quite low (1/4 x 1/157 = 1/628; 95% confidence intervals 1/1,250 - 1/174). Other FA(C) alleles may exist in this population and slightly raise this estimate. However, because IVS4 +4A->T accounts for the vast majority of FA in Jewish individuals, the carrier frequency of these

hypothetical additional FA(C) mutations would have to be considerably lower than that of the IVS4 +4A->T allele. If a family is concerned about recurrence despite the low risk, carrier testing could be carried out with a high degree of reliability in this population by ASO analysis of genomic DNA or by using chemical mismatch cleavage of mRNA from white blood cells or fibroblasts [6].

Acknowledgments

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Microcell mediated chromosome transfer maps the Fanconi anemia group D complementing gene to chromosome 3p

Michael Whitney [1,2], Matt Thayer [1], Carol Riefsteck [1], Susan Olson [1], Leslie Smith [1], Petra M. Jakobs [1], Robin Leach [4], Susan Naylor [4], Hans Joenje [5], and Markus Grompe* [1,3]

Department of Molecular and Medical Genetics[1], Department of Biochemistry[2], Department of Pediatrics[3], Oregon Health Sciences University, Portland, Oregon 97201, USA

Department of Cellular and Structural Biology[4], The University of Texas Health Sciences Center, San Antonio, Texas 98284, USA

Department of Human Genetics[5], Free University, Van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands

*To whom correspondence should be addressed

Summary

Fanconi anemia (FA) is an autosomal recessive disease characterized by progressive pancytopenia, skin hyperpigmentation, limb defects, and predisposition to leukemia. At the cellular level FA cells are hypersensitivity to cell killing by DNA crosslinking agents. Five complementation groups for FA are currently known but only one gene, FA(C) (Fanconi anemia group C), has been cloned. Here we describe the use of microcell mediated chromosome transfer to localize the FA(D) complementing gene to chromosome 3p. Exclusion mapping in three FA(D) families further mapped the FA(D) gene to between markers D3S1307 and D3S1619, which correspond to the chromosomal region between 3p22 and 3p26.

Introduction

Fanconi anemia (FA) is an autosomal recessive disorder characterized by progressive pancytopenia, short stature, thumb and radial ray defects, hyperpigmentation of the skin, and a predisposition to cancer [1, 2]. Cells derived from FA patients are hypersensitive to cell killing by DNA cross-linking agents such as mitomycin C (MMC) or diepoxybutane (DEB) [3]. FA cells show spontaneous chromosomal aberrations at a high frequency which include chromatid breaks, gaps, exchange figures and endoreduplication [4]. The increased frequency of chromosome breakage is even greater when FA cells are treated with DNA-crosslinking agents. Due to this sensitivity of FA cells to DNA crosslinking agents, it has been proposed that the defect in FA cells is in the repair DNA crosslinks. Additional cellular phenotypes of FA include oxygen sensitivity [5, 6], poor cell growth [7], and a G2 cell cycle delay [8, 9].

Somatic cell hybridization experiments have demonstrated the existence of at least 5 complementation groups for FA, termed A through E [10, 11]. Patients from the different complementation groups are clinically indistinguishable. One of the five FA genes, FA(C) has been cloned by cDNA complementation of the hypersensitivity of FA(C) cells to DNA crosslinking agents [12]. The FA(C) gene has been mapped to human chromosome 9q22 by in- situ hybridization and linkage analysis [10]. None of the other FA genes

have been mapped or cloned to date. The FA(C) protein has no significant homology to any known protein and its function remains to be determined. Mutations in FA(C) account for approximately 15 percent of FA patients, while the frequency of complementation groups A, B, D, E has been estimated at 50%, 5%, 15%, 15%, respectively [11, 13, 14].

The strategy of cDNA complementation, which was successful for identifying the FA(C) gene, has not yet been successful for cloning other FA genes. An alternative approach which includes linkage analysis followed by positional cloning is hindered, in FA, by genetic heterogeneity and the lack of a simple assay for determining the complementation group of a given FA family. In contrast to genetic linkage studies, a mapping approach using microcell mediated chromosome transfer relies on functional complementation to identify the disease bearing chromosome [15]. Therefore, genetically informative pedigrees are unnecessary. Here we report the successful use of this approach to map the gene for the FA(D).

Results

Localization of FA(D) gene to chromosome 3

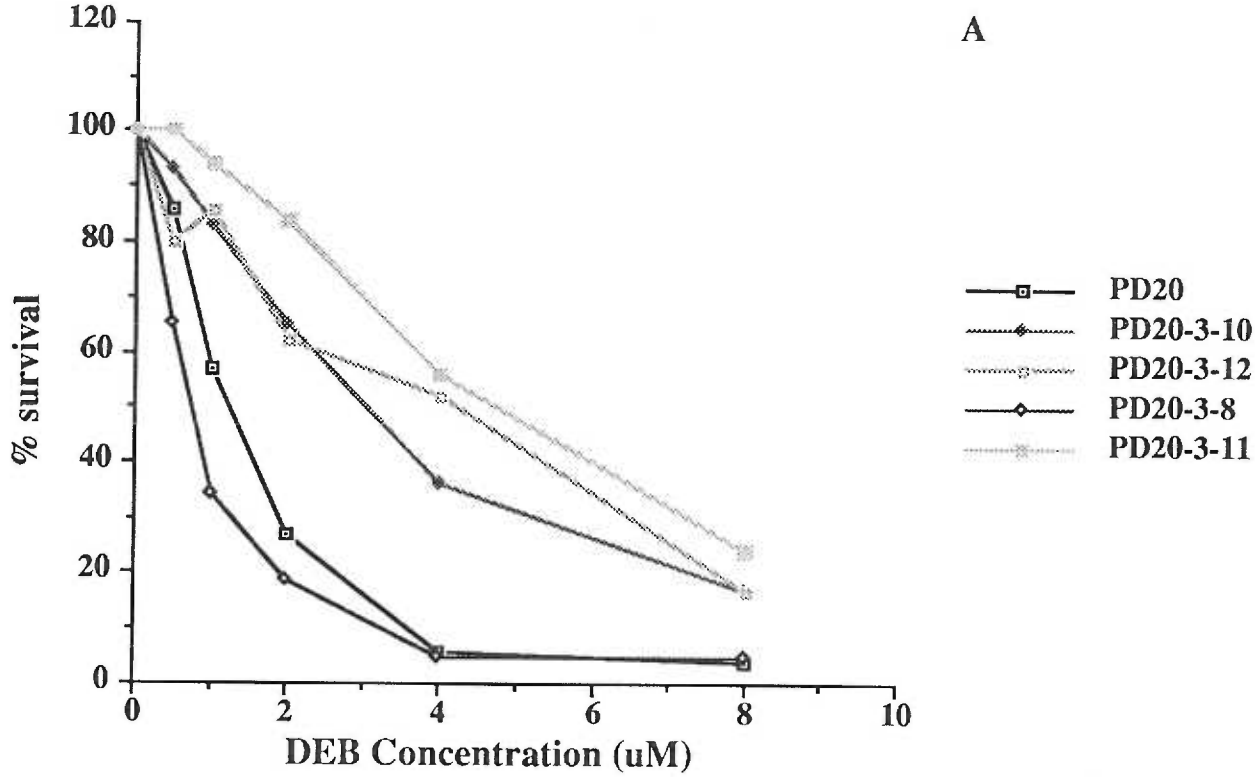
A new immortalized fibroblast cell line derived from an FA(D) patient, (PD-20) was used as a recipient for chromosome transfer. PD-20 cells retain all phenotypic characteristics of FA cells, including MMC and DEB sensitivity, as well as chromosome instability [16]. In a first group of experiments multiple murine A9 cell lines, containing single neomycin-marked human chromosomes, were used as

chromosome donors (see table 1). Microcells prepared from these donors were individually fused with PD-20 cells. After selection in G418 (geneticin), resistant clones, representing microcell hybrids, were picked and tested for sensitivity to cell killing by MMC and DEB. Multiple hybrids were analyzed from each fusion because donor chromosomes may fragment or partially delete during transfer. Multiple PD-20 microcell hybrids containing human chromosomes 4, 7, 8, 11, 14, 15, 17, and, 20 were tested and shown to be sensitive to both MMC and DEB (table 1). These chromosomes were therefore excluded as possible locations for the FA(D) gene.

