Cell, Vol. 117, 171–184, April 16, 2004, Copyright ©2004 by Cell Press

RAG Proteins Shepherd Double-Strand Breaks to a Specific Pathway, Suppressing Error-Prone Repair, but RAG Nicking Initiates Homologous Recombination

Gregory S. Lee,[1] Matthew B. Neiditch,[2] Sandra S. Salus,[1] and David B. Roth[1,*]
[1]The Skirball Institute of Biomolecular Medicine, Lab 2-10
and Department of Pathology
New York University School of Medicine
New York, New York 10016

Summary

The two major pathways for repairing double-strand breaks (DSBs), homologous recombination and nonhomologous end joining (NHEJ), have traditionally been thought to operate in different stages of the cell cycle. This division of labor is not absolute, however, and precisely what governs the choice of pathway to repair a given DSB has remained enigmatic. We pursued this question by studying the site-specific DSBs created during V(D)J recombination, which relies on classical NHEJ to repair the broken ends. We show that mutations that form unstable RAG postcleavage complexes allow DNA ends to participate in both homologous recombination and the error-prone alternative NHEJ pathway. By abrogating a key function of the complex, these mutations reveal it to be a molecular shepherd that guides DSBs to the proper pathway. We also find that RAG-mediated nicks efficiently stimulate homologous recombination and discuss the implications of these findings for oncogenic chromosomal rearrangements, evolution, and gene targeting.

Introduction

The integrity of the genome is under constant threat from both endogenous metabolic processes and exogenous DNA-damaging agents. Breaks in the phosphodiester backbone are particularly dangerous since they can lead to oncogenic chromosomal rearrangements through recombination. Single-stranded breaks (nicks) can initiate recombination in principle (Holliday, 1964; Meselson and Radding, 1975), but they have been widely believed to be simply religated. Repair of double-strand breaks (DSBs), on the other hand, clearly entails either homologous recombination or nonhomologous end joining (NHEJ), with all the attendant risks (Pâques and Haber, 1999).

Homologous recombination is generally thought to repair breaks made in meiotic prophase or during the S and G2 phases of the cell cycle, whereas NHEJ repairs DSBs generated in the absence of a sister chromatid (Goedecke et al., 1999; Pâques and Haber, 1999; Takata et al., 1998). This division of labor is not absolute, however. Several lines of evidence indicate that these pathways can act contemporaneously or even in concert: homologous recombination can occur during G1 (Fabre, 1978); NHEJ and homologous recombination compete for repair of transfected linear DNA molecules (Roth and Wilson, 1985); disabling one pathway increases the activity of the other (Pierce et al., 2001); and the two mechanisms can act in a coupled fashion to repair a DSB (Richardson and Jasin, 2000). Precisely how a particular repair pathway is selected to repair a given DSB, or even whether such a selection is made, has remained unknown. Yet it is reasonable to think that the choice of repair mechanism could serve as an important control point in various types of reactions involving DSB intermediates.

One model posits that the choice of pathway is essentially stochastic, determined by whether the broken ends happen to be bound by Ku or by, for example, Rad52 (leading to NHEJ or homologous recombination, respectively) (Goedecke et al., 1999; Van Dyck et al., 1999; Haber, 1999). Alternatively, it has been proposed that if the ends remain intact, they will be available for NHEJ, but 5′ strand resection will call homologous recombination into play (Frank-Vaillant and Marcand, 2002). Of course, these models simply push the key question back a step: what determines whether Ku or Rad52 binds a given DSB, or whether ends are subject to resection? Indeed, recent scanning force microscopy studies indicate that Rad52 and Ku actually prefer different DNA substrates and suggest that resection precedes Rad52 binding (Ristic et al., 2003). The identity of the "gatekeeper" molecule(s) that control this critical choice remains an open question. Adding to the puzzle is evidence suggesting that there may be other repair pathways, for example, the poorly characterized "alternative NHEJ" pathway that operates in the absence of classical NHEJ (i.e., Ku, XRCC4, DNA ligase IV, Artemis, and the DNA-dependent protein kinase catalytic subunit DNA-PKcs) (reviewed in Ferguson and Alt, 2001; Roth, 2003). We reasoned that if there are indeed mechanisms to guide broken DNA ends to an appropriate pathway, they would be most evident in recombination systems that introduce site-specific DSB and depend upon repair by a particular pathway to rejoin the DNA ends.

V(D)J recombination is one such system. A DNA rearrangement process that assembles antigen receptor genes during lymphocyte differentiation, V(D)J recombination relies on classical NHEJ to join site-specific DSB. As shown in Figure 1, recombination is initiated by the RAG-1 and RAG-2 proteins, which introduce nicks at recombination signal sequences (RSS); upon synapsis, the RAG proteins then convert these nicks to DSBs, leaving four ends (two hairpinned coding ends and two blunt signal ends) (Roth, 2003). Whereas signal ends are blunt ends that can be directly joined, the covalently sealed hairpins must be opened and processed before the coding ends can be joined. End-processing and joining are carried out by the classical NHEJ factors (Roth, 2003) along with the help of the RAG proteins themselves, which maintain the ends in a postcleavage complex in vitro (Agrawal and Schatz, 1997; Hiom and Gellert, 1998; Jones and Gellert, 2001) and in vivo (Qiu

*Correspondence: roth@saturn.med.nyu.edu
[2]Present address: Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544.

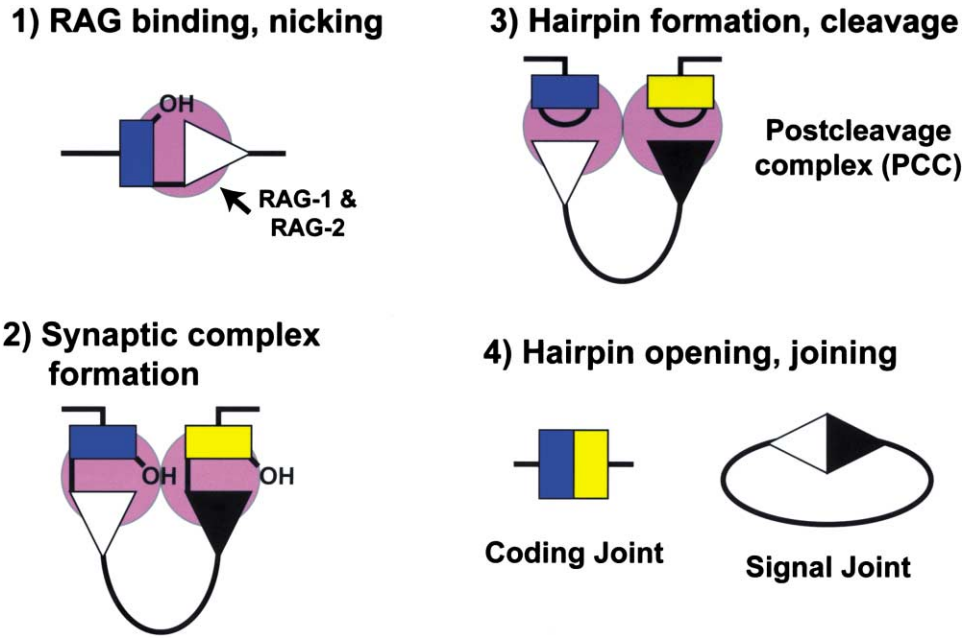

Figure 1. V(D)J Recombination

(1) The RAG proteins bind and create a single-strand nick at the border between the RSS (triangles) and coding sequence (boxes). (2) The RAG multimer must be bound to a pair of RSS, forming a synaptic complex, for the cleavage reaction to proceed. (3) The synaptic complex converts nicks to double-strand breaks via a transesterification reaction, generating blunt signal ends and hairpin coding ends (Roth, 2003), which are held in a postcleavage complex by the RAG proteins. (4) After cleavage, the nonhomologous end-joining (NHEJ) machinery ligates the signal ends to form a signal joint and opens and joins the hairpin coding ends to form a coding joint.

et al., 2001; Yarnall Schultz et al., 2001; Huye et al., 2002). Our in vivo studies of NHEJ mutants and mutant RAG proteins that have defects in joining led us to propose that the postcleavage complex serves as a scaffold for the four ends to facilitate repair (Zhu et al., 1996; Qiu et al., 2001; Yarnall Schultz et al., 2001; Brandt and Roth, 2002; Huye et al., 2002).

