doi:10.1016/j.jmb.2007.03.014 J. Mol. Biol. (2007) 370, 449–458

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Defective Signal Joint Recombination in Fanconi Anemia Fibroblasts Reveals a Role for Rad50 in V(D)J Recombination

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V(D)J recombination of immunoglobulin loci is dependent on the immune cell-specific Rag1 and Rag2 proteins as well as a number of ubiquitously expressed cellular DNA repair proteins that catalyze non-homologous end-joining of DNA double-strand breaks. The evolutionarily conserved Rad50/Mre11/Nibrin protein complex has a role in DNA double-strand break-repair, suggesting that these proteins, too, may participate in V(D)J recombination. Recent findings demonstrating that Rad50 function is defective in cells from patients afflicted with Fanconi anemia provide a possible mechanistic explanation for previous findings that lymphoblasts derived from these patients exhibit subtle defects in V(D)J recombination of extrachromosomal plasmid molecules. Here, we describe a series of findings that provide convincing evidence for a role of the Rad50 protein complex in V(D)J recombination. We found that the fidelity of V(D)J signal joint recombination in fibroblasts from patients afflicted with Fanconi anemia was reduced by nearly tenfold, compared to that observed in fibroblasts from normal donors. Second, we observed that antibody-mediated inhibition of the Rad50, Mre11, or Nibrin proteins reduced the fidelity of signal joint recombination significantly in wild-type cells. The latter finding was somewhat unexpected, because signal joint rejoining in cells from patients with Nijmegen breakage syndrome, which results from mutations in the Nibrin gene, occurs with normal fidelity. However, introduction of anti-Nibrin antibodies into these cells reduced the fidelity of signal joint recombination dramatically. These data reveal for the first time a role for the Rad50 complex in V(D)J recombination, and demonstrate that the protein product of the disease-causing allele responsible for Nijmegen breakage syndrome encodes a protein with residual DNA double-strand break repair activity.

Keywords: V(D)J recombination; Fanconi anemia; Rad50; fibroblast; Nijmegen breakage syndrome

Introduction

V(D)J recombination is a selective break/rejoining process that cleaves and rejoins chromosomal DNA to create novel recombinant immunoglobulin and T-cell receptor genes. This developmentally regulated and cell-type specific pathway is responsible for the generation of immunoglobulin diversity in vertebrates.[1–3] The process is initiated by the products of the recombination activating genes 1 and 2 (Rag1, Rag2), which together form a site-specific recombinase that binds to recombination signal sequences (RSS) present in the immunoglobulin

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Abbreviations used: RSS, recombination signal sequence; NHEJ, non-homologous DNA end-joining; RMN, Rad50/Mre11/Nibrin; FA, Fanconi anemia.

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and T-cell receptor genes, and introduces DNA double-strand breaks into chromosomal DNA of pre-B and pre-T cells.[4] The Rag1/Rag2 complex generates a pair of DNA double-strand breaks in the chromosome, thereby generating four new DNA ends. Two of these consist of 5′ phosphate, 3′ hydroxyl blunt ends and are referred to as signal ends. The other two consist of sealed hairpins and are called coding ends. The hairpins are opened by a nuclease called Artemis[5] before modification and rejoining to reconstitute the immunoglobulin or T-cell receptor locus.[6]

A fascinating aspect of the V(D)J recombination reaction is its dependence on the combined action of an enzyme that is selectively expressed in pre-B and pre-T cells, i.e. the heterodimeric site-specific nuclease comprised of the Rag1 and Rag2 proteins, as well as on a host of ubiquitously expressed enzymes that catalyze the non-homologous DNA end-joining (NHEJ) pathway of DNA double-strand break-repair.[7] These latter enzymes include DNA ligase IV,[8] and its binding partner Xrcc4,[9] the DNA end-binding heterodimer of Ku70 and Ku86,[10,11] Artemis,[5] the catalytic subunit of the DNA-dependent protein kinase,[12] and a more recently identified component identified as XLF[13] or Cernunnos.[14]

The NHEJ end-joining pathway is evolutionarily conserved, and *Saccharomyces cerevisiae* possess homologues of DNA ligase IV,[15] Xrcc4,[16] Ku70[17] and Ku86.[18] Recent work provides evidence[19] that Cernunnos is a homologue of the yeast DNA end-joining protein Nej1.[20] Interestingly, however, NHEJ in yeast functions in the apparent absence of homologues of Artemis and the catalytic subunit of DNA-dependent protein kinase. In addition, NHEJ in yeast displays an absolute requirement for a heterotrimeric complex of the Rad50, Mre11 and Xrs2 proteins.[21]