In a second group of experiments several microcell donors were combined and their corresponding chromosomes were transferred as pools. One of these pools contained human chromosomes 1, 2, 10, and 13 and a second consisted of chromosomes 3, 5, and, 20. Multiple hybrid clones were picked and analyzed for sensitivity to DNA crosslinking agents. Only the fusion which utilized human chromosomes 3, 5, and, 20 produced microcell hybrids resistant to both mitomycin C and DEB. One complemented hybrid from this fusion (PD20-PH-8) contained human chromosome 5. However, the complementation of PD20-PH-8 was later shown to segregate with a mouse chromosome and not chromosome 5 (data not shown). In contrast 6/7 microcell hybrids containing human chromosome 3 were found to be resistant to cell killing by both MMC and DEB. One of these 7 hybrid clones (PD20-3-8) remained sensitive to both crosslinking agents (Figure 1).

Donor Cells	Chromosome	Sensitive hybrids	Resistant hybrids
HA(4)	4	3	-
GM11714	5	6	-
10HSM-10	7	4	-
HA(8)	8	3	-
HDM-18	11	25	-
GM11715	15	7	-
L(17n)E9	17	9	-
GM13260	20	9	-
H(20)31-9	20	2	-
GM11689	13		
GM11712	2	13	-
GM11688	10		
GM13139	1		
GM11713	3		
GM11714	5	10	8
GM13260	20		
GM11713(fusion2)	3	0	18

Table 1. MMC sensitivity of FA(D) microcell hybrids. Donor cell lines are shown with the corresponding number of complemented and non-complemented hybrids indicated.


A
120
100
80
60
40
20
0
% survival
0
2
4
6
8
10
DEB Concentration (uM)
PD20
PD20-3-10
PD20-3-12
PD20-3-8
PD20-3-11

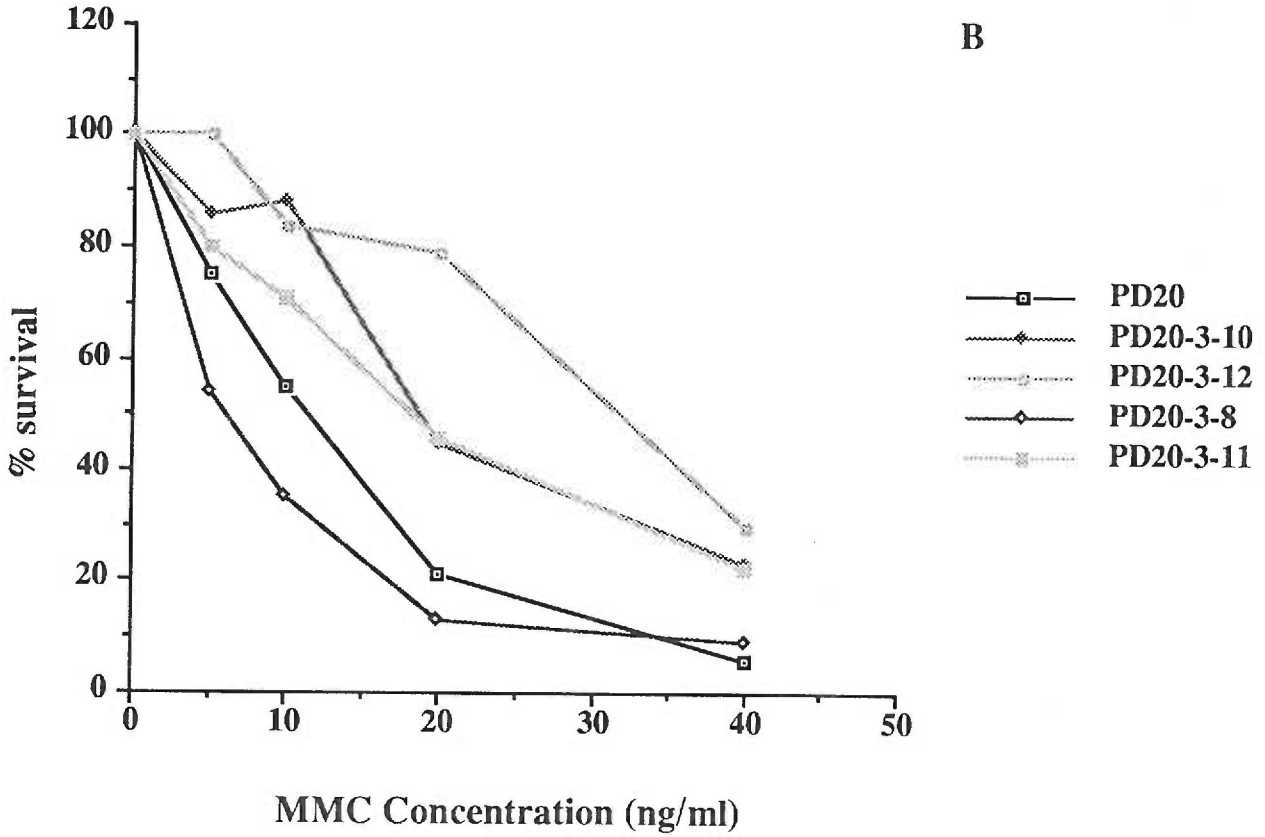

B
120
100
80
60
40
20
0
% survival
0
10
20
30
40
50
MMC Concentration (ng/ml)
PD20
PD20-3-10
PD20-3-12
PD20-3-8
PD20-3-11

Figure 1. Kill curves from PD-20 chromosome 3 microcell hybrids. A. DEB kill curves showing cellular sensitivity of three complemented PD-20 chromosome 3 microcell hybrids, PD-20-3-10, PD20-3-11, and PD20-3-12, as well as, one non-complemented hybrid PD20-3-8. The kill curves for parental PD-20 cells are also shown. B. MMC kill curves for the same cell lines as shown in A.

Next, these 7 chromosome 3 microcell hybrids were analyzed for phenotypic complementation by chromosome breakage analysis. This analysis, which is typically used for clinical diagnosis of FA [4], verified that the same 6/7 MMC resistant hybrids were cytogenetically corrected (see table 2). Table 2 shows chromosome breakage results for the parental cell line, PD20HP1, 8 corrected chromosome 3 hybrids, PD20-3-4, PD20-3-5, PD20-3-6, PD20-3-10, PD20-3-11, PD20-3-12, PD20-3-15, PD20-3-20. Also shown is the one non-complemented chromosome 3 microcell hybrid PD20-3-8 and a whole cell fusion between of group A and group D fibroblasts (PD20-GM6914). DNA florescence in-situ hybridization analysis with total mouse DNA demonstrated that 3/6 of these corrected hybrids contained no mouse DNA and, therefore mouse chromosomes could not account for the complementation of the FA(D) cells. Thus, only human chromosome 3 was able to complement the multiple cellular phenotypes associated with Fanconi anemia group D cells.

Cell line	DEB(100ng/ml)	DEB(200ng/ml)	MMC(40ng/ml)	MMC(60ng/ml)
PD20HP1	42	58	24	40
PD20-3-4	0	0	0	0
PD20-3-5	4	4	6	4
PD20-3-6	4	16	4	4
PD20-3-8	48	66	48	44
PD20-3-10	0	2	0	0
PD20-3-11	0	0	0	0
PD20-3-12	0	0	0	2
PD20-3-15	0	4	0	6
PD20-3-20	0	0	0	0
PD20-6914	0	0	4	18

Table 2. Chromosome breakage analysis of chromosome 3 microcell hybrids. Measured in percent of cells with radial formations

Deletion analysis of Chromosome 3 microcell hybrids

In order to further delineate the region of chromosome 3 responsible for complementation of the FA(D) cells we performed a microcell hybrid deletion analysis. Deletion analysis is possible because chromosomes transferred using microcell transfer frequently become deleted and this can be used to sublocalize complementing genes. The FA(D) gene containing region was expected to be present in complemented hybrids and deleted or mutated in non-complemented hybrids. Since both the correcting chromosome 3 and the recipient FA cells were of human origin polymorphic markers were used to distinguish the donor chromosome 3 from the two parental (PD-20) chromosomes. Of 80 chromosome 3 markers tested, 11 produced unambiguously different allele sizes for the donor and recipient chromosomes. These 11 markers were tested of the initial 7 chromosome 3 hybrids and only one of these hybrids, PD20-3-6 was found to be deleted for a single 3q marker, D3S1579. Because PD20-3-6 was a complemented hybrid this deletion must not contain the FA(D) gene. A deletion was not detected in the non-complemented hybrid PD20-3-8 using these 11 markers. Therefore, PD20-3-8 likely had a small deletion containing the FA(D) gene but not containing any of the polymorphic markers tested.