Although this idea has received support from another group (Tsai et al., 2002), the lack of a biochemical system capable of NHEJ-dependent V(D)J recombination has made it difficult to tease out the activities of the postcleavage complex. We reasoned that the postcleavage complex might shepherd coding and signal ends to the classical NHEJ machinery, thereby enabling proper joining and discouraging aberrant joining events. In order to test this hypothesis, we devised an in vivo system to determine the extent to which the postcleavage complex sequesters RAG-generated DSBs from an inappropriate repair pathway—homologous recombination. This experimental system has allowed us to test a large array of RAG mutants with various defects in joining as well as to study RAG-mediated nicks, whose fate has never been explored in living cells. Not only do our experiments confirm our model that the RAG proteins shepherd DSBs into the classical NHEJ pathway, but we have also found that RAG-mediated nicks can efficiently initiate homologous recombination.

Results

We constructed substrates containing a pair of defective cyan fluorescent protein (CFP) genes (Figure 2A) arranged so that homologous recombination initiated by nucleolytic cleavage in one of the two alleles can reconstitute a functional CFP gene (Figure 2B). To test both types of ends generated during V(D)J recombination, we employed substrates in which CFP is interrupted by RSS oriented so that cleavage generates either a pair of blunt signal ends (the SE substrate), a pair of covalently sealed hairpin coding ends (the CE substrate), or one of each in the case of a single RSS (12-RSS substrate). If these ends are repaired by joining, as in normal V(D)J recombination, the CFP gene will remain nonfunctional. If the ends are repaired by homologous recombination, however, then a functional copy of CFP will be formed, causing cells to fluoresce blue (Figure 2B). All substrates were tested in transient transfections into Chinese hamster ovary (CHO) fibroblasts, a model system widely used in studies of V(D)J recombination. Both full-length (FL) and truncated "core" versions of murine RAG proteins (Sadofsky et al., 1994a, 1994b) were used as indicated. Versions of core RAG proteins bearing point mutations are referred to by the amino acid(s) changed. As a negative control, we employed a RAG-1 mutant ("DDE mutant") in which three catalytic amino acids have been mutated. This mutant retains the ability to interact with RAG-2 and to bind DNA, but is catalytically inactive, unable to generate nicks or hairpins (Landree et al., 1999). The DDE mutant gave extremely low levels of homologous recombination (Figures 3A and 3B; Supplemental Figures S1 and S2 at http://www.cell.com/cgi/content/full/117/2/171/DC1) and was used to set background levels in flow cytometry assays. This background stimulation likely reflects repair of random breaks in the

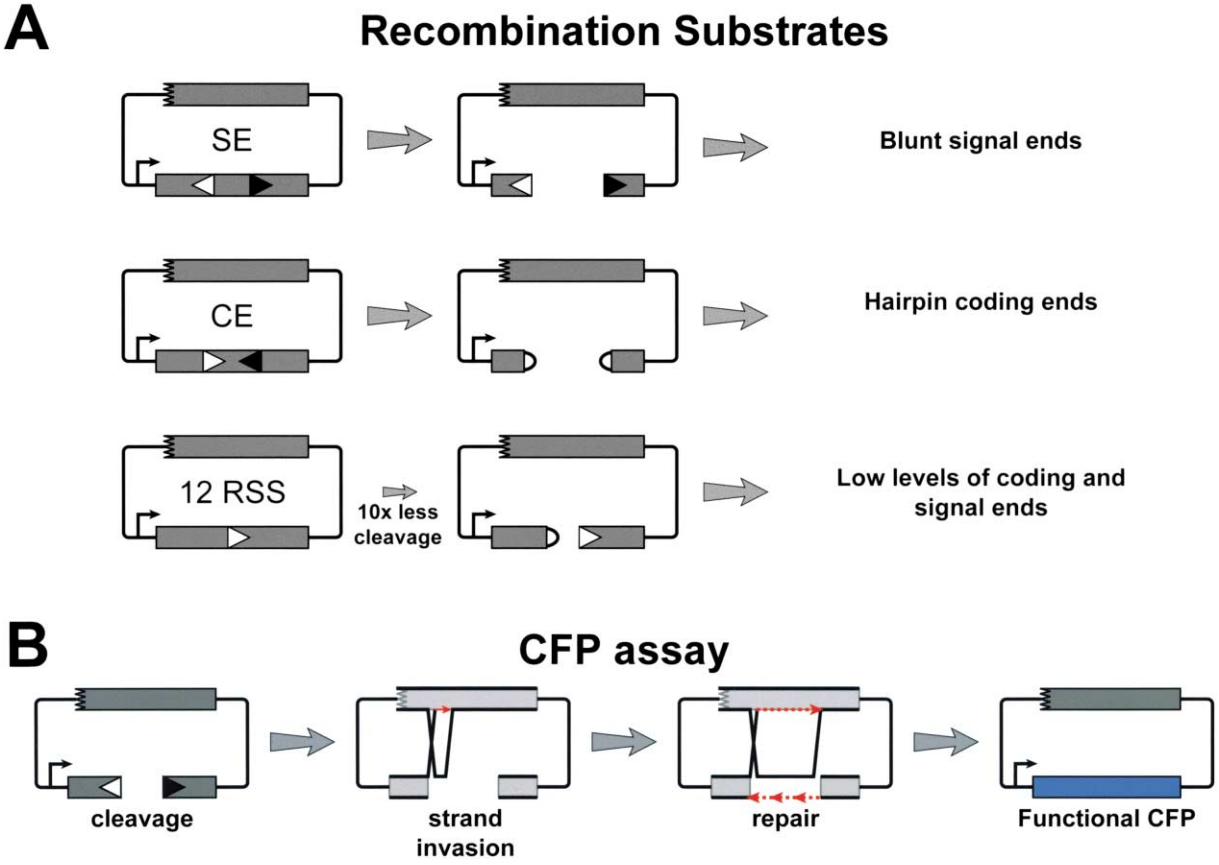

Figure 2. Homologous Recombination CFP Assay

(A) Substrates employed to detect homologous recombination contain two defective alleles of the *CFP* gene and differ only in the inserted sequence used to target nucleolytic cleavage of one allele. One substrate contains RSS configured so that cleavage produces blunt signal ends (SE); in another, cleavage produces hairpin coding ends (CE). A third substrate contains only a single RSS (12 RSS), which undergoes efficient nicking but poor DSB formation.

(B) A site-specific DSB generated in one *CFP* allele by the RAG proteins can stimulate homology-directed repair using the nonfunctional (N-truncated) allele, producing a functional *CFP* gene. Truncated, core versions of murine RAG proteins were used unless otherwise noted (see text for details).

transfected DNA, which are common (Wake et al., 1984). As an additional control, we tested a homologous recombination substrate with scrambled heptamer and nonamer elements (the "no RSS substrate") to ensure that nonspecific cleavage did not stimulate recombination. As expected, this substrate gave background levels of homologous recombination identical to the DDE mutant, as measured by flow cytometry (Figure 3B). As a positive control, we employed a similar substrate containing a site for the homing endonuclease, I-SceI, which stimulates homologous recombination in mammalian cells by introducing site-specific DSBs (Jasin, 1996; Moynahan et al., 2001); as expected, it produced robust homologous recombination (Figure 3B).

