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The molecular mechanism underlying formation of deletions in Fanconi anemia cells may involve a site-specific recombination

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ABSTRACT **Spontaneous and induced chromosomal breakage is an important cellular feature of Fanconi anemia (FA), an inherited DNA repair disorder characterized by progressive bone marrow failure, developmental abnormalities, and predisposition to leukemia. We have previously reported that in comparison to normal cells, there is a substantial increase in frequency of intragenic deletions at an endogenous locus (*HPRT*) in FA lymphoblasts. Taken together with the increased chromosomal instability, these observations indicated that the wild-type FA gene(s) plays an important role in the maintenance of the genomic integrity. To obtain information on the mechanism(s) underlying the genomic rearrangements in FA, the breakpoint sites of deletions in 11 FA-derived *HPRT*$^-$ mutants were analyzed. The results indicate that a significant proportion of deletions involving a loss of a given exon are identical and that two deletions of different size have the same 3′ breakpoint. Interestingly, it appears that in most of the mutants there is a common deletion signal sequence, which suggests that the mutations in the FA gene(s) may lead to an aberrant site-specific cleavage activity that might be responsible for the deletion proneness and the chromosomal instability characteristic of the FA pathology. From the similarity or even identity of the signal sequence at some of the breakpoints with the consensus heptamer which directs cleavage and joining in the assembly of immunoglobulin and T-cell receptor genes, we speculate that steps in common with the V(D)J recombinational process may be illegitimately involved in FA cells.**

Cells have evolved elaborate mechanisms to eliminate or to minimize the deleterious consequences of various types of genetic damage induced by physical and chemical agents. Each of these repair pathways consists of a well-orchestrated action of multiple proteins, some of which are highly specialized, whereas others seem to be common to other DNA processes such as replication and transcription (1, 2) or physiologically programmed V(D)J recombination (3–5), which is essential to the immune system for generating immunoglobulin and T-cell receptor diversity (6–8).

The importance of DNA repair mechanisms is illustrated in man by a group of genetic diseases in which the anomalies in the processing of specific DNA lesions are associated with a chromosomal instability and a predisposition to malignancy. Fanconi anemia (FA), one of these so-called DNA repair disorders (which include xeroderma pigmentosum, ataxia telangiectasia, and Bloom syndrome), is an inherited autosomal recessive disease characterized by progressive bone marrow failure, developmental abnormalities, and predisposition to leukemia. FA cells show a high level of chromosome breakage, either spontaneous or induced by DNA crosslinking agents such as mitomycin C, diepoxybutane, or photoactivated psoralens (9, 10). Four genetic complementation groups (A–D) have been described (11). The cDNA for the group C gene has been cloned (12). The function of the FA group C gene is, however, still unknown.

We have previously reported that in normal human lymphoblasts the vast majority of spontaneous and psoralen-photoinduced mutations at the hypoxanthine phosphoribosyltransferase (*HPRT*) locus are base substitutions. In contrast, in FA lymphoblasts the majority of spontaneous or psoralen-photoinduced mutants are the result of deletions of *HPRT* coding sequences (13–15). Moreover, the variant frequency at the glycophorin A (*GPA*) locus is significantly increased in erythrocytes from FA patients as compared with age-matched healthy donors (16). Since *GPA* variants reflect essentially structural deletions, rearrangements, and mutations leading to allele loss, the increased frequency of *GPA* variants seems to be consistent with the enhanced deletion frequency at the *HPRT* locus. Therefore, the increased deletion formation is an important feature of the FA phenotype, suggesting that FA gene(s) play a role in DNA metabolism and/or in the maintenance of the genomic integrity.

To obtain information on the mechanism(s) underlying the genomic rearrangements in FA, we examined the sequences at the breakpoint of deletions in two spontaneous and nine psoralen-photoinduced *HPRT*$^-$ mutants. The pattern of psoralen-photoinduced FA deletions suggests the action of a site-specific mechanism.