In order to obtain more information on the subchromosomal localization of the FA(D) gene, a second chromosome 3 fusion was performed and an additional 18 hybrids were generated. All of these hybrids were determined to be complemented by chromosome

breakage analysis. 2/18 of these new complemented hybrids, PD20-3-15 and PD20-3-20, were found to be deleted for multiple markers from the q arm of chromosome 3, (D3S1269, D3S1216, D3S1548, D3S1617, D3S1314) (figure 2). Thus, the FA(D) gene must lie between the telomeric region of 3p and the 3q region near the centromere. No deletions of in 3p were detected in any of the complemented hybrids.

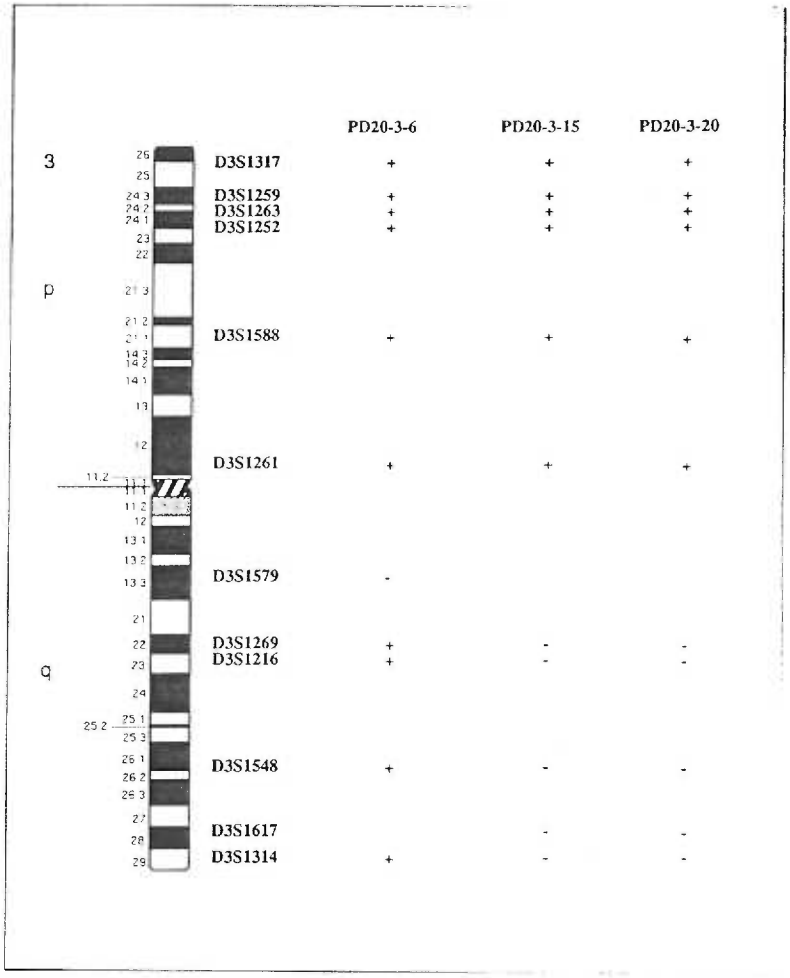


Figure 2. Deletion analysis of PD-20 chromosome 3 microcell hybrids. 12 informative markers which were tested on three complemented chromosome 3 microcell hybrids PD-20-3-6, PD20-3-15 and PD20-3-20.

Exclusion mapping of the FA(D) locus on Chromosome 3

Group D is a relatively rare complementation group of FA, and DNA samples were available from only 3 confirmed families. Because all 3 of these FA(D) families are small, it would be impossible to obtain statistically significant Lod scores from them. However, the localization of the FA(D) gene to chromosome 3 by functional complementation allows detailed positional information to be obtained through exclusion mapping. This strategy relies on the requirement that affected siblings in an autosomal recessive pedigree be genetically identical at the disease locus and that non-affected siblings have different allele on at least one chromosomes. These criteria were used, in conjunction with polymorphic markers, to generate an exclusion map for the FA(D) locus on chromosome 3.

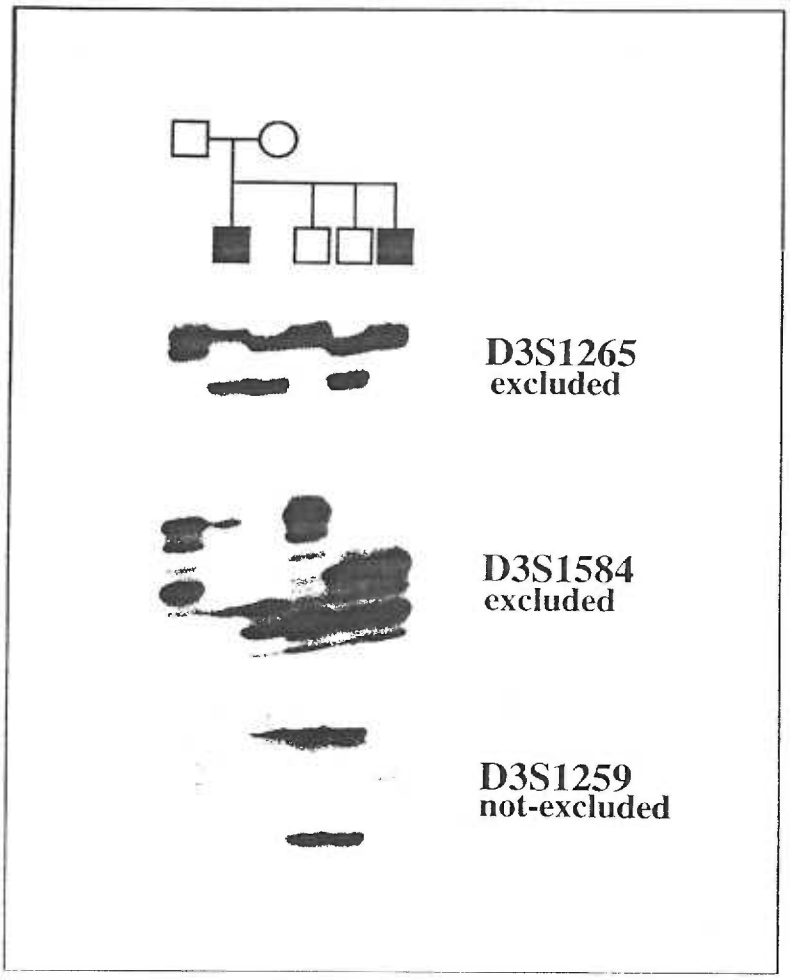


Figure 3A. Exclusion mapping of the FA(D) gene in the PD-20 family. The results for three polymorphic markers tested in the PD-20 family are shown. The corresponding allele sizes are positioned below the individual members in the pedigree as shown. From left to right they are: PD-19 (father), PD20 (affected sibling), PD-21 (mother), PD-22 (unaffected sibling), PD-23 (unaffected sibling), PD-24 (affected sibling).

A total of forty one informative chromosome 3 markers were analyzed in the PD-20 FA(D) family. The PD-20 family consisted of two affected and two unaffected children. Of the 41 markers tested 28 were excluded and 13 were not excluded (see figure 3).