We performed further controls to ensure that CFP expression indeed measures repair of the substrates by homologous recombination. First, substrates were recovered from sorted CFP-positive cells and sequenced; every functional copy of CFP recovered was perfectly repaired (data not shown), consistent with repair by homologous recombination. Second, RAG proteins (but not the DDE mutant) stimulated homologous recombination using a substrate configuration that measures intermolecular homologous recombination (a recipient plasmid contains the CFP allele with the cleavage site while a donor plasmid contains the truncated CFP allele). Importantly, omitting the truncated CFP allele from the "donor" substrate abolished formation of CFP-positive cells (data not shown), demonstrating that repair occurs by homologous recombination and not, for example, by exonucleolytic removal of the RSS from each end followed by perfect rejoining.

Unstable Postcleavage Complexes Stimulate Homologous Recombination

Elsewhere we have proposed that the joining step of V(D)J recombination could be impaired by any of several distinct mechanistic defects, such as an unstable postcleavage complex that releases ends prematurely, hyperstability (i.e., the complex retains the ends too avidly), or failure to recruit joining factors (Qiu et al., 2001; Yarnall Schultz et al., 2001; Brandt and Roth, 2002; Huye et al., 2002). Therefore, after establishing baseline levels of homologous recombination observed with wild-type

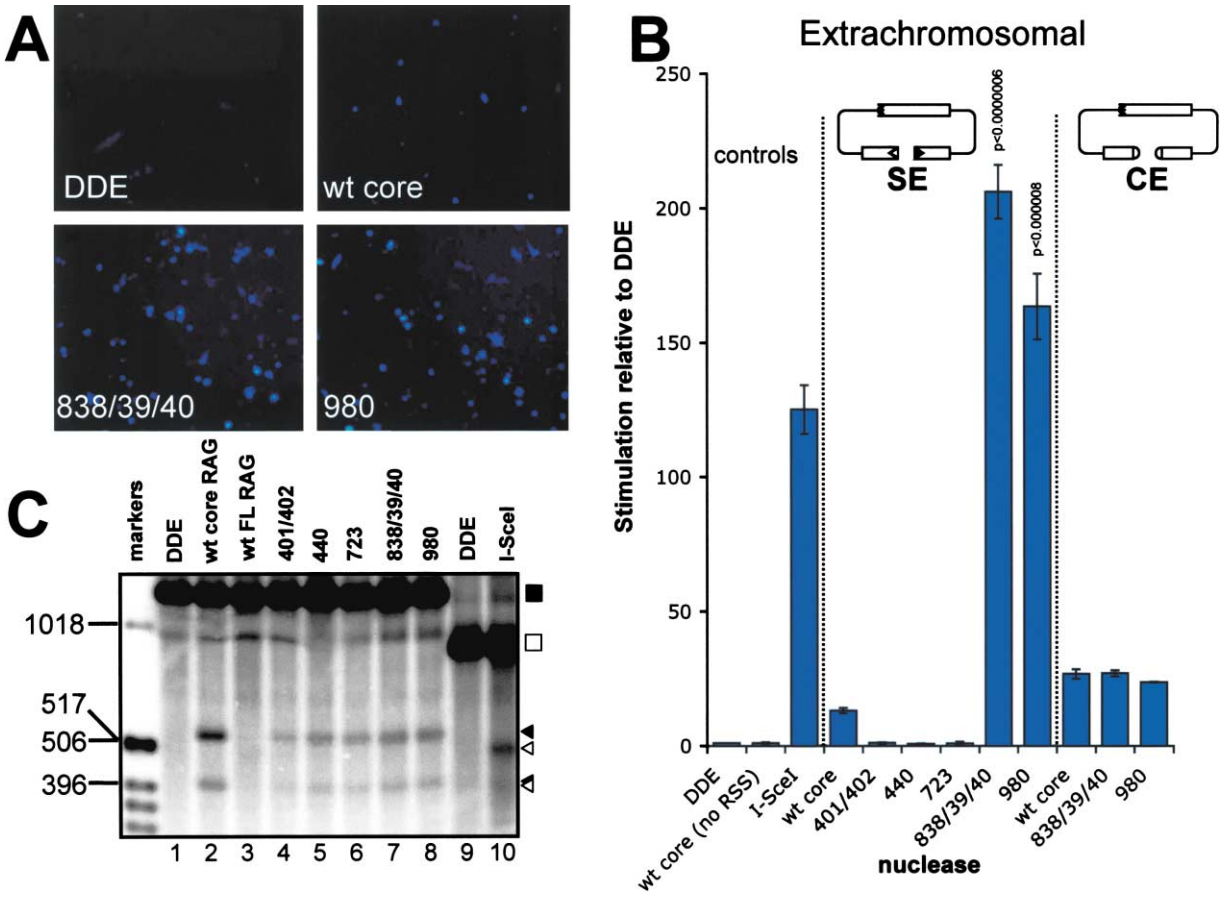

Figure 3. Two RAG Mutants Stimulate Robust Homologous Recombination on Extrachromosomal Substrates

(A) Fluorescent images of cells showing stimulation of homologous recombination by wild-type and joining-deficient RAG proteins (original magnification 100X).

(B) Relative stimulation of CFP expression by wild-type and joining-deficient RAG-1 proteins (R401A/R402A, R440A, S723A, R838A/K839A/R840A, K980A) quantitated by flow cytometry (see Experimental Procedures and Supplemental Figures for details). Two mutants, R838A/K839A/R840A and K980A, stimulated significantly more homologous recombination than wild-type core RAG proteins on the SE substrate ($p < 0.0000006$ and $p < 0.000008$, respectively). Because these mutants did not increase recombination on the CE substrate, the phenotype is not simply a result of increased nucleolytic activity. Error bars here and elsewhere represent standard error of the mean (SEM) from at least five experiments.

(C) Southern blot of DNA from transfections showing the levels of cleavage by wild-type and joining-deficient RAG proteins. DNA recovered from transfections by the Hirt method was digested with HpaI and NcoI and analyzed by Southern blotting. Expected fragment sizes of the SE substrate: uncleaved, 1229 bp (filled square); cleavage products, 557 and 416 bp (filled arrowheads). Expected fragment sizes of the I-SceI substrate: uncleaved, 903 bp (open square); cleavage products, 509 and 394 (open arrowheads). All mutant proteins showed decreased cleavage. Note that the uncleaved substrate bands (squares) serve as an internal control for relative transfection efficiencies and DNA recoveries between samples. Average DNA recovery varied less than 10% by phosphorimager quantitation (Supplemental Figure S4 online).

core RAG proteins, we used the CFP assay to test our collection of 15 mutants (six in RAG-1 and nine in RAG-2; see Supplemental Table S1 on *Cell* website) that can perform cleavage but are severely impaired in the joining phase of recombination (Huye et al., 2002; Qiu et al., 2001; Yarnall Schultz et al., 2001). We also tested a RAG-1 mutant, S723A, reported by another group to have a joining defect (Tsai et al., 2002). All of these mutants are expressed at wild-type levels in vivo (Supplemental Figure S3) and most have a mild cleavage defect (Supplemental Figure S4; see also Huye et al., 2002; Qiu et al., 2001; Yarnall Schultz et al., 2001).