MATERIALS AND METHODS

Mutant Collection. The spontaneous and induced *HPRT*$^-$ mutants were isolated (14) from FA HSC-62 lymphoblasts, of complementation group D (11). The induced mutants were isolated after treatment with 4,5′,8-trimethylpsoralen (Me_3Pso) associated with monochromatic UV radiation at 365 nm (mutants FTA13, FTA32, FTA34, FTA37, FTA40, FTA41, and FTA42) or 405 nm (mutants FTB15 and FTB18). Treatment with Me_3Pso plus 365-nm radiation results in formation of a mixture of monoadducts and interstrand crosslinks, whereas treatment with Me_3Pso plus 405-nm radiation induces only monoadducts. To reduce the frequency of the preexisting spontaneous *HPRT*$^-$ mutants to $2–4 \times 10^{-6}$ per survivor, cell stocks were treated prior to each mutagenesis experiment with CHAT medium (17). To ensure the independent origin of *HPRT*$^-$ mutants, the mutagenized population (7×10^7 cells per experimental point) was split into four to six flasks immediately after treatment. After a 7-day expression period, the cells were plated in 96-well plates (1 plate per flask) in selective medium containing 6-thioguanine. One mutant clone per plate was isolated. The doses of Me_3Pso plus UV radiation used for mutant isolation resulted in a 4- to 5-fold increase in

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Abbreviations: FA, Fanconi anemia; Me_3Pso, 4,5,′8-trimethylpsoralen.

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mutant frequency ($10–15 \times 10^{-6}$ mutant per survivor) in FA group D cells.

Sequence Analysis of Breakpoint Junctions. Southern blot hybridization and multiplex PCR (18) were used for structural analysis of genomic DNA (13, 14). The simultaneous amplification of nine *HPRT* exons using eight primer pairs, *a–h*, resulted in different-size fragments (Figs. 1 and 2). Exons 7 and 8 were amplified as a single fragment. PCR amplification with specifically designed primer pairs (Fig. 1) was used to explore the regions around the missing exon(s) in order to localize the deletion breakpoints. PCR was carried out (19) with 50 ng of genomic DNA per reaction and 20 pmol of each primer (GENSET, Paris) in 50 μl of 10 mM Tris·HCl pH 8.3/50 mM KCl/1.5 mM $MgCl_2$/0.01% gelatin/0.2 mM each dNTP with 2 units of *Taq* polymerase (Boehringer Mannheim). The reaction mixtures were heated at 94°C for 4 min and subjected in a Perkin–Elmer/Cetus thermocycler to 33 cycles of amplification consisting of denaturation (94°C, 1 min), annealing (the temperature being appropriate to the primer sets, usually 45–57°C, 1 min), and polymerization (72°C, 2 min), with the last cycle followed by an extension period (72°C, 5 min).

PCR was carried out in parallel with a deletion mutant and a wild-type *HPRT* clone. The PCR product yielded a single fragment of the expected size from both clones when the amplified fragment was not included in the deletion. Failure to see a PCR product only in a mutant clone indicated either that the amplified region was deleted in the mutant analyzed or that the deletion was extended into the primer's site(s). Attempts were then made with alternative primer pairs. To further localize the breakpoint junction, restriction mapping of the PCR product was used in some cases. The final product was concentrated, loaded on a 1% agarose gel, purified by a Qiaex gel extraction kit (Qiagen, Chatsworth, CA), and directly sequenced.

The primers designed for amplification across the breakpoint were used for sequencing with the *Taq* DyeDeoxy terminator (cycle) sequencing kit (Applied Biosystems). After the sequencing reactions, the excess dye terminators were removed by Quickspin Sephadex G-50 columns (Boehringer Mannheim). After drying, the samples were suspended in a 5:1 mixture of formamide and 50 mM EDTA (pH 8.0) and loaded on a denaturing 6% polyacrylamide gel on the Applied Biosystems automatic sequencer. Sequences were directly identified by the Applied Biosystems VERSION.SEQ software.