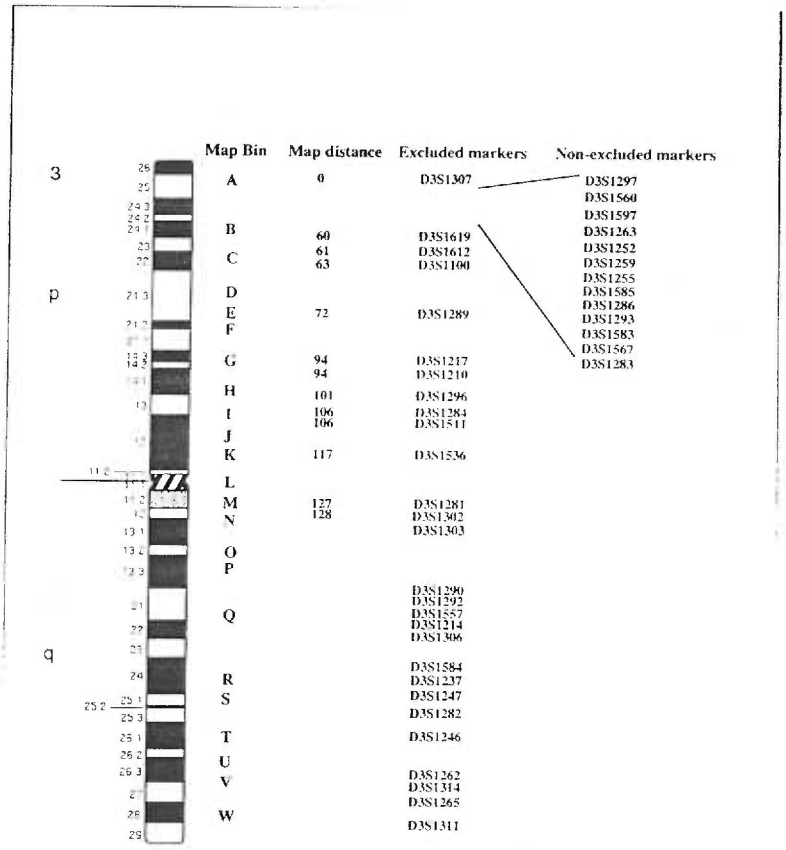


Figure 3B. Exclusion map for the FA(D) locus on chromosome 3. The result are shown for 41 informative microsatellite markers tested in the PD-20 FA(D) family.

All of the non-excluded markers were localized between D3S1619 and D3S1307. This region of homozygosity, between the two affected siblings spans from 3p22 to 3p26, and likely contains the FA(D) gene. Markers from this region of homozygosity were tested in two additional FA(D) families. The results obtained were consistent with a map position between D3S1619 and D3S1307, but the candidate region could not be narrowed further

Discussion

Over the past decade positional cloning strategies have led to the identification of more than 40 genes responsible for inherited human diseases [17]. For most of these genes the initial positional information was obtained through X-linked pedigrees, cytogenetic rearrangements, or linkage analysis from autosomal dominant inheritance. Alternatively, positional information was obtained by linkage analysis using large genetically informative families with autosomal recessive diseases. However, linkage analysis for rare, genetically heterogeneous diseases like Fanconi anemia is considerably more difficult. This is particularly true for rare complementation groups containing as few as one family. For these reasons we used microcell mediated chromosome transfer to identify the chromosomal location of the FA(D) complementing gene. This is a necessary first step for any strategy directed at positionally cloning the FA(D) gene.

The results presented here indicate the presence of an FA(D) complementing gene on chromosome 3p. None of the other human chromosomes tested, (see table 1) representing about 80% of the

genome, resulted in phenotypic complementation of FA(D) cells. Exclusion mapping further narrowed the FA(D) gene containing region to between 3p22 and 3p26. At least 25 known genes have been mapped to this region which spans approximately 50 cM. Two genes implicated in DNA repair, HHRAD23B and XP-C, map to this region and are currently being evaluated by us as candidate genes for FA(D) [18, 19]. As additional genes continue to be mapped to this region they will also become candidate genes for FA(D).

The information presented here could also prove useful for groups using cDNA complementation to identify the FA(D) gene. False positives are often identified by cDNA complementation and testing chromosomal localization could be used to identify reasonable candidates. Although the candidate region is large (50 cM) the odds of any given cDNA mapping to this region by chance is only about 1 in 60. Alternatively, the identification of the FA(D) gene could be achieved by using positional cloning strategies aimed at narrowing the FA(D) gene containing region to a clonable unit. These positional cloning approach should take advantage of the functional assay available for the FA(D) gene. One such approach would take advantage of the YAC contig which currently exists for the FA(D) candidate region to identify individual YACs which contain the FA(D) gene by functional complementation.

Due to the clinical and cellular characteristics of Fanconi anemia their corresponding gene products are likely to be involved with many cellular processes including DNA repair, cell cycle regulation, cancer progression as well as hematopoiesis. Despite the isolation of the FA(C) gene 3 years ago, still very little is known about cellular

function of FA(C) or the biochemical and physiological defects associated with FA. The isolation of additional FA genes may provide some additional clues to these cellular processes through sequence homology to known proteins an lead to a clearer understanding of Fanconi Anemia.

Materials and Methods

Chromosome breakage

For chromosome breakage analysis cell were plated on 100mm dishes and allowed to recover overnight. Cell were exposed the to MMC or DEB for a 24 hours. Cells were then treated with colcemid for 3 hours and placed in hypotonic media containing 25% FCS and dH2O. Cells were fixed to slides, stained with Wrights stain, followed by scoring cells for breaks, and radials per cell.

Cell lines

PD-20 is an FA(D) immortalized fibroblast cell line and was generated by the Oregon Health Sciences Fanconi Anemia cell repository. The human/mouse hybrid donor cells lines GM11713, GM11712, GM13139, GM11688, GM11714, GM13260, GM11689, GM11715, were obtained for the NIGMS Human Genetic Mutant Cell Repository. Donor cell lines HDM-18, and L(17n)E9 were obtained from Fournier and HA(8) was obtained from A.M. Killary. Murine hybrid cell lines were cultured in DMEM with 15% bovine calf serum (Hyclone). Human fibroblasts and microcell hybrids were cultured in α-MEM and 10% fetal bovine serum.

Microcell fusion

Microcell fusions were performed as described by Fournier [15, 20]. Briefly, donor cells were split onto 150mm dishes and allowed to recover for 24 hours. Colcemid (0.06 ug/ml) was added and cells were incubated for 24-48 hours. Micronucleated cells were then trypsinized and allowed to sit down on 'bullets' coated with crosslinked concanavalin A. Bullets were then placed into 50ml centrifuge tubes containing DMEM and 10ug/ml cytochalasin B and centrifuged at 14krpm for 30' at 37°. The resulting pellets were resuspended in DMEM and filtered through an 8 um filter. The microcells were then mixed with 100ug/ml phytohemagglutinin P (PHA-P, Difco) and added to a monolayer of PD-20 fibroblasts. After 15 minutes cells and microcells were fused with 50% PEG [21] for 1', washed with serum free DMEM media and allowed to grow overnight in DMEM with 15% fetal bovine serum. The next day cells were split 1:10 into selective media containing 250ug/ml G418 and HAT (hypoxanthine, aminopterin, thymidine). Clones were then picked, expanded and independently analyzed.

MMC and DEB Kill curves

Kill curves were generated by use of an MTT assay [22] for cellular viability after a 5 day exposure to MMC or DEB. Approximately 2000 cells were seeded into six wells of a 24 well microtiter dish. MMC or DEB was added to final concentrations of 0, 5, 10, 20, 40, 80 ug/ml for MMC or 0, .5, 1, 2, 4, 8 nmol/ml for DEB. After incubation for five days, 500ug/ml MTT final concentration was added and cells were incubated 4 hours at 37°C. The MTT containing media was then aspirated and 1 ml of 5% triton was added. After the blue precipitate was completely dissolved absorbance at 580nm was measured and plotted against the concentration of DEB or MMC.

Dinucleotide repeat analysis

Oligonucleotide primers for the microsatellite markers were obtained from Research Genetics. PCR was performed as described in the publication and mapping of each primer set. Resulting PCR products were electrophoresed through a 6% polyacrylamide gel, transferred onto Hybond N+ and probed with an end labeled 70 base pair CA repeat oligo. DNA from the PD20 family and the microcell hybrids was prepared as described in [23].