Only two of the sixteen mutants, RAG-1 K980A and RAG-1 R838A/K839A/R840A, stimulated robust homologous recombination compared to wild-type RAG controls, as seen by fluorescence microscopy (Figure 3A and data not shown). Flow cytometry confirmed dramatic stimulation: both mutants consistently produced 10–15 times as much homologous recombination as wild-type core RAG controls (the relevant control since the mutants are on a core background) ($p < 0.000008$, Figure 3B). We also tested the RAG-1 R838A/K839A/R840A mutant with the signal end substrate integrated into the CHO genome. This mutant consistently generated higher levels of homologous recombination than wild-type core RAG-1 controls ($p < 0.0004$, Figure 4). The ability of these two mutants to stimulate homologous recombination is even more impressive in light of their mild cleavage defect in vivo: R838A/K839A/R840A and K980A produce 2.5-fold lower levels of signal ends

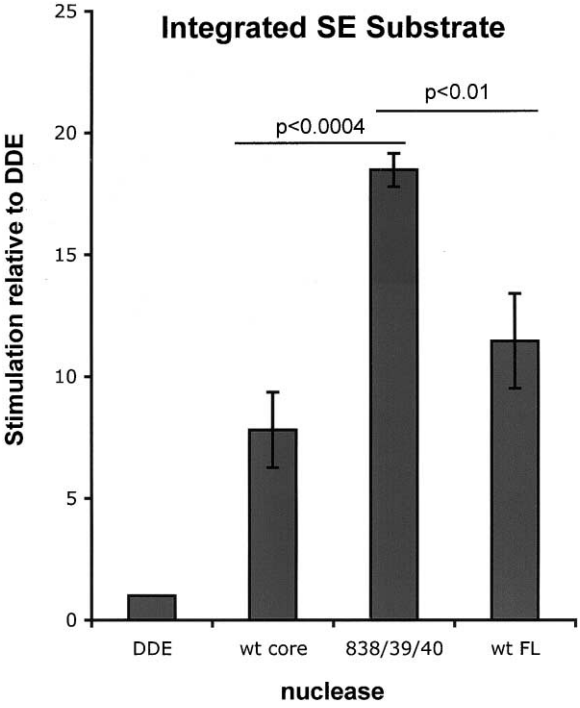

Figure 4. RAG Mutant Stimulates Homologous Recombination on Integrated Substrate

The joining-deficient RAG-1 protein, R838A/K839A/R840A, stimulated more homologous recombination than the wild-type RAG proteins ($p < 0.0004$, $p < 0.01$ for core and full-length, respectively) in a cell line with an integrated SE substrate. Two independent cell lines gave similar results.

in vivo than wild-type RAG proteins (Figure 3C and Supplemental Figure S4). Thus, on a per-end basis, these mutants stimulate ~25 to 35 times more homologous recombination than wild-type RAG proteins.

The high levels of homologous recombination produced by the two joining-deficient mutants were not observed with a coding-end substrate and are therefore specific for signal ends (Figure 3B, right side of graph). This most likely indicates that covalently sealed coding ends do not trigger homologous recombination. The significance of the low levels of homologous recombination stimulated by wild-type RAG proteins on the two substrates will be discussed below.

The data shown in Figures 3 and 4 suggest that these two RAG-1 joining mutants form unstable postcleavage complexes that fail to shepherd signal end intermediates to NHEJ, leaving the free signal ends to initiate homologous repair. These results further imply that the wild-type RAG proteins (both core and full-length versions) prevent the signal ends from accessing the homologous recombination machinery. A comparison with results obtained from I-SceI lends weight to this conclusion: whereas I-SceI generates fewer broken ends (~2.5-fold less) than wild-type core RAG proteins (Figure 3C), I-SceI stimulates homologous recombination ~9 times better (Figure 3B). Thus, the (fewer) I-SceI-generated ends are far more readily available to the homologous recombination machinery than ends produced by wild-type RAG proteins—at least 20-fold on a per-end basis.

The situation is reversed with the RAG-1 K980A and RAG-1 R838A/K839A/R840A mutants, which produce virtually the same levels of broken ends as I-SceI (Figure 3C): these mutants stimulate significantly higher levels of homologous recombination than I-SceI (Figure 3, $p < 0.05$ and $p < 0.0007$, respectively). Why would this be the case? In vitro work has shown that after cleavage by I-SceI, only one DNA end remains associated with the enzyme (Perrin et al., 1993). The most parsimonious explanation for these data is that the RAG postcleavage complex affords the greatest sequestration of DNA ends; I-SceI restricts the accessibility of ends to some degree (retaining one); and the two unstable RAG mutants fail to retain the DNA ends at all. The fact that the mutants stimulate roughly twice as much homologous recombination as I-SceI, which retains only one set of ends, dovetails nicely with this explanation.

To test the hypothesis that the two joining-deficient RAG mutants prematurely release the signal ends in vivo, we examined the stability of the mutant postcleavage complexes in vitro. After cleavage in vitro, purified RAG proteins remain tightly bound to the signal ends; disrupting this association requires harsh treatments, such as phenol extraction or heating to high temperatures (70°C) (Jones and Gellert, 2001; Leu et al., 1997; Ramsden et al., 1997), which presumably denature the bound RAG proteins. Capitalizing on previous work showing that the signal ends become available for joining by ligase only after the RAG proteins are removed (for example, by incubation at 70°C) (Jones and Gellert, 2001; Leu et al., 1997; Ramsden et al., 1997), we were able to assess postcleavage complex stability in a straightforward manner: we allowed RAG cleavage to occur, incubated the reactions at a series of temperatures ranging from 37°C–70°C to allow disruption of the postcleavage complex, then measured levels of signal end ligation (schematized in Figure 5A). As expected, wild-type postcleavage complexes do not release significant proportions of the signal ends at 37°C, 45°C, or 50°C; only at 70°C do we see quantitative end release, as measured by the ability of the two ends to be ligated (Figure 5B). (Quantitative end release by treatment at 70°C was verified in other experiments by comparison to treatment with SDS/proteinase K.) In sharp contrast, postcleavage complexes formed by K980A or R838A/K839A/R840A showed maximal release of the signal ends even at the lowest temperature tested, 37°C (Figure 5B). Repeating this experiment with different protein preparations and adding a loading control (a second set of PCR primers that amplifies a short segment from the plasmid backbone) obtained the same results (Figure 5C). Because the mutant RAG proteins have a ~5-10-fold cleavage defect in vitro (Huye et al., 2002 and data not shown), they produce fewer ligatable ends than wild-type RAG proteins, which is reflected in the data. Data from the experiment shown in Figure 5C are quantitated in Figure 5D. The convergence of the results from the CFP in vivo assay and end release in vitro lead us to conclude that these two mutants form unstable postcleavage complexes that fail to properly retain the signal ends.

It is interesting that none of our other 13 joining-deficient RAG-1 and RAG-2 mutants stimulated homologous recombination better than wild-type RAG proteins