Because the genes encoding Rad50,[22] Mre11,[23] and Nibrin,[24] the mammalian homologue of the yeast Xrs2 protein, are essential, it has proven difficult to address directly whether these proteins have a role in DNA repair in mammalian cells. However, several recent lines of evidence are consistent with the interpretation that they do. First, Donahue and Campbell showed recently that antibody-mediated inhibition of the Rad50/Mre11/Nibrin (RMN) protein complex rendered mammalian somatic cells hypersensitive to the cytotoxic effects of induced DNA damage, and inhibited extrachromosomal plasmid end-joining dramatically *in vivo*.[25] Second, it has been shown recently that conditional inactivation of the Nibrin gene in murine cells reduced chromosome stability and enhanced cellular sensitivity to ionizing radiation-induced cell death.[26,27] Third, biochemical evidence supports a role for the RMN protein complex in DNA end-joining *in vitro*. For example Zhong *et al*. showed that anti-Rad50 antibody blocked plasmid end-joining in mammalian nuclear cell extracts.[28] In addition, studies performed using yeast proteins revealed that the purified Rad50 complex stimulated DNA ligase IV-dependent plasmid end-joining.[29] Furthermore, analysis of plasmid end-joining catalysed by fractionated protein extracts derived from mammalian cells revealed that plasmid end-joining was enhanced by the addition of a fraction enriched for the Rad50 complex.[30] Fourth, a recent description of a plasmid DNA end-joining defect in cells from patients suffering from Nijmegen breakage syndrome, which is associated with partial loss-of-function alleles of the gene encoding Nibrin.[31] Fifth, Chen *et al*. showed that Nibrin localized within freshly harvested thymocytes at sub-nuclear foci.[32] Their data indicated that Nibrin foci formed in a V(D)J recombination-dependent manner, and these authors proposed that the foci represented newly formed signals ends.

Interestingly, analysis of cells from mice harbouring a conditional inactivation of the Nibrin gene highlighted a previously unknown role of the RMN complex in immunoglobulin rearrangements. Kracker *et al*. showed that Nibrin has a role in immunoglobulin class-switch recombination.[26] Reina-San-Martin *et al*. reported similar observations.[27] These results are consistent with the finding that immunoglobulin class switch recombination occurs aberrantly in cells from patients with hypomorphic mutations in the Mre11 gene.[33] Over-expression of Nibrin is associated with increased frequency of somatic hypermutation and gene conversion in human and chicken somatic cells.[34] Finally, Clatworthy *et al*. showed recently that signal joint V(D)J recombination, which can be induced in transgenic yeast strains expressing Rag 1 and Rag 2,[35] was dependent on the RAD50, MRE11 and XRS2 genes.[36] Taken together, these findings provide support for the hypothesis that the RMN complex participates in V(D)J recombination in mammalian somatic cells.

Intriguing support for this hypothesis comes from two separate studies of cells from the cancer predisposition/DNA repair defect disorder Fanconi anemia (FA). First, Smith *et al*. showed that V(D)J recombination in immortalized lymphoblasts from FA patients is associated with abnormal rearrangements, primarily involving deletions at the sites of rejoining.[37] Second, Donahue and Campbell showed that Rad50-dependent DNA end-joining, which has a dominant role in plasmid end-joining *in vivo* is deficient in fibroblasts from FA patients.[25] This is consistent with an earlier study documenting aberrant Rad50 function in cells from FA patients.[38]

To shed further light on this question, we performed three series of experiments. First, we used a plasmid recombination assay to compare V(D)J recombination in diploid fibroblasts from FA patients to that observed in fibroblasts from normal donors. Second, we used this same system to evaluate the consequence of antibody-mediated inhibition of RMN function on V(D)J recombination in normal cells. Third, we examined V(D)J recombination in an immortalized fibroblast strain from a patient afflicted with Nijmegen breakage syndrome. The results presented below demonstrate that the rejoining of coding ends occurs aberrantly in

fibroblasts from FA patients, and provide direct evidence that the Rad50 protein complex participates in V(D)J recombination in mammalian cells. In addition, our results indicate that the Nibrin protein encoded by the major disease-causing allele responsible for Nijmegen breakage syndrome retains some residual DNA repair function.

Results

V(D)J recombination in fibroblasts from Fanconi anemia patients and normal donors

To test the hypothesis that V(D)J recombination occurred aberrantly in fibroblasts from FA patients, we used a plasmid based recombination assay (Materials and Methods).[39–41] The assay is depicted in Figure 1, in which the substrate plasmid (upper image of the Figure) is recombined during V(D)J recombination (indicated by the vertical arrow) to generate a recombinant plasmid (the lower image in the Figure). The plasmids used in these experiments have been engineered such that V(D)J recombination, directed by appropriate recombination signal sequences (depicted by the white rectangles), deletes a prokaryotic transcription silencer element (depicted as a bold **X**). The consequence of this rearrangement event is that the recombinant plasmid harbours a functional chloramphenicol acetyl transferase gene (depicted in Figure 1 as a striped rectangle). Because both the substrate and recombinant plasmids harbour a functional ampicillin-resistance gene (depicted as a shaded rectangle), bacteria harbouring either of these plasmids are resistant to ampicillin. In contrast, bacteria harbouring the recombinant plasmid are resistant to both ampicillin and chloramphenicol.