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Mice with a targeted disruption of the Fac gene exhibit phenotypic characteristics of Fanconi anemia

Michael Whitney [1,2], Carol Riefsteck [1], Susan Olson [1],
Malcolm Low [4], and Markus Grompe [1,3]

Department of Molecular and Medical Genetics [1],
Department of Biochemistry [2], Department of Pediatrics [3],
Oregon Health Sciences University, Portland Oregon 97201,
Vollum Institute [4], Portland, Oregon 97201

Summary

Fanconi anemia (FA) is a genetically heterogeneous autosomal recessive disease characterized by progressive pancytopenia, thumb and radial abnormalities, skin hyperpigmentation, short stature and a predisposition to leukemia. The FA(C) (group C) gene is the only gene, of the five known complementation groups, that has been cloned. The FA(C) gene product has no significant sequence homology with any known protein and its function has not been determined. A murine homologue(Fac) of the human FA(C) gene has been identified and shown to be 67% identical and 78% similar to the human sequence. We have now used embryonic stem cell technology to create a disruption of the murine Fac gene. Mice homozygously deleted for Fac exon 9 were generated and phenotypically characterized. Homozygous mice are viable with no apparent developmental deformities. Complete blood counts on 6 week old mice appear normal. Female Fac deficient mice do show a marked reduction in fertility. In addition, primary skin fibroblasts derived from homozygous mice show chromosome breakage in response to DNA crosslinking agents similar to that seen in FA patients. These findings indicate a FA like phenotype and mutant mice are currently being further characterized.

Introduction

Fanconi anemia (FA) is an autosomal recessive disease characterized by childhood onset progressive pancytopenia and a predisposition to acute myeloid leukemia [1]. Developmental deformities are also displayed which include short stature, thumb and radial ray defects, as well as kidney and renal abnormalities [2]. Cells derived from FA patients are hypersensitive to cell killing by DNA crosslinking agents such as, Mitomycin C (MMC) and diepoxybutane (DEB) [3]. Spontaneous chromosomal aberrations are observed at an increased frequency in FA cells and include chromatid breaks, gaps, exchange figures and endoreduplication. This increased frequency of chromosomal aberrations becomes even more apparent if growing FA cells are treated with DNA crosslinking agents. Due to this sensitivity of FA cells to DNA crosslinking agents, and their apparent chromosome instability, the defect in FA has been proposed to be in the repair of DNA crosslinks. It has alternatively been suggested that the FA defect is in the regulation of embryonal and postembryonal development, especially of the hematopoeitic stem cell [2].

Currently, there are five known complementation groups for FA suggesting the presence of at least five genes [4, 5]. Of these complementation groups only the gene defective in FA(C) patients has been cloned [6]. The cloning of the human FA(C) gene by functional complementation provided no hints to the functional defect in FA as

it was not homologous to any protein of known function. Recent studies with antibodies generate against the FA(C) protein suggest that the FA(C) protein is localized primarily in the cytoplasm. It has therefore been suggested that FA(C) does not have a direct role in DNA repair [7, 8]. A murine homologue of the human FA(C) gene has been identified and has amino acid identity of 67% and similarity of 78% with the human protein [9]. It has further been shown that the murine FA(C) homologue (Fac) can functionally complement the hypersensitivity of human FA(C) cells to DNA crosslinking agents.

No animal model for FA exists to provide a system in which the complex developmental deformities in FA can be studied. An animal model for FA could also provide a system in which novel treatments for FA could be tested. These novel therapies would include pharmacological treatments as well as gene therapy approaches targeted at correcting hematopoeitic stem cells [10]. It is for these reasons that we generated mice which are deficient for the murine homologue of the human Fanconi anemia group C gene. Phenotypic similarities between Fac deficient mice and human patients are presented.

Results

Generation of Fac Deficient Mice

The murine Fac gene was previously identified by low stringency cross hybridization with human FA(C) [9]. A λFix mouse 129Sv genomic library was screened with a full length mouse Fac cDNA probe. One genomic clone (λphage gmFac7) obtained from this screening was mapped and shown to contain Fac exons 8, 9, and, 10 (see figure 1). λphage gmFac7 was then used to construct a targeting vector designed to disrupt the murine Fac gene. In this targeting construct Fac exon 9 was replaced with a neomycin resistance expression cassette. Specifically, the 1.1 kb *EcoRI* fragment containing exon 9 and the 1.4 kb *EcoRI/BamHI* fragment immediately downstream, were replaced with the *BamHI/EcoRI* fragment from PGKNeobpA [11]. In addition, PGKTK [11] was cloned into the *Not I* site of the SK+ polylinker outside the 3.9kb 3' genomic flank of the construct. This targeting vector was electroporated into embryonic stem cells and clones doubly resistant to G418 and Gancyclovir were selected [11]. Four correctly targeted clones were identified from 77 G418/Gancyclovir resistant clones. Targeting was determined by Southern blot analysis, using the 1.4kb exon 8 containing *EcoRI/BamHI* fragment as a probe, on *BglII* digested genomic DNA (see figure 2).