RESULTS

The analysis of 17 spontaneous and 70 psoralen-photoinduced *HPRT*$^-$ mutants derived from FA lymphoblasts of complementation group D revealed that 51 had structural rearrangements, mostly intragenic deletions (13–15). There is a clear difference between the spectrum of deletions observed in spontaneous and in psoralen-photoinduced mutants (Table 1). The deletions involving exon 3 or exon 9 were the most frequent among the induced mutants, whereas more than half of spontaneous deletions involved exon 4. To investigate the mechanism underlying the deletion events in FA cells, we analyzed the breakpoint junctions in 11 mutants (9 photoinduced by psoralen treatment and 2 spontaneous); their multiplex PCR patterns are shown in Fig. 2. Among the 9 induced mutants, 6 involve either exon 3 loss (FTA37, FTA40, FTA41, FTB15, and FTB18) or exon 2 and 3 loss (FTA32) and 3 involve exon 9 loss (FTA13, FTA34, and FTA42). These deletions are absent among the spontaneous mutants (Table 1). To localize the breakpoint junctions, we used PCR amplification of genomic DNA with several oligonucleotide primer pairs located in introns flanking the missing exon(s) (Fig. 1).

A Significant Proportion of the FA Deletions Involving a Given Exon Are Identical. Only one of the FA deletion