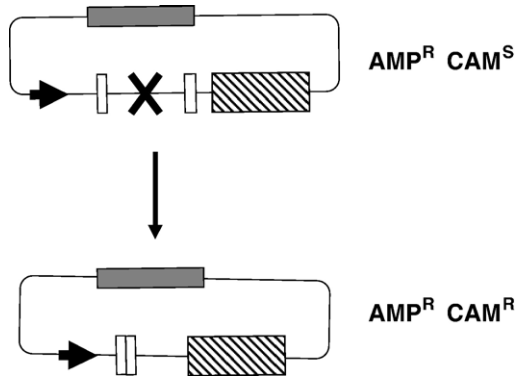

Figure 1. An illustration of V(D)J recombination of an extrachromosomal plasmid substrate. The upper image depicts the substrate plasmid pKSV(D)JSV40ori (described in Materials and Methods) and the lower image depicts a plasmid product that has undergone V(D)J recombination. The substrate plasmid confers resistance to ampicillin (AMP^R), but not to chloramphenicol (CAM^S). In contrast, the recombinant plasmid confers resistance to both ampicillin and chloramphenicol (AMP^R, CAM^R). Shaded box; ampicillin-resistance gene; striped box; chloramphenicol-resistance gene; arrowhead; bacterial promoter for chloramphenicol gene; white rectangles; V(D)J recombination signal sequences; bold **X**, prokaryotic transcriptional stop signal. (See Materials and Methods for details of the assay.)

To measure the frequency of V(D)J recombination, the substrate plasmid is electroporated into the recipient cells, low molecular mass DNA is recovered from these cells 48 h later, and this material is used to transform antibiotic-sensitive *Escherichia coli*. (Because the Rag1 and Rag2 genes are not expressed in human fibroblasts, the recombination substrate must be co-transfected into the cells along with expression plasmids encoding the Rag1 and Rag2 genes, see Materials and Methods.) The bacteria are subsequently plated on medium containing ampicillin, or ampicillin plus chloramphenicol. Dividing the number of dual antibiotic-resistant clones (resulting from bacteria transformed by recombinant plasmid) by the number of single antibiotic-resistant clones (resulting from bacteria transformed by recombinant OR non-recombinant plasmid) gives the frequency of V(D)J recombination that occurred in the mammalian cells. Depending on the structure and orientation of the recombination signal sequences, the frequency of rejoining of signal ends or coding ends can be measured. Furthermore, DNA sequence analysis of the plasmids recovered from the bacteria can be used to gain additional insight into the precise structure of the rejoined ends.

A series of experiments were performed in which we examined the frequency with which signal ends were rejoined in a diploid fibroblast strain of cells from an FA patient from the FAA complementation group, as well as in a diploid fibroblasts belonging to the FAC complementation group. In addition, signal end rejoining was examined in FAA and FAC fibroblasts that had been transduced with retroviruses encoding the respective wild-type FANC cDNAs (referred to in Table 1 as FAA corrected and FAC corrected), and in two strains of diploid fibroblasts from normal donors. As the data in Table 1 reveal, the frequencies with which signal joint rejoining occurred in these different cell strains were remarkably similar. Using analysis by the χ^2 test revealed that the slight differences observed were not statistically significant ($P > 0.1$). It is noteworthy that these frequencies are similar to that previously reported in a different diploid strain of human fibroblasts from a normal donor (0.0021).[25] The recombination frequencies are also in close agreement with that reported by Smith *et al*. (0.0017).[37] Therefore, the data presented in Table 1 support the conclusion that the rejoining of signal ends formed during V(D)J recombination occurs with wild-type frequency in fibroblasts derived from FA patients.

Antibody-mediated inhibition of V(D)J recombination

We showed earlier that co-electroporation of antibodies can be used to block DNA repair and recombination of introduced plasmid substrates

Table 1. V(D)J signal joint recombination occurs at wild-type frequency in fibroblasts from Fanconi anemia patients

	Number of colonies		
Cells	Ampicillin^R chloramphenicol^R	Ampicillin^R	V(D)J recombination frequency
HT1080	18	7760	0.0023
CCL 153 (WT)	14	6480	0.0022
PD.715.F (WT)	13	5696	0.0023
PD.720.F (FAA)	9	5152	0.0022
FAA Corrected	11	6112	0.0018
PD.551.F (FAC)	11	5920	0.0019
FAC Corrected	9	4768	0.0019

The numbers of colonies obtained represent pooled data obtained from three or more independent experiments. The recombination frequencies obtained from these replicate experiments did not differ significantly (data not shown).

selectively.[25,42] Control experiments presented in those reports demonstrates that introduction of antibodies into intact mammalian cells mimics the effects of antisense expression or expression of dominant-negative alleles in blocking protein function selectively. It is particularly noteworthy that introduction of anti-DNA ligase IV antibody inhibited cellular plasmid V(D)J recombination to levels observed in cells expressing antisense DNA ligase IV.[25]

The data in Table 1 suggest that co-introduction of antibodies specific for NHEJ proteins such as DNA ligase IV, and Ku86 would inhibit V(D)J recombination in both the normal diploid fibroblasts, as well as in FA fibroblasts. One would further predict that introduction of antibody specific for the Fancd2 protein would have no effect on V(D)J recombination of signal joints in either cell type. The data presented in Figure 2 are consistent with these predictions. Introduction of antibodies specific for DNA ligase IV, Ku86 or Xrcc4 reduced the frequency of rejoining of signal joints dramatically in both HT1080 and FAA cells. In all cases, the results were statistically significant (Student's *t*-test. $P < 0.001$). In contrast, co-electroporation of anti-Fancd2 antibody had no significant effect on the frequency with which signal joints present on the substrate pKSV(D)JSV40ori plasmid were rejoined.