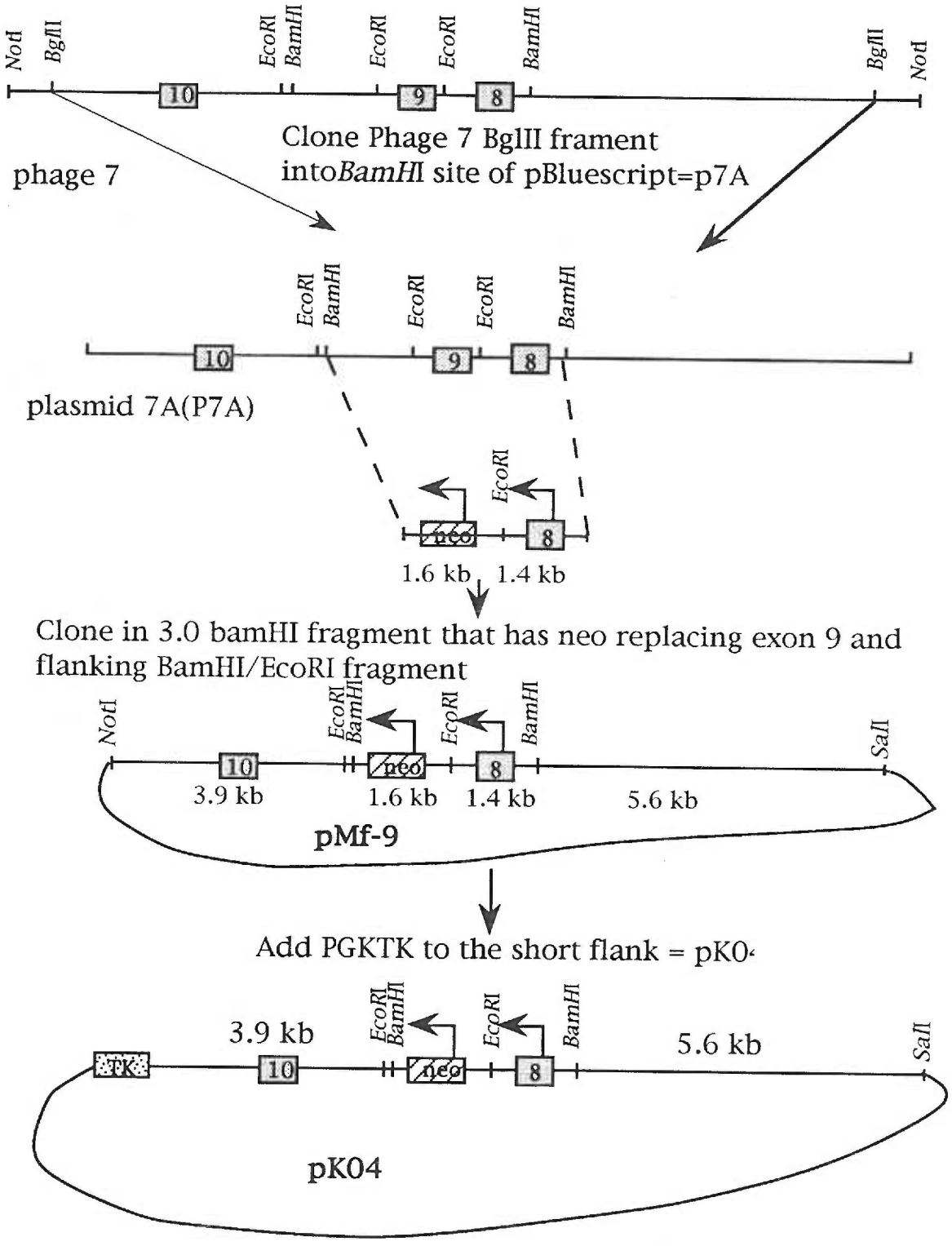

Figure 1. Construction of FAC targeting plasmid

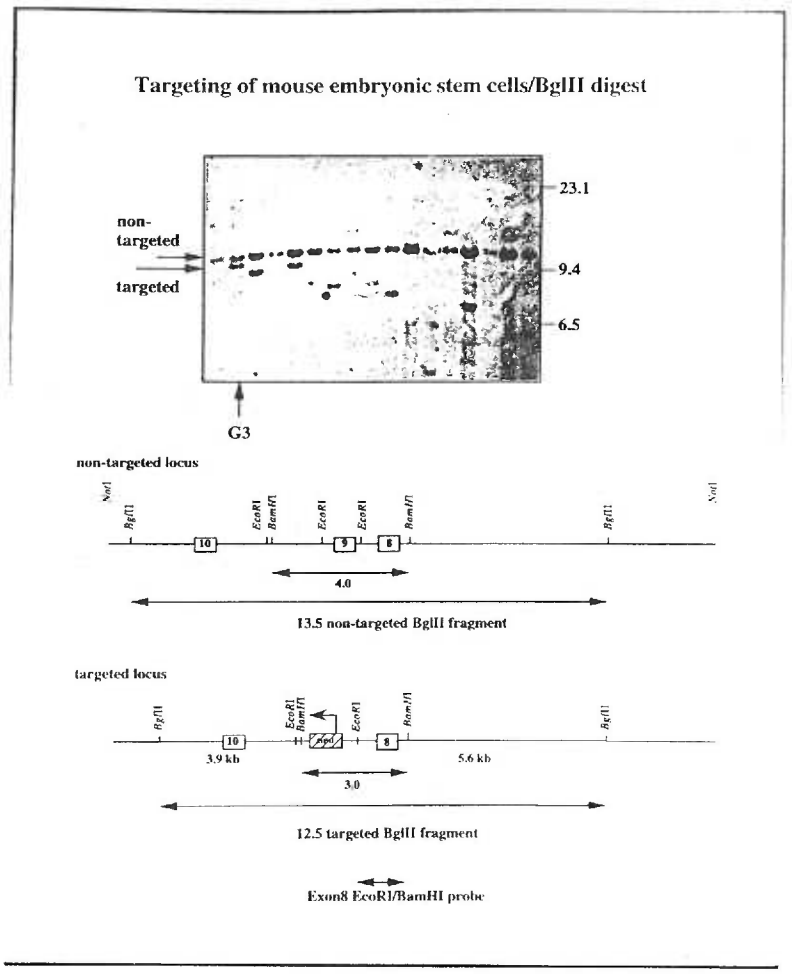

Figure 2. Targeted disruption of the murine Fac gene(*BglII* digest). BglII digested genomic DNA probed with the exon 8 containing 1.4 kb EcoRI/BamHI fragment.

Because the exon 8 probe was contained within the targeting construct a second Southern blot was done using an *NheI* digest on the four clones which appeared to be properly targeted. For the *NheI* digest a probe was used from the 5' flank outside the region contained within the targeting construct (see figure 3). No secondary insertions or rearrangements were detected in any of the four targeted clones.

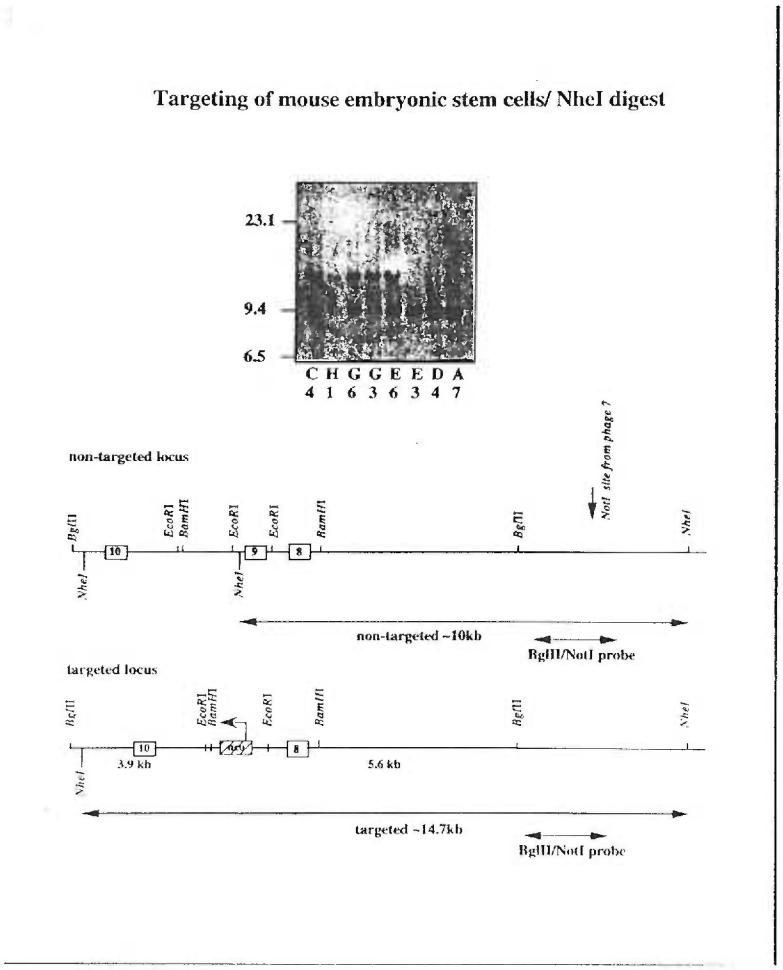

Figure 3. Targeted disruption of the murine Fac gene (*NheI* digest). *NheI* digested genomic DNA probed with the 5' flanking *NotI/BglII* fragment

Chimeric mice were generated by microinjection of C57BL blastocysts with a single targeted clone FacH1, followed by transplantation into pseudopregnant females [12]. Heterozygous founders were generated from chimeric mice and bred to produce homozygous Fac deficient mice.

Hematopoeitic analysis

Complete blood counts which included hemoglobin, hematocrit, red blood cell count, white blood cell count, and platelet counts were used to analyze Fac deficient mice as well as normal litter mate controls. There were no statistically significant differences detected between the Fac deficient mice when compared with their normal litter mate controls at 6 weeks of age. We are continuing to monitor these mice as hematopoeitic abnormalities may develop in at an older age.

Reduced fertility of Fac deficient mice

Although there were no apparent hematopoeitic abnormalities in the Fac deficient mice at 6 weeks of age it was noticed that Fac homozygous mutant females (Fac-/Fac-) appear to have significantly smaller litters than their homozygous wild type (Fac+/Fac+) or heterozygous (Fac+/Fac-) litter mates. We therefore, set up breedings to test the level of reduced fertility in these mice. A small pilot experiment was set up using 4 (Fac-/Fac-) and 4 (Fac+/ Fac-) female mice. Each female mouse was crossed with Fac+/Fac+ male mice and, the number of litters, and size of litters was monitored over a 3 month period. For the (Fac+/ Fac-) females there was a total of 11 litters with an average size of 7.8 pups. For the (Fac-/Fac-) females, for the same period, there were only 4 litters with an average size of 1.5. These results indicate there is a reduction in litter size as well as a reduction in the total number of litters.

Chromosome instability in Fac deficient mice

Primary mouse fibroblast cultures were established from 17 day embryos derived from two homozygous deficient, two heterozygous and one homozygous wild type embryo. The primary fibroblasts were cultured in DMEM containing 15% fetal bovine serum, and gentamicin. These five primary fibroblast cell lines were tested by chromosome breakage analysis (see figure 4). The two fibroblast cell lines (MPF-3 and MPF7), which were derived from Fac homozygous deficient mice, showed increased chromosome breakage over homozygous wildtype (MPF-4) and heterozygous controls (MPF-5 and MPF-1) at every concentration of mitomycin C tested. One homozygous mutant fibroblast cell line, MPF-7, showed spontaneous chromosome breakage at a significant level.