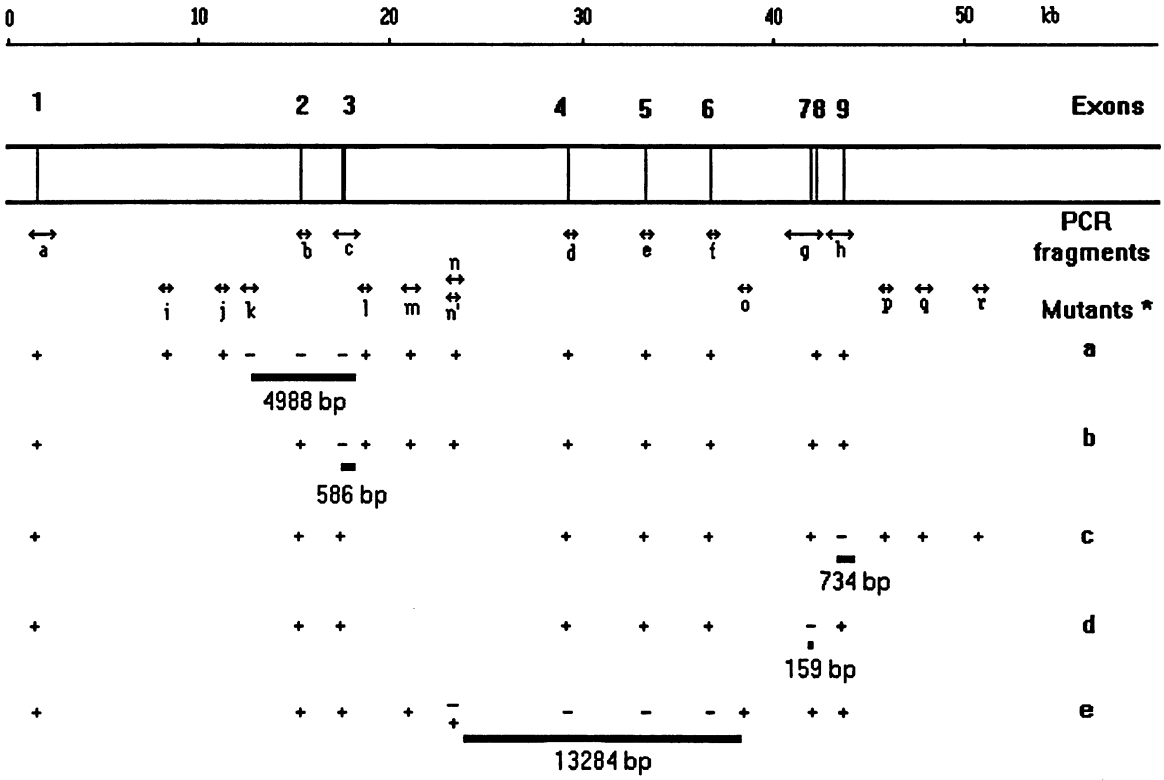

FIG. 1. Structure of the human *HPRT* gene. Positions of exons, sites of PCR primers, and amplified fragments (double-headed arrows) are represented. The presence (+) or absence (−) of PCR product and the final sites of deletion (solid boxes) are shown for each class of mutants. (*a*) FTA32 deleted exons 2 and 3. (*b*) FTA37, FTA40, FTA41, FTB15, and FTB18 deleted exon 3. (*c*) FTA13, FTA34, and FTA42 deleted exon 9. (*d*) FSp23 deleted exon 7. (*e*) FSp11 deleted exons 4, 5, and 6.

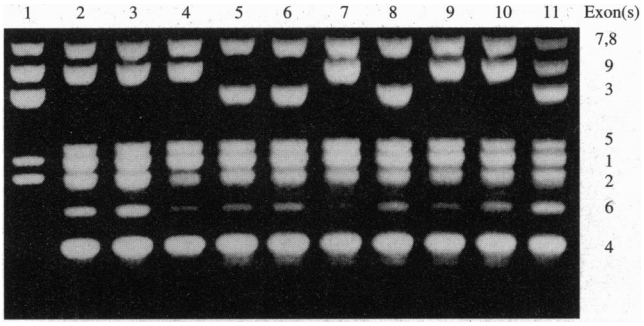

FIG. 2. Multiplex PCR analysis of *HPRT*$^-$ mutants derived from FA lymphoblasts HSC62, of complementation group D. Lane 1, mutant FSp11, missing exons 4–6; lane 4, mutant FTA32, missing exons 2 and 3; lanes 2, 3, 7, 9 and 10, mutants FTB15, FTB18, FTA37, FTA40, and FTA41, missing exon 3; lanes 5, 6, and 8, mutants FTA13, FTA34, and FTA42, missing exon 9; lane 11, wild-type cell clone. Mutant FSp23 (not shown) presents multiplex patterns similar to wild type except that the fragment corresponding to amplification of exons 7 and 8 is smaller.

mutants involves a deletion of exons 2 and 3. PCR amplification with the primer sets *j* and *l*, localized in the indicated regions in intron 1 and 3, respectively (Fig. 1), yielded amplified fragments of the expected size from the mutant FTA32 as well as from the wild-type *HPRT* clone, whereas primers *k* gave a product only from the wild-type cells. The primer combination *k*/*l* was then checked to amplify the deletion junction. The sequence analysis showed a deleted fragment of 4988 bp, flanked in the wild-type sequence by AT repeats (Fig. 3*a*). Note that there is a strong psoralen photobinding site, TATAT, at the 5′ breakpoint of this deletion.

The size of PCR products from five mutants missing exon 3 suggested that the deletions involved were similar. Subsequent sequence analysis revealed that the deletions in all five mutants were identical. The deletion encompassed 586 bp of the *HPRT* sequence including 39 bp from the 3′ end of exon 3. The deletion has a 4-bp direct repeat (CATT) at the breakpoints, one copy of which is preserved in the novel junction (Fig. 3*b*). Surprisingly, the breakpoint at the 3′ end of this deletion was located only 12 bp from the breakpoint involved in mutant FTA32, the mutant missing exons 2 and 3.

As with the previous group of mutants, sequence analysis of the three *HPRT*$^-$ clones missing exon 9 revealed a deletion of 734 bp which was the same in all three mutants (Fig. 3*c*). A short sequence repeat (AAGA) was present at the two breakpoints of the deleted fragment. The upstream breakpoint was located within the 3′ end of an *Alu* family repeat.