Rejoining of signal joints in FA fibroblasts is error-prone

Smith *et al*. reported that lymphoblasts from FA patients rejoined signal joints at wild-type efficiency, but that the fidelity of the rejoining reaction was reduced by approximately fivefold, compared to that observed in rearranged plasmids recovered from wild-type lymphoblasts.[37] To determine whether signal joint rejoining was similarly imprecise in fibroblasts from FA patients, we examined recombinant plasmid DNA recovered from HT1080 cells, and from an FAC fibroblast strain. In addition, we examined recombinant plasmids recovered from an HT1080 cell line that over-expresses the FANCC allele

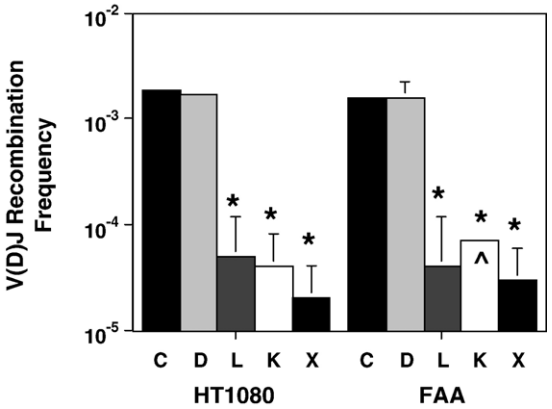

Figure 2. Antibody-mediated inhibition of V(D)J recombination of signal joints in HT1080 and FAA cells. V(D)J recombination assays were performed as described in the legend to Figure 1 in the HT1080 cell line or in diploid fibroblast strain PD.720.F derived from an FA patient (complementation group FAA), in the presence of 0.4 μg of antibody as indicated. C, non-specific IgG; D, anti-Fancd2 antibody; L, anti-DNA ligase IV antibody; K, anti-Ku86 antibody; and X, anti Xrcc4 antibody. Results depict the mean±SEM of three or more independent experiments. *$P < 0.001$, Student's *t*-test.

L554P, and from HT1080 cells that were transfected with anti-Fancd2 antisera. We, and others, have established that over-expression of this FANCC allele functions as a dominant negative, conferring a FA-like phenotype on human cells.[42–44] Because precise rejoining of signal ends on plasmid pKSV(D)JSV40ori generates a novel ApaLI site, initial analysis was performed by restriction endonuclease digestion. Precisely rejoined plasmids will yield three fragments upon digestion with ApaLI (Figure 3, lane marked Pr), while ApaLI digestion of an imprecisely rejoined plasmid (Figure 3, lanes marked Im) yields two bands. As the results depicted in Table 2 reveal,

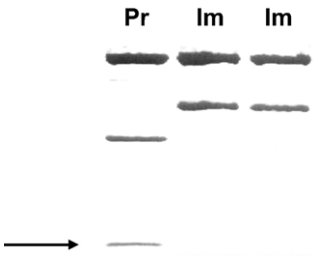

Figure 3. Restriction endonuclease analysis of plasmids harboring recombinant signal joints. Plasmid DNA was recovered from bacterial clones harbouring recombinant pKSV(D)JSV40ori plasmid recovered from human cells, digested with the restriction endonuclease ApaLI, and resolved by electrophoresis on a 0.8% agarose gel. The Figure depicts a negative image of an ethidium bromide-stained gel showing the products of digestion of three independently obtained clones. ApaLI digestion of precisely rejoined plasmids (Pr) yields three fragments, the smallest of which is indicated by an arrow, whereas digestion of imprecisely rejoined plasmids (Im) yields two fragments.

rejoining of signal ends formed during V(D)J recombination in wild-type cells was very precise; imprecise rejoining was detected in only 1.1% of the clones analysed. In contrast, rejoining of these substrates in diploid fibroblasts from an FA patient (PD.551.F FAC), which occurred with wild-type efficiency, was imprecise in 8.7% of instances,a nearly eightfold increase over that observed in wild-type cells.

The frequency with which imprecise rejoining of signal ends occurred in the L554P-expressing HT1080 cells (9.7%), and in HT1080 cells that had been co-electroporated with antisera specific for the Fancd2 protein (9.5%) was nearly identical with that observed in the FAC cells (8.7%). Analysis by the χ^2 test revealed that in all three cases, the observed increase in imprecise rejoining in these cells was statistically significant ($P < 0.02$) when compared to that detected in control experiments carried out in wild-type cells. The finding that anti-Fancd2 antibody reduced the fidelity of the recombination reaction significantly is noteworthy, given that introduction of this same antiserum had no effect on the overall frequency of signal joint rejoining (Figure 2). These data support the conclusion that rejoining of signal ends occurs in an error-prone manner in FA fibroblasts. Smith *et al*. observed that the fidelity of signal end rejoining was reduced in immortalized lymphoblasts from FA patients, although the finding was not statistically significant.[37] However, their data indicated that the background level of imprecise rejoining in a lymphoblast cell line from a normal donor was somewhat higher than that seen in fibroblasts.