		Mitomycin C ug/ml					
Cell line	Fac genotype	0	4	8	10	20	40
		%radial formations					
MPF-3	-/-	0	12	38	36	82	92
MPF-7	-/-	8	20	18	46	86	86
MPF-1	-/+	0	0	0	0	10	26
MPF-4	+/+	0	0	0	0	16	32
MPF-5	-/+	2	2	2	0	10	26

Figure 4. Chromosome breakage of Fanconi anemia group C mouse primary fibroblasts. Chromosome breakage measured as percent cells with radial formations. MPF-3 and MPF-7 are homozygous deficient and show increased breakage at all concentration of MMC. Heterozygous MPF-1 and MPF-5 show the same breakage levels as the homozygous wildtype control MPF-4

Methodology

Generation of Fac targeted ES clones

A λFix. AB129 mouse genomic library was screened with a full length Fac cDNA which was generated by PCR. The cDNA probe was generated using primers MG060 5' ctgctcctagagatggctcagg 3' , and MG065 5'ggtgcccacattctgtactacc3' and mouse liver cDNA as template. The targeting construct was made with two uninterrupted regions of homology of 3.9 and 7kb. Internally, the 1.1 kb *EcoRI* fragment containing Exon 9 and the 1.4 kb *EcoRI/BamHI* fragment just downstream of that were replaced with a pgkNeo insert. In addition, a HSV thymidine kinase expression cassette (PGKTK) [13] was cloned into the Not I site of the SK+ polylinker outside the 3.9kb genomic region of mouse Fac. The targeting vector was then electroporated into embryonic stem cells and clones were selected for G418 resistance and resistance to Gancyclovir. Four correctly targeted clones were identified from 77 G418/Gancyclovir resistant clones as determined by Southern blot analysis. Two Southern blots were done, one with a probe contained within the targeting construct (Exon8 containing *EcoR1/BamHI* fragment) and, a second (5' *BglII/NotI* fragment from phage gmFac7) on the 5' flank outside the region contained within the targeting construct. AB129 embryonic stem cells were cultured in DMEM with 15% fetal bovine serum, penicillin, streptomycin, mercaptoethanol, and leukemia inhibitory factor (LIF). They were plated on a feeder layer of SNL cells which had previously been mitotically inactivated with MMC [11].

Generation of Fac deficient mice

Chimeric mice were generated by microinjection of C57BL blastocyst with two targeted ES clones (FacH1, FacG3), followed by transplantation into pseudopregnant females [12]. Four highly chimeric mice were generated from clone FacH1 and an additional two from clone FacG3. Heterozygous Fac deficient founders were produced from clone (FacH1) and bred to produce homozygous Fac deficient mice.

Hematopoeitic analysis

Blood for hematopoeitic analysis was harvested by retroorbital bleed into heparinized capillary tubes. Complete blood count analysis were done by Northwest Veterinary Diagnostic Services.

Chromosome breakage

For chromosome breakage analysis cells were plated on 100mm dishes and allowed to recover overnight. Cell were then treated with various concentration of DEB or MMC for a 24 hour period followed colcemid for 3 hours. Cells were then fixed to slides, stained with Wright's stain, and 50 cell were scored for breaks and radial formations.

Discussion

Animal models are useful tools for the study of human diseases. They provide a model system for both the study of novel treatments, and for experiments aimed at understanding the functional defect and pathology of the disease. With the recent advent of embryonic stem cell technology and targeted homologous recombination it has become possible to generate mice deficient for any previously cloned mouse gene. This technique is therefore useful for the study genes of unknown function as well as genes which were previously well characterized. The phenotypic characteristics of mice generated by this technique are sometimes exactly as predicted, but can also be unexpected. This is because phenotypic expectations are usually based upon an incomplete set of primary evidence. Here we report the use of this technique to generate mice deficient for the murine homologue of the Fac gene, which when defective in humans causes Fanconi anemia. The predicted phenotype would therefore be one that mimics the human disease.

Human FA is characterized by a high level of phenotypic heterogeneity, both in severity, and age of onset. This phenotypic heterogeneity may be due to a number of factors including, genetic background, environmental factors. Therefore, the phenotypic expectation for mice disrupted for the Fac gene is somewhat unknown. An additional variable in the production of mouse models for human diseases, by targeted gene disruption, is the considerable genetic differences between mice and humans.

The results of the preliminary characterization of Fac deficient mice indicate there are some phenotypic similarities with human FA. The hematopoeitic abnormalities seen in human patients have not been detected in the Fac deficient mice at 6 weeks of age. This may not be surprising because the hematopoeitic abnormalities associated with human FA are progressive and sometimes not detected until their fourth or fifth decade of life. We are therefore continuing to monitor the Fac deficient mice for hematological abnormalities.

The Fac deficient mice show no apparent developmental abnormalities. However, since the developmental abnormalities associated with FA are very inconsistent and variable in severity their absence in Fac deficient mice may not be that surprising. The mice may have developmental deformities which are more subtle than their human counterparts. We are currently planning to test the Fac deficient mice for these more subtle developmental abnormalities.

The major phenotypic similarity that has been detected so far between the Fac deficient mice and human FA patients is chromosome instability. This chromosome instability was detected in fibroblast cell lines derived from homozygous Fac deficient mice. Chromosome instability, which is tested with chromosome breakage analysis, is the earliest detectable characteristic associated with human FA. This result indicates that the exon 9 deletion has disrupted Fac function and it may therefore be likely that these Fac deficient mice will develop the additional phenotypes associated with FA at an older age.

The unexpected phenotype detected in Fac deficient mice is a reduction in female fertility. It is as yet unclear what biological defect is causing this apparent phenotype. It is possible that the reduced fertility represents a developmental defect of the ovaries, uterus or other part of the female reproductive system. We are currently testing whether the reduced number of offspring is due to embryo loss or reduced release of eggs by the mother.

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Discussions and Conclusions

Information obtained from the study of human genetic disease can be useful for both understanding basic mechanisms of human biology, as well as for understanding the clinical features and possible treatments for the disease. The work presented here relates to both of these areas of the human genetic disease Fanconi anemia (FA). The phenotypic characteristics of Fanconi anemia are very interesting as defects are displayed in multiple developmental and cellular processes. It is therefore, difficult to explain how these defects which include, developmental and hematological abnormalities, increased cancer risk, and sensitivity of cells to DNA damaging agents, can all be accounted for by a single gene disorder. This is probably one reason why its has been suggested that the FA defect is most likely a defect in DNA repair. The additional abnormalities associated with FA may then be secondary consequences of this fundamental defect in DNA repair. The high level of phenotypic heterogeneity in FA could therefore depend on the accumulation of mutations in a variety of different genes. Although this possible explanation fits for FA, until the other FA genes are cloned and characterized the functional defect will remain unknown. The elucidation of the basic defect in FA promises to provide insights into mechanisms of repair, cell cycle regulation or hematopoeitic stem cell biology, and carcinogenesis.

The isolation of additional FA genes is high priority in FA research as thier identification may provide additional clues of FA biology.

Toward the identification of new FA genes we have presented here results that map the FA(D) gene to chromosome 3p22-3p26. This mapping represents a significant advance towards the identification of the FA(D) gene and will hopefully lead to the cloning of the gene. A number of known genes have been mapped to this region of chromosome 3. Two of these genes HHRAD23B, and XP-C, have been implicated in or shown to be involved in DNA repair. They are therefore currently being investigated as candidate genes for FA.

As for the clinical characterization of FA, our results have shown that a single splice mutation (IVS4+4A->T) in the FA(C) gene accounts for the majority (83%) of FA among Ashkenazi-Jewish people. We have also estimated that the carrier frequency of this mutation in this population is about 1:60. These findings have important clinical implications as they provide useful information for genetic counseling in FA families of Jewish descent. Additionally, due to the high morbidity and mortality of FA, many families are interested in prenatal diagnosis and carrier testing. The identification of these and other common mutations allows the generation of simple assays for this testing. We have also shown that mutations in FA(C) account for a minority(~15%) of FA cases.