Table 1. Type of spontaneous and psoralen-photoinduced deletions in FA cells of complementation group D

Missing exon(s)	No. of mutants	
	Spontaneous	Induced
1	1	2
2	1	0
2, 3	0	1
3	0	9
4	5	2
4, 5	0	5
4–6	4	0
6	2	0
7	1	0
7, 8	0	1
7–9	2	5
9	0	4
1–4	0	2
1–6	1	0
1–9	0	3
Total	17	34

Two spontaneous deletions were also analyzed. The mutant FSp23 is due to loss of exon 7. The deletion is relatively small, 159 bp, and a single cytosine residue is common to each breakpoint (Fig. 3*d*). The mutant FSp11 is due to loss of exons 4–6. PCR amplification with a combination of primer pairs *n*′ and *-o* gave a 1700-bp product; this defined an ≈13,000-bp deletion. The deleted fragment of 13,284 bp is flanked in the wild-type sequence by AG repeats (Fig. 3*e*).

The Pattern of FA Deletions Suggests the Action of a Site-Specific Mechanism. In spite of their independent origin, including recovery after different psoralen phototreatments, deletions involving exon 3 in the mutants FTA37, FTA40, and FTA41 (isolated after treatment with Me_3Pso plus 365-nm radiation) and FTB15 and FTB18 (isolated after treatment with Me_3Pso plus 405-nm radiation) are identical. In the three mutants missing exon 9, the deletions are also identical. Moreover, in spite of the large size of intron 3 (13.4 kb) of *HPRT*, it is striking that in the deletion involving loss of exons 2 and 3 (Fig. 3*a*), the 3′ breakpoint (nt 17,345; ref. 20) was located only 12 bp from the 3′ breakpoint (nt 17,333) in the deletion involving loss of exon 3 (Fig. 3*b*). These observations seem to be consistent with deletional events generated by a site-specific DNA recombination.

Analysis of the sequences at the junctions in FA deletions revealed similarities with the conserved heptamer [CAC(T/A)GTG] which directs gene rearrangements in the immune system (7, 8). A perfect heptamer, CACTGTG, lies three bases upstream of the 3′ end of the deletion involving loss of exons 2 and 3 (Fig. 3*a*). The same perfect heptamer is at the 3′ breakpoint of the deletions involving loss of exon 3 (Fig. 3*b*). A search for similar sequences within 15 bp of the other deletion breakpoints revealed the presence of heptamer-like sequences, although they are less conserved. The motif CACATCA, observed 12 bp upstream of the 3′ end of the exon 9 deletion (Fig. 3*c*), and the motif CACGAAG, observed at the 3′ breakpoint in the deletion involving exon 7 (Fig. 3*d*), have four bases in common with the conserved heptamer sequence. Fifteen bases upstream of the 3′ end of the deletion involving loss of exons 4–6 is the sequence ATCAGTG, in which the last five bases are identical to those of the consensus heptamer. This motif is separated by 21 bp from a nonamer, GGCTTTTCT (Fig. 3*e*), which has seven bases in common with the consensus nonamer motif (7, 8). At the 5′ breakpoints in the deletion involving exon 3 (Fig. 3*b*) and exon 7 (Fig. 3*d*), the heptamers GACTGTA and CATAGTC, respectively, have 5 bases in common with the conserved sequence. At the 5′ breakpoints of deletions involving loss of exon 9 (Fig. 3*c*) and loss of exons 4–6 (Fig. 3*e*), the GTGACAG and GTGTCTC sequences match six bases of the consensus heptamer, although the sequence is inverted. Within 15 bp of the 5′ end of the deletion involving loss of exons 2 and 3, there is apparently no sequence similar to the conserved consensus heptamer. Of interest is the addition of one non-germline nucleotide at the junction of this deletion (Fig. 3*a*).