Sequence analysis of imprecisely rejoined plasmids recovered from L554P cells (Figure 4) indicated that rejoining was associated with deletions extending beyond the RSS heptamers on one or both ends (indicated by the negative numbers in parentheses). In one instance (clone 2), the recombinant plasmid contained sequences on both ends that are ordinarily deleted during V(D)J recombination (indicated by positive numbers in parentheses). In all cases, the site of the deletion was flanked by direct sequence repeats varying in length from one to three nucleotides (indicated by underlining in Figure 4). Smith *et al*. also observed that direct repeat sequences were commonly associated with aberrantly processed V(D)J recombination events.[37]

Table 2. V(D)J signal joint recombination is error-prone in cells lacking a functional Fanconi anemia signalling pathway

	Imprecise events	Precise events	Imprecision (%)
HT1080	2	179	1.1
PD.551.F (FAC)	4	42	8.7*
HT1080 + anti-Fancd2 antibody	7	67	9.5**
HT1080 L554P	7	65	9.7**

The numbers of colonies obtained represent pooled data obtained from two or more experiments. The recombination frequencies obtained from these replicate experiments did not differ significantly (data not shown). *$P < 0.02$, χ^2; **$P < 0.005$, χ^2.

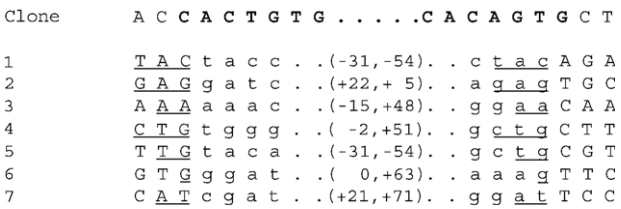

Figure 4. Sequence analysis of recombinant plasmids encoding imprecisely rejoined signal ends. Plasmid DNA containing imprecisely rejoined signal ends (identified by screening for the absence of an ApaLI site (see Figure 3) were subjected to DNA sequence analysis. The heptamer sequences of RSS23 and RSS12 are indicated in bold. Underlining indicates direct repeat sequences. Upper case letters indicate sequences present in recombinant clones, and lower case letters represent nucleotides deleted during the recombination reaction. Numbers in parentheses indicate the number of bases deleted (if negative) or retained at the RSS sites.

V(D)J recombination of plasmids containing coding joints in L554P and wild-type cells

We utilized the plasmid transfection strategy outlined above to compare V(D)J recombination of coding joints in HT1080 cells as well as in HT1080 cells expressing the dominant negative L554P allele of the FANCC gene. On the basis of the results described above, as well as those reported by Smith *et al*.,[37] we anticipated that coding joint rejoining would occur at wild-type frequency in cells expressing the L554P allele. Results from a series of experiments ($N = 4$) revealed a slight (but not statistically significant) increase in the frequency with which an introduced plasmid harbouring a coding joint underwent V(D)J recombination in the L554P-expressing cells, compared to non-transgenic HT1080 cells (Figure 5). The absence of a detectable difference in the frequency with which signal joints were rejoined in these two cell lines (not shown) suggests that the slightly elevated frequency of coding joint rejoining observed in the L554P cells is not biologically meaningful.

Smith *et al.* showed that coding joint rejoining in lymphoblasts from FA patients was aberrant, resulting in large deletions not detected in recombinant plasmids recovered following V(D)J recombination events in control cells.[37] We next performed DNA sequence analysis of coding joints formed during V(D)J recombination in both HT1080 and L554P cells. The results of this analysis revealed that in the majority of cases (90% for both L554P and HT1080 cells) recombination events were associated with short insertions or deletions immediately flanking either or both RSS sequences. Analysis of products recovered from the two cell lines revealed a single instance each where a non-germline nucleotide had been inserted at the site of rejoining. We also recovered a single clone from each of the cell

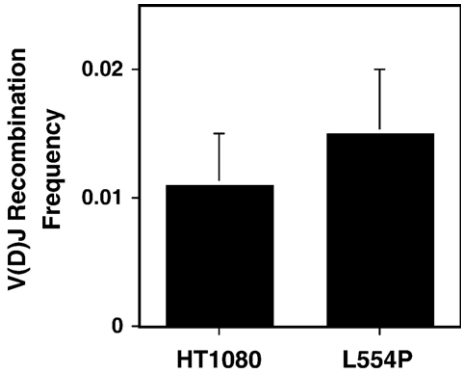

Figure 5. The frequency of V(D)J recombination of coding joints is not reduced in cells expressing a dominant negative FANCC allele. V(D)J recombination assays were performed using plasmid pGG51 as described in Materials and Methods. Experiments were performed on non-transgenic HT1080 cells (HT1080) and on HT1080 cells stably expressing the L554P allele of the FAC gene (L554P). Data represent the mean±SEM, $N=4$.