In another project directed at understanding the functional defect in FA we have used 'knockout' technology to create mice deficient in the Fac gene. The availability of an animal model for FA would provide a model for the study novel treatments, as well as for experiments directed at understanding the defects and pathology of the disease. The Fac deficient mice we have generated are still quite young and the major phenotypic similarity they share with human

FA, is cellular chromosome instability. This chromosome instability, which is tested for by chromosome breakage analysis, was detected in fibroblast cells derived from homozygous Fac deficient mice. Chromosome breakage is the earliest detectable characteristic of human FA. It may therefore be likely that the Fac deficient mice will develop the additional phenotypes associated with FA at an older age.

Appendix A

Identification of a rabbit homologue of the human Fanconi anemia group C gene by degenerate oligonucleotide RT-PCR

Mike Whitney and Markus Grompe

Introduction

The use of genetically and developmentally well characterized animal systems, such as yeast or drosophila, can sometimes lead to a functional understanding of pathways which are more complex and more difficult to study in more highly evolved species. Therefore, we made a significant attempt to identify Fanconi anemia group C homologues from these species. One approach to identify homologous given genes is through the use of low-stringency DNA cross-hybridization [1]. This strategy relies on a relatively large region homology between the chosen probe and the unknown target sequence. An alternative approach utilizes degenerate oligonucleotide PCR which can detect sequence homologies which would be missed by DNA cross hybridization. For this approach degenerate oligonucleotides are designed which represent for all

possible DNA sequences for a given peptide sequence. Peptide sequences of 6-8 highly conserved amino acids, from a given protein are selected with the hope that these peptide sequences will be conserved in additional species. Two such peptide sequences are selected, which are separated by 30-200 amino acids, and used to design degenerate oligonucleotide primers for PCR.

Currently, FA(C) homologues have only been identified from mice and human. Therefore, degenerate oligos were designed to conserved protein region between these two genes. As additional homologues of the FA(C) gene are identified, degenerate oligos can be redesigned to more highly conserved regions of the gene. The mouse and human FA(C) sequences are only modestly conserved at 67% amino acid identity and 79% similarity [2, 3]. This sequence homology is quite low when compared to other DNA repair genes such as xeroderma pigmentosum groups A and B which are 85% and 96% identical, respectively [4, 5]. Although the homology between the human and mouse Fac sequences are not that high, the mouse Fac gene has been shown to functionally complement the MMC and DEB hypersensitivity of human FA(C) cells (HSC536) [2].

Materials and Methods

RNA preparation and degenerate oligo RT-PCR

Total rabbit RNA was isolated from tissue using the RNAzol method. First strand cDNA was made using 10 ug RNA as template. Reverse transcriptions using 0.5 ug random hexamers were performed in a volume of 40 λ using a BRL kit (Life Technologies,

Inc., Gaithersburg, Maryland). One microliter of this cDNA was used as template for RT-PCR with FA(C) degenerate primers. Oligonucleotides primers used were MG69 (5'atgaattcgtnga(tc)ga(ag)atgtt(tc)(ca)g3') and MG 74 (5'taggatccgt(ag)tangg(ga)aa(ga)tangt3') or oligo MG74 and MG75 (5'atgaattctn(tc)tnga(ga)acnga(tc)ggngc3'). For the MG69/MG74 reactions 600ng of oligo MG69 and 200ng of oligo MG74 were used. For MG74/MG75 reactions 200ng MG74 and 400ng of oligonucleotides MG75 were used. Amplifications were done in Cetus buffer with 2mM dNTPs and 2 units AmpliTaq. Cycling conditions were 5x(94 30" 46 30" 72 2') followed by 35x(94 30" 55 45" 72 2').

Cloning of the PCR products

Rabbit Fac PCR products were then digested with EcoRI and BamHI and gel isolated from a 2% Nusieve agarose gel. Bluescript vector was prepared by digestion with *BamHI* and *EcoRI* followed by gel isolation. Prepared vector and PCR fragment were then ligated in one-for-all buffer (Pharmacia), with 1mM ATP, 1mM DTT, and 2 units of DNA ligase. Ligations were then transformed into XL1 blue competent cells. DNA was prepared from resulting white colonies using the boiling mini-prep method. Mini-prepped DNA was then digested with *EcoRI* and *BamHI* to test for insert size.

Sequencing and analysis of rabbit FA(C) sequence

Cloned PCR products of the predicted size were sequenced using universal forward and reverse primers. Sequence comparison and alignments were performed using Intelligenetics.

Results and Discussion

Degenerate primers MG69/MG74 and MG74/MG75 were used in a PCR amplification with mouse, chicken and rabbit cDNA. PCR products of identical size were seen for both of these PCR reaction in the rabbit and mouse amplification. There was no product detected in the chicken RT-PCR amplifications. This indicated that the PCR products amplified from the rabbit cDNA may represent a homologue of the FA(C) gene. To clone these PCR products they were cut with *EcoRI* and *BamHI*, gel purified and ligated into a *BamHI/EcoRI* cut Bluescript vector. Six amplicilin resistant colonies were grown up for DNA isolation followed by digestion with *EcoRI* and *BamHI*. For the MG69/MG74 rabbit PCR product one of the six mini-prepped plasmids gave the expected size fragment. For the MG74/MG75 rabbit PCR product 5/6 miniprepped plasmids produced the expected size *EcoRI/BamHI* fragment. Fragments were then sequenced using dideoxy-chain termination sequencing using vector universal forward and reverse primers. The one PCR product from the PCR reaction using primers MG69/MG74 was determined to be related to the human and mouse Fac sequences. The four PCR products from the MG74/MG75 were sequence and were all identical to each other. They were also similar to the human and mouse FA(C) sequences.

The larger of the two PCR fragments identified by this approach was 160 base pairs long. The predicted amino-acid sequence for this cDNA shows significant similarity to the two known homologues of the FA(C) gene (see figure 1). Full length cDNAs are currently being identified for this rabbit homologue of the FA(C) gene.

The identification of this rabbit homologue helps demonstrate that the FA(C) gene is likely conserved in all mammalian species. The rabbit FA(C) homologue has similar homology to the human sequence as that of the murine homologue. The complete sequencing and three-way alignment with the rabbit sequence could indicate regions of the FA(C) sequence which are more highly conserved. As additional homologue are cloned it may be possible to redesign degenerate oligos which will allow the amplification of FA(C) homologues in lower vertebrate as well as invertebrate species.

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HUMANFAC      1 GTtGATGAGATGTTCAGGtgtgCACTCCTGGAaACCGATGGgGCCCtgGAAaTcatAGCCa
                || ||||||||||||||||    |||||||||| |||||||| ||||  |||| |   ||||
RABBITFAC     1 GTgGATGAGATGTTCAGGcccaCACTCCTGGAgACCGATGGaGCCCcaGAAgTagcAGCCg

consensus       GT-GATGAGATGTTCAGG----CACTCCTGGA-ACCGATGG-GCCC--GAA-T---AGCC-


HUMANFAC     62 CTaTtCAgGTGTTtACGcagTGCTTtGTaGAAGCTCTGGAGaaaGcAagCaAGCAGCtGcg
                || | || ||||| |||   ||||| || |||||||||||| | |  | |||||| |
RABBITFAC    62 CTcTcCAaGTGTTcACGagaTGCTTcGTgGAAGCTCTGGAG  GaAgaCgAGCAGCcGaa

consensus       CT-T-CA-GTGTT-ACG---TGCTT-GT-GAAGCTCTGGAGaaaG-A--C-AGCAGC-G--


HUMANFAC    123 GTTTGCaCTCAAGACcTACTTtCCtTACACttcTCC
                ||||||  |||||||| ||||| || |||||   |||
RABBITFAC   120 GTTTGCtCTCAAGACttTACTTcCCaTACACggaTCC

consensus       GTTTGC-CTCAAGAC-TACTT-CC-TACAC---TCC


RABBITFAC     1 VDEMFRptLLETDGApEvaAalQVFTrCFVEALE edeQpkFALKTYFPYTd
                ||||||  |||||||  |  |  |||| |||||||    |  ||||||||||
HUMANFAC      1 VDEMFRcaLLETDGAlEiiAtiQVFTqCFVEALEkaskQlrFALKTYFPYTs

consensus       VDEMFR--LLETDGA-E--A--QVFT-CFVEALEk---Q--FALKTYFPYT-
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Figure 1. Sequence alignment of rabbit and human FA(C) gene. DNA and protein alignments are shown

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