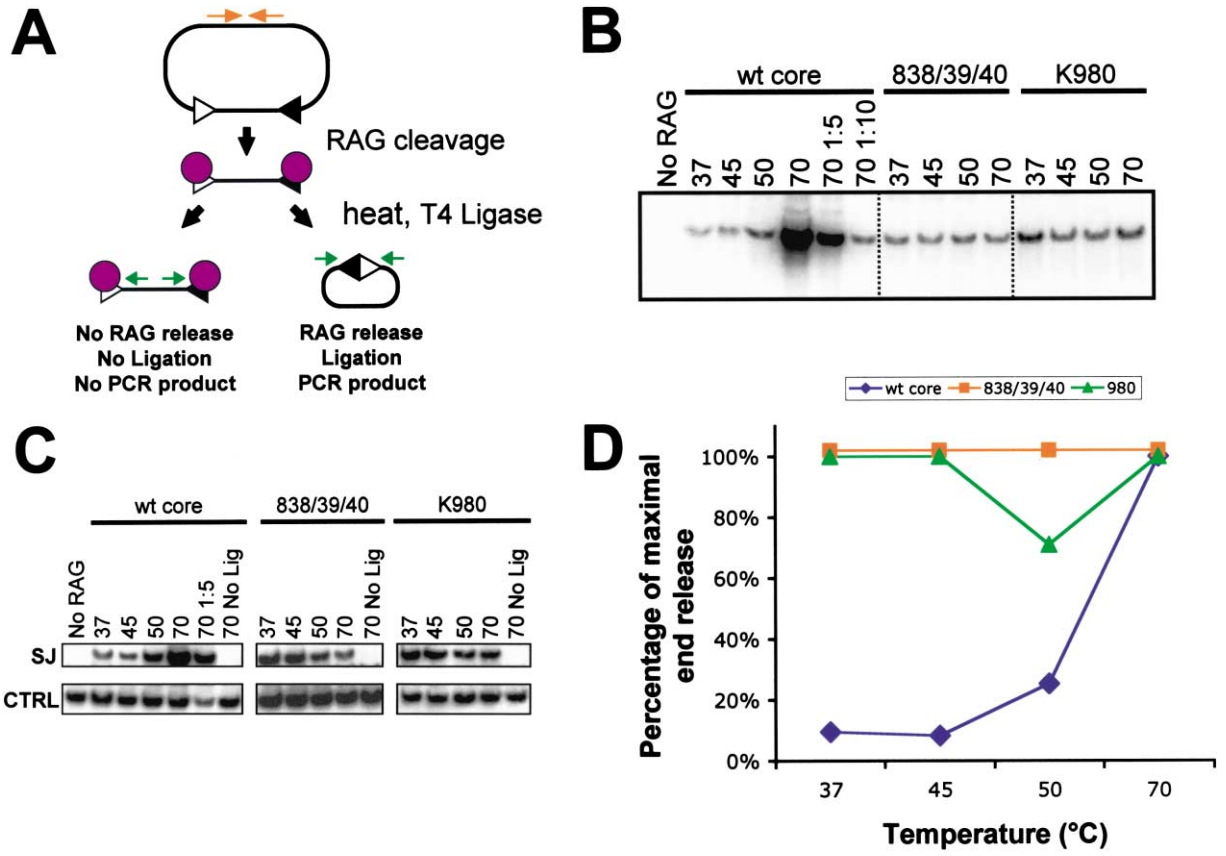

Figure 5. Joining-Deficient RAG Mutants Form an Unstable Postcleavage Complex

(A) A plasmid recombination substrate (pJH290) was subjected to in vitro cleavage reactions using purified RAG proteins. After cleavage, reactions were treated at various temperatures to measure the stability of RAG postcleavage complex binding to the signal ends (see Experimental Procedures for details). Signal end release allows ligation and generation of a PCR product.

(B) Southern blot showing signal end release by the RAG proteins as measured by the availability of ends for ligation. Wild-type RAG proteins showed little end release below 70°C, whereas joining-deficient mutant RAG proteins showed maximal levels of end release even at the lowest temperature tested. As expected, the modest cleavage defect of the mutant RAG proteins (see Figure 3C) resulted in 10-fold lower levels of signal ends overall than wild-type (Huye et al., 2002).

(C) Southern blot (from a second experiment using a different protein prep) showing signal end release by the RAG proteins (SJ). As in (B), wild-type RAG proteins showed little end release below 70°C, whereas joining-deficient mutants showed maximal end release even at the lowest temperature tested. The nonspecific control PCR products (CTRL) indicate that differences observed were not due to fluctuations in amounts of template or gel loading.

(D) Graph showing the percentage of maximal release of signal ends. Amounts of signal joint PCR product (SJ) in each lane were normalized according to the amount of control PCR product (CTRL). Percentage of maximal release was calculated as the intensity of each band over the intensity of the band generated by treatment at 70°C.

(Figure 3B and data not shown). Indeed, many actually yielded lower levels of homologous recombination than wild-type RAG proteins. This is the case with the S723A mutant, which had been reported to display defect in post-cleavage complex stability in vitro (Tsai et al., 2002). When we examined the S723A mutant in vitro, it did not show an increased tendency to release signal ends (Supplemental Figure S5). This difference in results may be explained by the fact that, in the previous study, the assay examined the stability of the coding end-signal end interaction, while we measured the RAG protein-signal end interaction. Our evidence indicates that this RAG-1 mutant, like most of our joining-deficient RAG mutants, does not increase the accessibility of the signal ends to other recombination pathways in living cells.

The Postcleavage Complex Protects Ends from Alternative NHEJ

What do these mutants tell us about the wild-type post-cleavage complex? Can we infer from the behavior of unstable postcleavage complexes—inappropriately allowing DNA ends to participate in homologous recombination— that the wild-type complex shepherds ends to the NHEJ pathway? Such an inference would be much stronger if we could examine what happens when a wild-type postcleavage complex is deprived of its normal NHEJ protein partners for completing the joining step of the reaction. As luck would have it, we and others have already performed such experiments.

Cells and extracts deficient for classical NHEJ components (Ku, XRCC4, etc,) (Baumann and West, 1998; Ka-

botyanski et al., 1998; Roth, 2002; Verkaik et al., 2002) are still able to perform end joining very efficiently, enough to allow cell survival by virtue of an ill-defined pathway known as "alternative NHEJ" (Roth, 2003). The factors that participate in alternative NHEJ are not known, but junctions formed by this repair pathway characteristically display excessive deletions and reliance on short sequence homologies (microhomologies) (Roth and Wilson, 1986). Alternative NHEJ is also quite error-prone and has been implicated in spontaneous and V(D)J recombination-induced chromosome aberrations that display the hallmark microhomologies (Ferguson et al., 2000; Zhu et al., 2002). Considering the dangers inherent in using a translocation-prone mechanism for joining V(D)J recombination intermediates, it would seem prudent for the postcleavage complex to sequester the ends from this pathway. And, indeed, this seems to be the case: whereas the efficiency of end joining of transfected linear plasmid substrates is virtually unaffected in Ku80- and XRCC4-deficient cell lines (Kabotyanski et al., 1998; Verkaik et al., 2002), joining of ends generated by RAG-mediated cleavage of essentially the same plasmid substrates is decreased more than a hundred-fold (Han et al., 1997, 1998; Taccioli et al., 1993). This result had been quite puzzling, but our present data suggest a plausible explanation: the postcleavage complex prevents RAG-generated breaks from accessing the alternative NHEJ pathway.

Our analysis of the joining mutants K980A and R838A/K839A/R840A makes this model even more compelling. These two mutants, which are the only joining-deficient mutants to appreciably stimulate homologous recombination over wild-type levels in the present study, are also the only ones to produce joints with short sequence homologies and excessive deletions (Huye et al., 2002; Qiu et al., 2001; Yarnall Schultz et al., 2001)— signatures of alternative NHEJ. These observations provide further evidence that the postcleavage complex shepherds V(D)J recombination intermediates away from not only homologous recombination but also from alternative NHEJ mechanisms. We speculate that RAG mutants deficient in this shepherding function would dramatically increase the incidence of oncogenic chromosome translocations in developing lymphocytes.

RAG-Mediated Nicks Efficiently Stimulate Homologous Recombination

We were intrigued by the observation that wild-type core and full-length RAG proteins stimulated some homologous recombination on extrachromosomal and integrated substrates (Figures 3 and 4, and see below). We considered two possible explanations for this phenomenon: either some signal ends escape from even wild-type postcleavage complexes, or other V(D)J recombination intermediates, such as nicks, might be initiating recombination. Nicks have long been hypothesized to be capable of initiating homologous exchange (Holliday, 1964; Meselson and Radding, 1975), but compelling evidence that this occurs in vivo has proved difficult to obtain.

The first hint that nicks might be the initiating lesions in our CFP assay came from the results in Figure 3B. The coding and signal end substrates are identical except for the orientation of the RSS: cleavage of the SE substrate produces blunt signal ends, but cleavage of the CE substrate yields DNA hairpins that must be opened before strand invasion can occur. The fact that the two substrates yielded similar levels of homologous recombination with wild-type core RAG proteins is counterintuitive if DSBs are the major initiators since there is an extra step involved in making the covalently sealed coding ends available for recombination that would likely decrease the efficiency of the reaction. Yet it is precisely what we would expect if nicks initiate homologous recombination since the two substrates should be equally susceptible to nicking.