lines in which the deletion breakpoints at each end were relatively far removed from the RSS heptamer sequences. These recombinants may have resulted from mis-processed V(D)J recombination intermediates, or may have arisen *via* another DNA repair or recombination process. Irrespective of which pathway is responsible for the generation of these clones, it seems clear that there is no obvious difference between the structure of recombinant coding joints recovered from either wild-type or L554P-expressing cells. Thus, in contrast to what was observed with rejoining of signal ends, there does not appear to be any defect in coding end rejoining in fibroblasts in which FANCC gene function has been inactivated.

Role of Rad50 in V(D)J recombination

We observed recently that the plasmid end-joining deficiency in FA cells could be replicated in wild-type cells through antibody-mediated inhibition of Rad50, or of other members of the RMN protein complex.[25] These results support the interpretation that plasmid end-joining in wild-type cells relies on the DNA tethering activity of the Rad50 complex. The finding that V(D)J recombination of signal joints is aberrant in FA cells raised the prospect that this reaction, too, could depend on the tethering function of the Rad50 complex. This latter possibility was proposed to explain the recent finding that V(D)J recombination of introduced plasmid substrates in yeast is dependent on Rad50 gene function.[36]

We therefore used antibody-mediated inhibition to test the hypothesis that interfering with Rad50 function in mammalian somatic cells would reduce the fidelity of signal end rejoining. As outlined in Table 3, introduction of anti-Rad50 antibody along with the recombination substrate reduced the fidelity of rejoining by greater than 20-fold. Similarly, introduction of antibody specific for Mre11 or Nibrin resulted in a 20–25-fold increase in the frequency with which imprecise signal end rejoining occurred. Co-introduction of antibodies specific for all three antigens resulted in a nearly 30-fold increase in the frequency with which imprecisely rejoined recombinant plasmids were recovered. It is noteworthy that introduction of non-specific antiserum had no effect on the fidelity of the signal joint rejoining reaction (Table 3), and had no effect on the overall frequency with which V(D)J recombination occurred in these cells (Table 2 and data not shown).

The finding that antibody-mediated inhibition of Nibrin function reduced the fidelity of signal joint recombination substantially is apparently at odds with a report showing that fibroblasts from an Nijmegen breakage syndrome patient rejoined signal joints present on a plasmid recombination substrates with a fidelity that was indistinguishable from that observed in cells from a normal donor.[45] We therefore examined V(D)J recombination of a signal joint containing plasmid substrate in a simian virus 40 (SV40) immortalized fibroblast cell line (NBS-ILB1) obtained from a Nijmegen breakage syndrome patient (see Materials and Methods). We confirmed that the frequency and fidelity of V(D)J recombination in these cells did not differ from that observed in HT1080 cells (data not shown).

A possible explanation for this apparent paradox is that the allele responsible for creating the NBS phenotype encodes a Nibrin protein that retains residual function, rather than no function. This interpretation is consistent with the finding that introduction of the cDNA encoding the human disease-causing allele of Nibrin rescued the embryonic lethality observed in mice in which the endogenous Nibrin gene had been inactivated.[46] Thus, it is conceivable that there is sufficient residual Nibrin function in cells from patients with Nijmegen breakage syndrome to ensure that rejoining of signal ends occurs with wild-type fidelity. If this hypothesis is correct, one would anticipate that introduction of anti-Nibrin antibody into NBS-ILB1 cells would increase significantly the frequency with which imprecisely rejoined signal joints are recovered. An additional experiment was therefore performed in

Table 3. Antibody-mediated inhibition of the RMN complex reduces the fidelity of V(D)J signal joint recombination

	Imprecise events	Precise events	Imprecision (%)
Control IgG	2	179	1.1
Anti-Rad50	22	64	25.6*
Anti-Mre11	16	49	24.6*
Anti-Nibrin	17	40	29.8**
Anti-Rad50 + Anti-Mre11 + Anti-Nibrin	19	43	30.6**

The numbers of colonies obtained represent pooled data obtained from two or more experiments. The recombination frequencies obtained from these replicate experiments did not differ significantly (data not shown). * $P<10^{-5}$, χ^2; ** $P<10^{-6}$ χ^2.

which anti-Nibrin antibody was co-electroporated along with the V(D)J recombination plasmid into NBS-ILB1 cells. Plasmid DNA was subsequently recovered from these cells and used to transform *E. coli*. As anticipated on the basis of the results presented above, inclusion of anti-Nibrin antibody had no effect on the frequency of V(D)J recombination in these cells (data not shown). However, analysis of plasmid DNA recovered from 20 bacterial colonies containing recombinant plasmids revealed that four of them (20%) contained imprecisely rejoined signal ends. This frequency of imprecise rejoining does not differ significantly ($P > 0.05$, χ^2) from that observed in control cells in which anti-Nibrin antibody was co-electroporated with the recombination substrate plasmid (see Table 3).