In addition, in psoralen-photoinduced deletions, at the breakpoint (Fig. 3*a*) or close to the putative heptamers (Fig. 3 *b* and *c*), the preferred sites for psoralen photoadditions (TATAT, TAT, TTT) (21, 22) can be observed.

DISCUSSION

Understanding the mechanisms underlying genomic rearrangements in human pathological states is especially important because chromosomal translocations and large or intragenic deletions constitute a major class of disease-related genetic events. Several mechanisms have been suggested to account for deletion formation in mammalian cells: homologous recombination between repetitive sequences, as well as nonhomologous recombination between regions with short blocks of junctional homology, stretches of alternating purines and pyrimidines, and homopurine and homopyrimidine tracts

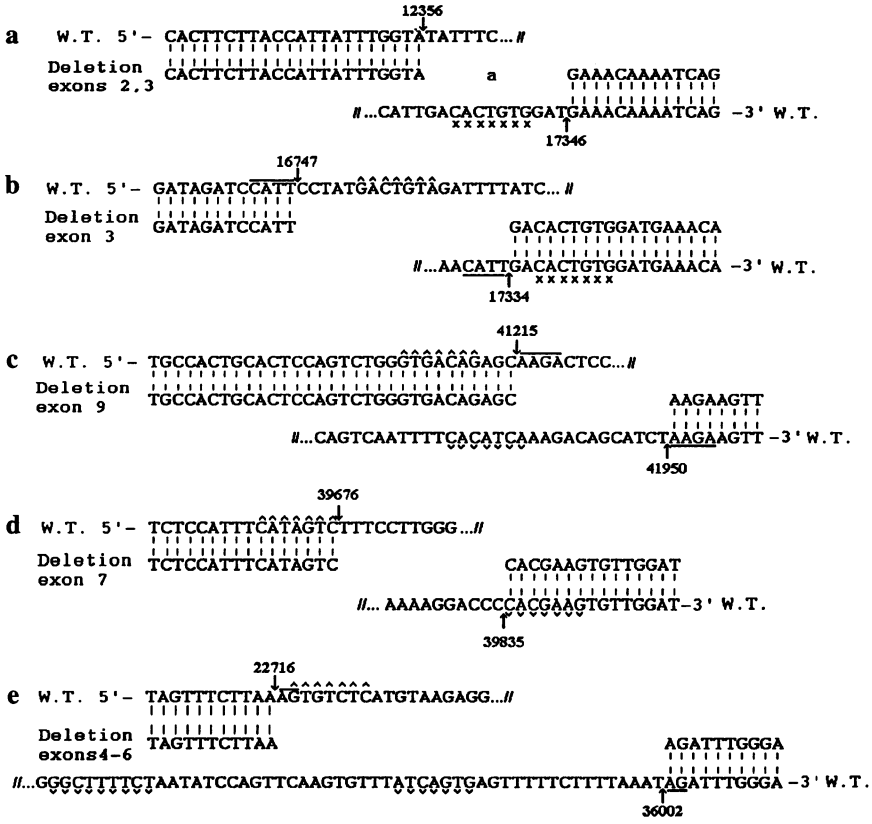

FIG. 3. Sequences of breakpoint junctions in *HPRT*$^-$ mutants in FA. Junction sequences are aligned with corresponding 5′ and 3′ wild-type (W.T.) sequences. Vertical bars between bases show identity. The perfect heptamers are marked xxxxxxx. The cryptic heptamers are marked ^ ^ ^ ^ ^ ^ ^. The junctional direct repeats are indicated by horizontal lines, and the observed break sites by vertical arrows and corresponding base number of the wild-type sequence (20). (*a*) Mutant with exons 2 and 3 deleted: FTA32. (*b*) Mutants with exon 3 deletion: FTA37, FTA40, and FTA41, isolated after treatment with Me_3Pso plus 365-nm radiation, and FTB15 and FTB18, isolated after treatment with Me_3Pso plus 405-nm radiation. (*c*) Mutants with exon 9 deletion: FTA13, FTA34, and FTA42. (*d*) Spontaneous mutant with deletion of exon 7: FSp23. (*e*) Spontaneous mutant with deletion of exons 4–6: FSp11.