To determine whether nicking could indeed lead to homologous recombination, we employed four RAG-1 nick-only mutants that efficiently perform single-strand nicking but cannot form DSB in vivo (they produce $<1\%$ of the levels of DSB-formed wild-type RAG proteins, as measured by sensitive ligation-mediated PCR assays) (Huye et al., 2002). All of these "nick-only" mutants (R855A/K856A, R894A, K890A, and R972A/K973A) stimulated homologous recombination at least as efficiently as their wild-type counterparts on the extrachromosomal SE substrate (Figure 6A); three out of four were expressed at wild-type levels according to Western blot (Supplemental Figure S3 online). Similar results were obtained with a RAG-2 nick-only mutant, K38A/R39A (Qiu et al., 2001) (data not shown). The nick-only mutants do not produce DSB detectable by Southern blotting (Figure 6B and longer exposure beneath) or ligation-mediated PCR (Huye et al., 2002). These same nick-only mutants proved even better than wild-type full-length and core RAG proteins at stimulating homologous recombination on an SE substrate stably integrated into the genome (Figure 7, right side).

Our next set of experiments took a complementary approach. Instead of using nick-only RAG proteins, we used what is, in effect, a nick-only substrate: a single 12-RSS, which readily undergoes nicking in vitro (Cuomo et al., 1996; Ramsden et al., 1996) but yields very poor DSB formation in vivo (Steen et al., 1997). Confirming our model, the 12-RSS substrate gave the same levels of homologous recombination as the SE substrate in extrachromosomal assays (Figure 6A) and yielded robust homologous recombination with full-length and core RAG proteins as an integrated substrate (15-fold over the DDE control; Figure 7, left side). Even when the nick-only mutants were assayed on the single RSS substrate, they were able to stimulate homologous recombination as efficiently as the full-length or core wild-type RAG proteins (Figure 7). Since neither of two different conditions that block DSB formation by the RAG proteins diminished the efficiency of homologous recombination, singly or in combination, we conclude that most of the homologous recombination observed with wild-type RAG proteins in this system is elicited not by DSB, but by nicks.

This conclusion receives further support from the lack of correlation between levels of DSB and homologous recombination in various situations. Whereas full-length RAG proteins produce ten-fold fewer signal ends than core proteins (Figure 3C, Supplemental Figure S4; see

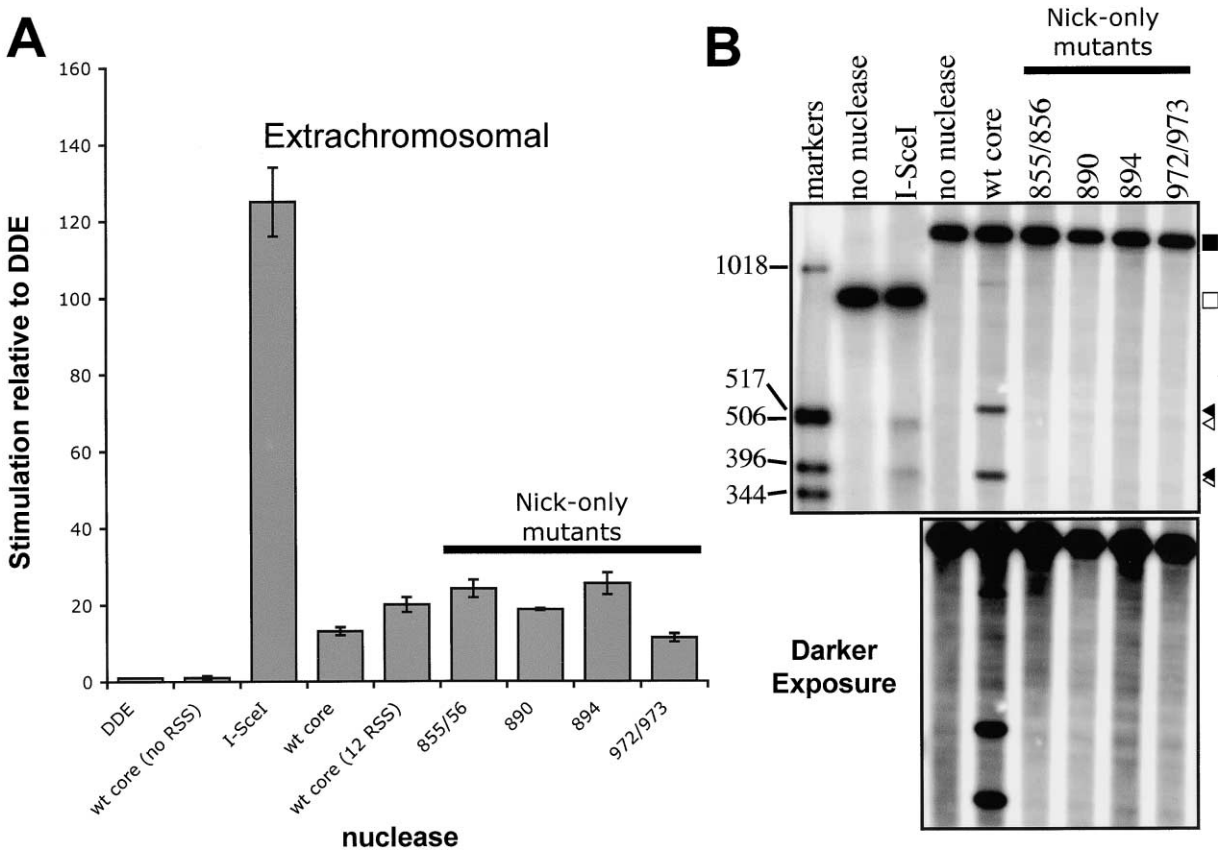

Figure 6. RAG-Generated Nicks Stimulate Homologous Recombination on Extrachromosomal Substrates

(A) Graph showing relative stimulation of CFP expression by wild-type core and nick-only mutant RAG-1 proteins (R855A/K856A, K890A, R894A, and R972A/K973A) on SE substrate, unless otherwise noted.

(B) Southern blot of HpaI- and NcoI-digested DNA recovered from cells expressing wild-type core and the same nick-only mutant RAG proteins. Cleavage products are described in Figure 2D. As expected (Huye et al., 2002), no detectable DSB formation was observed with nick-only RAG mutants. This finding was confirmed by overexposing the blot, which revealed no cleavage products generated by the nick-only RAG mutants (shown below panel B).

also Steen et al., 1999), both versions of the RAG proteins yield similar levels of homologous recombination (Figures 4 and 7). Furthermore, as noted above, I-SceI produces lower levels of DSB than core RAG proteins yet generates significantly higher levels of homologous recombination (Figure 3B), again highlighting the discordance between levels of DSB and homologous recombination initiated by the RAG proteins. These data strengthen the notion that nicks, rather than DSB, are the major RAG-induced initiators of homologous recombination in this system.

Is it possible, then, that the two unstable postcleavage complex mutants (K980A and R838A/K839A/R840A) stimulate homologous recombination not by releasing DNA ends but by nicking at extremely high levels? Three lines of evidence argue convincingly against this possibility. First, these two mutants do not nick more than wild-type proteins in vitro (Huye et al., 2002), but they stimulate far more homologous recombination than wild-type proteins or nick-only mutants. Second, neither of these mutants stimulated recombination more than wild-type core RAG proteins on the single-RSS substrate (data not shown), which allows robust nicking but does not support efficient double-strand break formation. Third, neither of these mutants stimulated recombination above wild-type levels on the CE substrate (Figure 3B), as would be expected if they were more proficient at nicking. We therefore conclude that the two mutants that destabilize the postcleavage complex in vitro stimulate homologous recombination in vivo by making the normally sequestered signal ends available.