Discussion

Using plasmid-based assays we showed that diploid fibroblasts from FA patients rejoin both coding and signal ends formed during V(D)J recombination at frequencies identical with those observed in fibroblasts from a normal donor. While the structure of rejoined coding joints does not appear to differ between FA and wild-type cells, our data show that the fidelity of signal end rejoining in fibroblasts from FA patients is reduced by nearly tenfold compared to that observed in wild-type fibroblasts. The error-prone rejoining phenotype was reversed in FA cells that had been transduced with a retrovirus expressing the appropriate FA gene product. Reduced fidelity of signal end rejoining was observed also in wild-type cells expressing a dominant negative allele of the FANCC gene, as well as in wild-type cells in which an antibody against the Fancd2 protein was introduced along with the recombination substrate. In contrast, there was no apparent reduction in either the efficiency or fidelity of coding end rejoining in fibroblasts expressing a dominant negative allele of the FANCC gene. The absence of any apparent defect in rejoining of coding ends in fibroblasts derived from FA patients provides a plausible explanation for the fact that these patients do not suffer from immunodeficiency.

On the basis of previous findings that fibroblasts from FA patients have defects in RMN complex function,[25,38] one would anticipate that inhibition of RMN function would lead to error-prone signal end rejoining in normal fibroblasts. Consistent with this hypothesis, we observed that the introduction of antibodies specific for the components of the RMN complex significantly reduced the fidelity of signal end rejoining in wild-type cells. These results support the interpretation that the Rad50/Mre11/Nibrin protein complex plays a role in ensuring high-fidelity rejoining of signal ends during V(D)J recombination in mammalian cells.

These findings contribute to an emerging body of evidence that the RMN complex participates in immunoglobulin gene rearrangements. As was outlined in Introduction, there have been a number of reports of a previously undetected role for the RMN complex in class-switch recombination, somatic hypermutation, and gene conversion.[26,27,33,34] Identification of a role for the RMN complex in V(D)J recombination in mammalian fibroblasts is also consistent with recent work by Clatworthy *et al*. showing that recombination of signal joints located on an plasmid introduced into yeast was dependent on exogenously supplied Rag1 and Rag2 function as well as on the products of the yeast NHEJ genes Rad50, Mre11 and Xrs2.[36]

Our results indicate that RMN complex function is required to ensure precise rejoining of signal ends during V(D)J recombination in human fibroblasts. However, this interpretation seems inconsistent with a previous study of V(D)J recombination of plasmid substrates in fibroblasts from patients with Nijmegen breakage syndrome. Yeo *et al*. reported that these cells, which are homozygous for a five base-pair deletion within the coding region of the Nibrin gene, rejoined signal ends with wild-type efficiency and fidelity, a finding we confirmed in the NBS-ILB1 cell line, which is also derived from a Nijmegen breakage syndrome patient.[45] The key to understanding this seeming paradox appears to lie in the recent finding that introduction of Nibrin cDNA encoding the five base-pair deletion responsible for human disease rescued the embryonic lethality observed in mice in which the endogenous Nibrin gene had been inactivated.[46] The simplest interpretation of this finding is that the human disease allele retains some residual function. Consistent with this interpretation, we showed that introducing anti-Nibrin antibody into NBS-ILB1 cells reduced the fidelity of signal joint rejoining significantly. Thus, it appears that the absence of a detectable defect in rejoining of signal ends in cells derived from Nijmegen breakage syndrome patients is due to residual activity associated with the disease-causing allele of Nibrin.

Our data raise the intriguing question of why antibody-mediated inhibition of the RMX complex, which reduces the efficiency of plasmid end-joining in human fibroblasts dramatically,[25] produces such a subtle defect in rejoining the double-strand breaks formed during V(D)J recombination. The most likely explanation would appear to be that mammalian somatic cells possess one or more factors that render RMN complex function largely redundant during V(D)J recombination. While the identity of this factor or factors is not known, our current understanding of the molecular biology of the V(D)J recombination suggests a possible candidate that could tether signal and coding ends together during the reaction, thereby substituting for the RMN complex. The Rag1/Rag2 heterodimer remains associated with signal ends formed during V(D)J recombination,[47] which could conceivably function to stabilize them. This model is consistent with evidence suggesting that Rag1/Rag 2 remains bound to linearized V(D)J reaction intermediates and recruits the Ku heterodimer. The Ku heterodimer is postulated to displace the Rag1/Rag2 complex from the DNA and subsequently recruit the

Xrcc4/DNA ligase IV complex that catalyses phosphodiester bond formation.[1,2] We propose that, while the presence of the Rag1/Rag2 complex is sufficient to ensure efficient rejoining of signal ends, the RMN complex is required to ensure that the subsequent rejoining of these ends occurs in an error-free manner. Our data suggest that when the RMN complex is not able to participate in the reaction, this rejoining becomes error-prone.