(for review, see ref. 23). Structural rearrangements at the *HPRT* locus account for 75% of mutations detected in fetal T lymphocytes derived from umbilical cord blood (24). Moreover, ≈50% of these *HPRT*$^-$ mutants are due to intragenic deletions of exons 2 and 3. Sequence analysis of the break sites has revealed that these deletions are mediated by a V(D)J recombination-like activity in normal fetal cells (19). In contrast to newborns, this type of V(D)J-mediated deletion accounts for only 2% of all mutations detected at the *HPRT* locus in T lymphocytes of adults (25). In other words, during pre-T-cell development an illegitimate V(D)J recombination constitutes the major mechanism of mutation at the *HPRT* housekeeping gene.

An important feature of FA pathology is an increased genomic instability. This is reflected at the *HPRT* locus by a high proportion of deletions among the spontaneous and induced mutations. Indeed, the deletion events represent up to 65% and 78% of spontaneous mutations occurring at the *HPRT* locus in FA lymphoblasts of complementation group D (ref. 13) and group A (data not shown), respectively. The deletion events represent only 18% of spontaneous mutations arising in normal human lymphoblasts (13). Preliminary experiments using extrachromosomal constructs indicated that the majority of spontaneous mutants arising in a target gene (*lacZ*) were due to deletions when the substrates were replicated in cells belonging to the four FA complementation groups. Moreover, in comparison with age-matched healthy controls, the frequency of *GPA* variants N0 and M0 (resulting essentially from allele loss) increased ≈30-fold in erythrocytes of 12 FA patients of the MN blood type (16). Therefore, the deletion proneness seems to be a common characteristic of all FA complementation groups.

We previously reported the sequence analysis of psoralen-photoinduced mutants in normal and FA complementation group D lymphoblasts (14, 15). In normal lymphoblasts, the frequency of psoralen-photoinduced deletions was very low: 0% when mutants were isolated after treatment resulting in psoralen monoadditions and 10% when mutants were isolated after psoralen photoadditions including interstrand crosslinks. In FA group D lymphoblasts, >60% of *HPRT*$^-$ mutations induced by all psoralen treatments were gene rearrangements. Sequence analysis of the breakpoint junctions in *HPRT* deletions can be expected to provide informations on the molecular basis of deletional events in FA cells. The 11 mutants analyzed in this study are representative of the frequency with which these deletions are observed in our spontaneous and psoralen-photoinduced mutant collection.

Sequence analysis of the breakpoint junctions in FA deletion mutants revealed the following features. (*i*) A significant proportion of deletions involving a given exon were identical. Moreover, there was only a 12-bp distance between the 3′ breakpoints in two different deletions (586 bp and 4988 bp; Fig. 3) located in the large intron 3. (*ii*) The 12-bp sequence found between the 3′ breakpoints of these two last deletions (loss of exon 3 and of exons 2 and 3; Fig. 3) contained a heptamer perfectly matched to the conserved heptamer sequence which is the essential element of the recognition signal sequences directing V(D)J recombination. Cryptic heptamer-like sequences were found at or close to all but one of the listed 5′ and 3′ breakpoints (Fig. 3 *b–e*). (*iii*) At one of the breakpoints (Fig. 3*a*), the addition of one non-germline-encoded nucleotide was observed. By assuming that the *HPRT* deletions might derive from cleavage events at or close to the described heptamer sequences, a loss of several nucleotides (0–15 bp) was observed at the termini for which the heptamer or heptamer-like sequences were in the deleted fragments (Fig. 3 *a, b, c,* and *e*). (*iv*) In most of the mutants short (2–4 bp) sequence homologies at each breakpoint can be noted, one copy of which is retained in the novel junctions. (*v*) In mutants isolated after psoralen phototreatments, the preferred sites for psoralen photoadditions (TATAT, TAT, TTT) were at or close to the breakpoints and/or to the putative heptamers.