Discussion

A Molecular Shepherd: The RAG Postcleavage Complex

The functions of the RAG postcleavage complex have been a fairly long-standing enigma. In particular, there has been confusion over whether the complex allows DNA ends to interact with other factors such as the NHEJ proteins or whether it sequesters the ends so as not to elicit cellular DNA damage responses. There has been no direct evidence either way in vivo, and the results of indirect analyses are contradictory. V(D)J recombination does not appear to activate p53-dependent DNA damage checkpoints (Guidos et al., 1996; Jones and Gellert, 2001; Nacht et al., 1996); on the other hand, V(D)J recombination intermediates can be sensed by

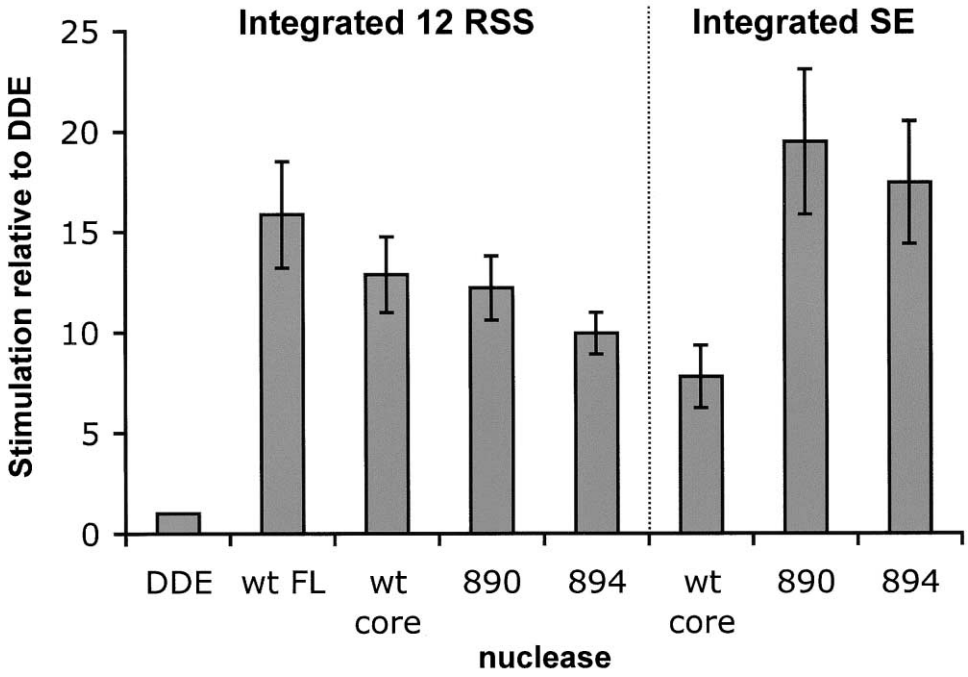

Figure 7. RAG-Generated Nicks Stimulate Homologous Recombination on Integrated Substrates

Graph showing the relative stimulation of CFP expression by full-length and wild-type core RAG proteins, as well as nick-only mutant RAG proteins, in a cell line containing the single RSS substrate stably integrated in the chromosome. To the right we show the nick-only mutants stimulating homologous recombination on an integrated SE substrate.

repair machinery, efficiently stimulating focus formation by NBS1 and γ-H2AX in thymocytes (Chen et al., 2000) and interacting with ATM (Perkins et al., 2002).

In our studies, the wild-type postcleavage complex did not allow signal ends to stimulate homologous recombination, but the two joining mutants that form an unstable postcleavage complex release these blunt ends and stimulate even higher levels of homologous recombination than the gold standard, I-SceI. It is significant that these same two mutants also form coding joints that display all the characteristics of alternative NHEJ (Huye et al., 2002). The fact that mutations in the RAG genes can cause joining defects that so closely resemble those of NHEJ mutants had led us to propose in previous work that the RAG proteins, like NHEJ factors, help prevent aberrant handling of broken ends, reducing the incidence of oncogenic chromosome rearrangements—i.e., that the RAG proteins serve as both a recombinase and a genome guardian in developing lymphocytes (Brandt and Roth, 2002; Huye et al., 2002; Roth, 2003). We have now provided strong in vivo evidence that the RAG postcleavage complex serves a more dynamic role, guiding the DNA ends to the proper NHEJ repair pathway (Figure 8A). The RAG proteins are thus an archetype for a new category of molecule: molecular shepherds that guide DSB to a particular repair pathway.

We propose that mutations that destabilize the postcleavage complex, allowing premature release of signal or coding ends, could cause genomic instability in developing lymphocytes (Figure 8B). It is worth noting that one of the amino acids altered in one of our mutants that releases signal ends (R838) corresponds to an amino acid mutated (R841W) in a form of human-inherited immunodeficiency, atypical SCID/Omenn syndrome (Villa et al., 2001). It is also striking that the two mutations that increase the accessibility of signal ends, K980A and R838A/K839A/R840A, both reside near the C terminus of the catalytic core of RAG-1. This region of RAG-1 could be especially important for retaining the signal ends in the postcleavage complex, and these two mutants could prove to be useful tools for stimulating homologous recombination.

Evidence that Nicks Initiate Homologous Recombination

Previous studies have implicated site-specific nicks in stimulating homologous recombination in *S. cerevisiae* (using a coliphage nicking enzyme) (Strathern et al., 1991) and during mating type switching in fission yeast (Arcangioli, 1998). In neither instance could it be determined whether nicks themselves or subsequent DSB stimulate homologous recombination; in the case of *S. pombe* mating type switching, it was suggested that the initiating lesions are DSB generated from the nicks by DNA replication (Arcangioli and de Lahondes, 2000). We now present five lines of evidence suggesting that RAG-generated nicks initiate homologous recombination in mammalian cells.

First, wild-type RAG proteins produced similar levels of homologous recombination on both the CE and SE substrates, despite the fact that coding end hairpins must be opened before the coding ends can be available for recombination. Second, nick-only RAG mutants

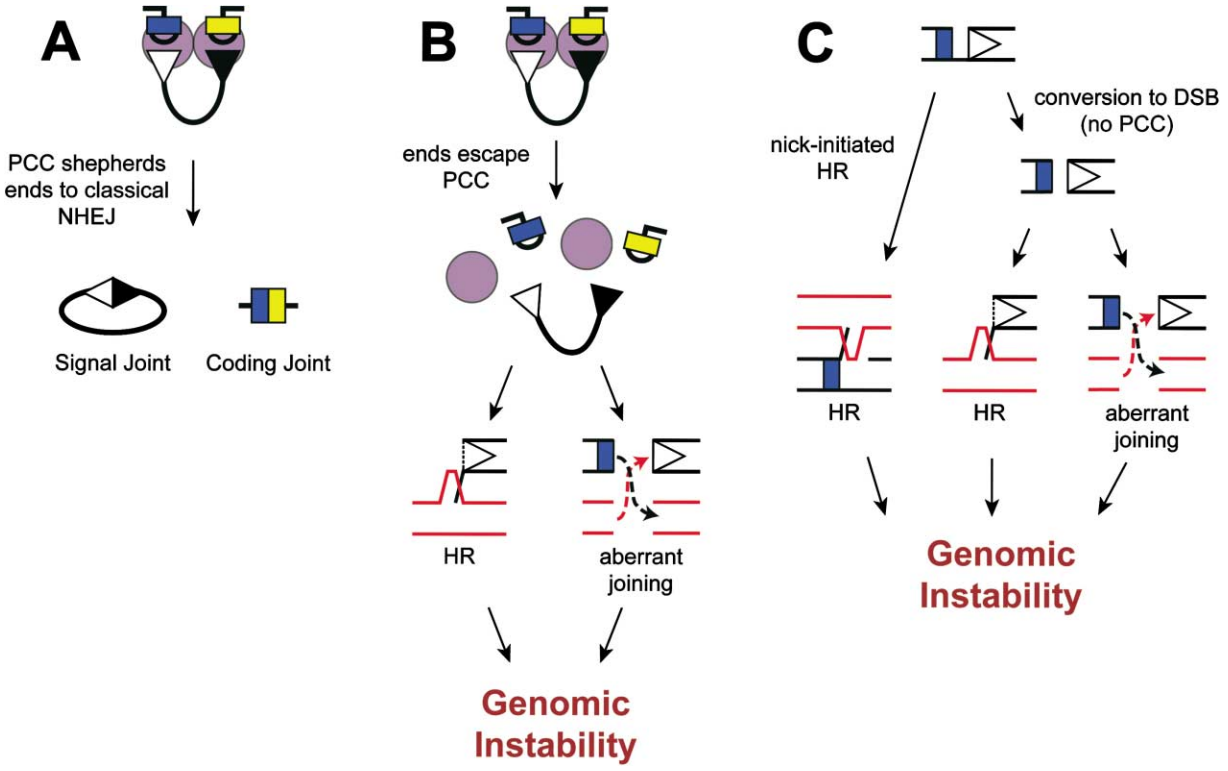

Figure 8. Models for RAG-Mediated Genomic Instability

(A) In normal V(D)J recombination, the postcleavage complex shepherds the broken DNA ends to the classical NHEJ repair pathway, protecting the genome from potentially dangerous aberrant recombination events.