Materials and Methods

Cell culture and plasmid constructs

Cells were maintained in a humidified 5% (v/v) CO_2-containing atmosphere at 37 °C. Human diploid fibroblast strains derived from FA patients were obtained from the Oregon Health Sciences University Fanconi Anemia Cell Repository. PD.720.F cells are human diploid fibroblasts belonging to complementation group A, and PD.551.F cells are human diploid FA fibroblasts belonging to complementation group C. Complementation group assignment of FA cell strains was performed at the Fanconi Anemia Cell Repository. The Fanconi Anemia Cell Repository provided derivatives of the PD.720.F and PD.551.F cell strains that are infected with retroviruses encoding the Fanca and Fancc cDNAs, respectively, and are referred to as FAA corrected and FAC corrected. PD.715.F is a normal human diploid fibroblast cell strain derived from normal donors, and was provided by the Oregon Health Sciences University Fanconi Anemia Cell Repository. HT1080 cells were derived from a spontaneous human fibrosarcoma,[48] and were obtained from the American Type Culture Collection Cell Repository. The normal diploid human fibroblast cell strain CCL153 was obtained from the National Institute of General Medical Sciences Cell Repository. Cell line NBS-ILB1,[49] an SV40-immortalized diploid fibroblast cloned derived from fibroblasts obtained from a patient with Nijmegen breakage syndrome were kindly provided by Patrick Concannon. Dr Maureen E. Hoatlin, OHSU provided a plasmid encoding the patient-derived dominant negative L554P allele of the Fanconi anemia complementation group C gene. This plasmid was used to transform HT1080 cells, and a stable clone was isolated. Detailed characterization of this clone[50] confirmed previous findings[44] that expression of the L554P FANCC allele induced a Fanconi anemia-like phenotype on HT1080 cells.

Plasmids pRAG1[51] and pRAG2[52] (gifts from Dr David Baltimore, California Institute of Technology, Pasadena, CA) encode the *RAG-1* and *RAG-2* genes, respectively. The plasmids are comprised of the Rag1 and Rag2 cDNAs cloned into the mammalian expression vector pCDM8. Plasmid pRSVEdl884 encodes the SV40 large T-antigen but lacks an SV40 origin of replication.[53] Plasmids pKSV(D)JSV40ori (derived from pJH200[39]) and pGG49 (identical with pSJ[40]), were used to measure signal joint recombination, and plasmid pGG51[41] was used to measure coding joint recombination.

V(D)J recombination

Recombination assays were performed essentially as described.[25] Approximately one million cells were electroporated using a BTX (Holliston, MA) ECM 600 device set for 300 V and 960 μF (4mm gap) in a volume of 500 μl in the presence of 10 μg of the V(D)J recombination substrate plasmid, 5 μg each of plasmids pRAG1 and pRAG2, and 5 μg of plasmid pRSVEdl884. Where indicated, antibodies (0.4 μg) were present during electroporation. Rabbit monoclonal anti-Ku86, rabbit monoclonal anti-DNA ligase IV and rabbit monoclonal anti-Xrcc4 antibodies were obtained from Serotec Ltd (Raleigh, NC). Rabbit polyclonal anti-Fancd2, rabbit polyclonal anti-Rad50, rabbit polyclonal anti-Mre11 and rabbit polyclonal anti-Nbs1 antibodies were obtained from Novus Biologicals, Inc. (Littleton, CO). Following incubation for 48 h, the plasmids were recovered from cells and digested with the restriction endonuclease DpnI. The frequency with which V(D)J recombination occurred in the substrate plasmids was determined by transforming this recovered DNA into DH10B electrocompetent reporter bacteria. The design of the recombination substrate plasmids are such that, upon V(D)J recombination, a portion of plasmid DNA flanked by recombination signal sequences is deleted. As a consequence, a prokaryotic silencer element that is flanked by the recombination signal sequences is deleted, thereby permitting transcription of a chloramphenicol acetyl transferase gene that renders bacteria resistant to the antibiotic chloramphenicol. Since both the non-recombinant and recombinant plasmids encode a functional beta-lactamase gene, bacteria harbouring either recombinant or non-recombinant plasmid are resistant to ampicillin. Thus, the frequency of V(D)J recombination that occurs within the mammalian cells is calculated by dividing the number of chloramphenicol and ampicillin-resistant bacterial colonies by the total number of ampicillin-resistant bacterial colonies obtained when the recovered plasmid DNA is transformed into sensitive bacteria. (The other plasmids used in this assay do not confer antibiotic resistance and are not detected by this assay.) Precise rejoining of signal ends generated during V(D)J recombination of plasmids pKSV(D)JSV40ori and pGG49 results in the generation of a novel ApaLI recognition sequence. Imprecisely rejoined signal ends, identified by ApaLI digestion of plasmid DNA recovered from chloramphenicol-resistant bacteria, were subjected to DNA sequence analysis, as were representative plasmids (derived from pGG51) containing recombinant coding joints.

Acknowledgements

This work was supported in part by a National Institutes of Health Grant AG16678. A. D. was supported by an American Heart Association Northland Affiliate Predoctoral fellowship.

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Edited by J. Karn

(Received 11 July 2006; received in revised form 2 March 2007; accepted 5 March 2007)
Available online 15 March 2007