That the deletions in independent mutants involving a given exon are identical and that two deletions of different sizes have the same breakpoint indicate that these FA rearrangements may be due to a site-specific mechanism. The presence of a

perfect heptamer motif at the 3′ breakpoint in deletions involving exon 3 and exons 2 and 3 allowed us to speculate that a V(D)J-like site-specific cleavage activity may be involved in the FA deletion events at the *HPRT* locus. In view of this hypothesis, the question arises whether the heptamer-like sequences observed at the other breakpoints are relevant with respect to such a directed activity.

The minimum sequence requirement for V(D)J cleavage and joining has been extensively studied (26, 27). For exogenous substrates introduced into a pre-B-cell line, a nonamerless signal allowed recombination at a reduced but significant frequency whereas a heptamerless signal did not. Within the heptamer, the three base pairs closest to the cutting site appear to be critical in initiating and completing the normal V(D)J reaction. Base substitutions at these positions significantly decrease the normal V(D)J activity.

The presence of the perfect heptamer at the 3′ breakpoint in *HPRT* deletions involving exon 3 may explain the relatively high occurrence of these deletions among the FA mutants. Interestingly, at the 3′ breakpoints of all *HPRT* deletions, the three bases closest to the putative cutting sites in the proposed heptamers matched precisely with the consensus heptamer sequence. At the 5′ breakpoints of the analyzed deletion junctions in FA, the heptamer-like motif either was not present (Fig. 3*a*) or did not conform well to the conserved consensus sequence with respect to the normal V(D)J recognition/cleavage events. Indeed, either the proposed heptamer sequences at the 5′ break site have six bases in common with the consensus heptamer but the sequence is inverted (Fig. 3 *c* and *e*), or they have five bases matched with the consensus heptamer but the minimum base requirements in positions closest to the cutting sites are not conserved (Fig. 3 *b* and *d*). It may be asked whether the presence of these sequences at the breakpoints in FA deletions may reflect some aberrancies of a V(D)J-like recognition/cleavage in FA cells. Under this assumption one plausible explanation for psoralen-photoinduced deletions at the *HPRT* locus in FA cells might be the association of specific sequence motifs close to the sequences modified by psoralen photoadditions which would then serve as signals for a site-specific cleavage. This step followed by a search for short homology (direct repeats) would define the subsequent end joining. In good agreement with previous reports (28, 29), the higher representation of some type of junctions (Fig. 3 *b* and *c*) might be explained by the presence of 4 bp of homology in participating ends which might align preferentially.

V(D)J recombination is a multifactorial process, normally restricted to the lymphoid cells and subject to developmental control (30, 31). The lineage and developmental stage specificity seems to be established by regulation of *RAG1* and *RAG2* transcription (32). The other components required for the complete reaction seem to be recruited among the ubiquitously expressed cellular genes. Some of these components may have a function in other DNA processes. The V(D)J machinery may share common factors with a mechanism involved in the repair of double strand breaks. Indeed, a 2- to 3-fold decrease in the rate of double-strand break repair has been observed in cells from mice with the *scid* defect (3), in which V(D)J recombination activity is altered (33, 34). Moreover, cells from rodent double-strand break repair mutants are impaired in the rearrangements of V(D)J recombination substrates after introduction of the *RAG1* and *RAG2* genes (4, 5). Therefore, some components of the pathway involved in repair of double strand breaks participate also in the V(D)J recombination reaction. The data reported in the present study suggest that mutations in the FA gene may lead to an aberrant site-specific cleavage activity which will be responsible for the deletion proneness of FA. An increased amount of small polydisperse circular DNA in five FA cell lines has been observed (35). This type of extrachromosomal circular DNAs seems to emerge as the products of V(D)J recombination activity (36). Our work raises the question whether a site-specific cleavage activity in FA cells has steps in common with an illegitimate V(D)J recombination process.

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