(B) If the broken DNA ends created during V(D)J recombination escape the postcleavage complex, they become free to participate in homologous recombination with an ectopic partner after some end processing (indicated by a dotted line at the end of the RSS triangle) followed by crossing over (HR). Alternatively, the ends could join with a break on another chromosome created by other means (aberrant joining). We propose that the poorly characterized alternative NHEJ pathway is involved in such aberrant joining events and could promote chromosomal translocations.

(C) RAG-generated nicks might also lead to genomic instability, either by initiating homologous recombination with an ectopic partner or through conversion to a DSB that could then initiate HR or participate in other aberrant joining reactions. These DSB might be particularly reactive, as they would lack the protection of the RAG postcleavage complex. Note that these models are not mutually exclusive.

stimulated homologous recombination as effectively as wild-type RAG proteins, despite their inability to form detectable DSB. Third, wild-type RAG proteins produced similar levels of homologous recombination on both the SE substrate and the single RSS "nick-only" substrate, which does not support efficient DSB formation in vivo. Fourth, if RAG-mediated DSB were the initiating lesions, there should be a correlation between DSB formation and stimulation of homologous recombination. Yet full-length RAG proteins, which generate far lower levels of signal ends than the core proteins (in agreement with previously published work [Steen et al., 1999]), produced similar levels of homologous recombination on integrated substrates as core RAG proteins (Figures 4 and 7). Fifth (but related to the last point), I-SceI generates fewer DSBs than the RAG proteins, but we observe substantially *more* homologous recombination with I-SceI than with wild-type RAG proteins (Figure 3B).

Finally, we cannot rule out the possibility that nicks are being converted to DSB by some non-RAG means such as DNA replication or nonspecific nuclease activity. As suggested by in vitro experiments (Grawunder and Lieber, 1997), nicks might be protected from repair through the continued presence of RAG-1 and RAG-2, allowing cell cycle progression and formation of a replication-induced DSB. This could occur in the presence of normal cell cycle checkpoints because the continued presence of RAG proteins could mask the nick; of course, the situation might be worsened in cells defective for proper checkpoint controls. In another scenario, replication-mediated conversion of nicks to DSB could result from mis-expression of RAG proteins during S phase, which might occasionally occur in developing lymphocytes. If such DSB are formed, they are likely to be short-lived, extensively processed, or both since we have been unable to detect them by Southern blotting (Figure 6B) or by sensitive ligation-mediated PCR assays (Huye et al., 2002). Furthermore, if nicks were being efficiently converted into DSB, one might expect our nick-only mutants to give rise to coding and/or signal joints, but none were observed by PCR-based (Huye et al., 2002) or sensitive fluorescent reporter assays (G.L. and D.R., unpublished observations). Thus, although our experiments do not prove that nicks initiate homologous recombination directly, they do raise the possibility that

early models of nick-induced homologous recombination (Holliday, 1964; Meselson and Radding, 1975) may be relevant for mammalian cells. If nicks can induce homologous recombination without passing through DSB intermediates, the ability of mutant RAG proteins to generate highly reactive, site-specific nicks in living cells could have interesting practical applications, such as in gene targeting experiments. As discussed below, however, even if the homologous recombination we observe is caused by conversion of some nicks to DSB (albeit at a low frequency that escapes our current detection methods), the ability of these RAG-induced lesions to stimulate homologous recombination has interesting implications for genomic instability.

New Models for Oncogenic Rearrangements in Developing Lymphocytes

If nicks are converted to DSB by some nonspecific (non-RAG-mediated) mechanism, the resulting DSB would, by definition, lack the protection of a RAG postcleavage complex. Given the results we obtained with the two RAG mutants that prematurely release ends, such DSB would be more likely to engage in aberrant joining events than normal signal or coding ends (Figures 8B and 8C). Thus, RAG-mediated nicks—whether they stimulate recombination directly or after conversion to DSB—can no longer be considered inconsequential intermediates that pose no threat to genomic stability.

The Alt group has speculated that RAG-generated DSB might initiate homologous recombination between similar sequences located on nonhomologous chromosomes and thereby cause oncogenic translocations (Ferguson and Alt, 2001; Zhu et al., 2002) (see Figure 1, Ferguson and Alt, 2001). Although we are unaware of evidence supporting this notion, our data indicate that such events could be stimulated by RAG-generated nicks—whether they are first converted to DSB lacking the protection of a postcleavage complex or not (Figure 8C). Our results also suggest that RAG-mediated DSB could initiate homologous recombination or aberrant repair by other pathways in the case of certain mutations or conditions that destabilize the postcleavage complex (Figure 8B). Of course, free DSB—generated either by release of ends from the postcleavage complex or by conversion of RAG-mediated nicks to DSB through passage of a replication fork—could participate in oncogenic rearrangements by mechanisms other than homologous recombination, such as alternative NHEJ (Figures 8B and 8C).

The recombinogenic potential of RAG-induced nicks sheds new light on a long-standing puzzle regarding the genesis of chromosomal translocations. The presence of RSS-like sequences ("pseudo-RSS") at translocation breakpoints in lymphoid neoplasms has been noted for many years and led to the hypothesis that the RAG proteins might mistake these sequences for true RSS and thereby initiate V(D)J recombination with the wrong substrate (reviewed in Roth, 2003). The chief weakness of this "substrate selection error" model has been that many of the pseudo-RSS deviate so far from the consensus that they form extremely poor substrates for DSB formation by the standard V(D)J recombination mechanism (Raghavan et al., 2001; Ramsden et al., 1996; Steen et al., 1997). Moreover, the breakpoints are often not located precisely at these pseudo-RSS, as would be expected if breakage were mediated by a standard RAG cleavage event. These facts have led many to consider the presence of nonconsensus RSS near translocation breakpoints to be merely coincidental (Lewis, 1994; Tycko and Sklar, 1990; Vanasse et al., 1999), but our studies provide another possible explanation for these observations: RAG-generated nicks could be the initiating lesions. Although many pseudo-RSS, including isolated heptamer and nonamer sequences, do not support DSB formation, they form excellent substrates for nicking (Ramsden et al., 1996). Furthermore, nicks at pseudo-RSS can occur several nucleotides from the normal cleavage site (Cuomo et al., 1996; Ramsden et al., 1996), and further processing of unprotected DSB that derive from RAG-induced nicks could explain breakpoints located a short distance from the pseudo-RSS. We certainly do not propose that all translocations arise via RAG-mediated nicking, but it is conceivable that the RAG proteins might nick a subset of pseudo-RSS and initiate aberrant rearrangements. The ability of full-length RAG proteins to robustly stimulate homologous recombination from an integrated 12 RSS substrate (Figure 7) raises the possibility that RAG-induced nicks at isolated RSS or pseudo-RSS could contribute to aberrant rearrangements.

Evolutionary Implications of RAG-Initiated Homologous Recombination

A widely accepted model for the evolution of the somatically rearranging antigen receptor genes invokes an ancestral, RAG-mediated transposition event that interrupted a preexisting, nonrearranging receptor gene, leading to a split gene that is assembled by transposon excision (V(D)J recombination) (Agrawal et al., 1998; Hiom et al., 1998; Lewis and Wu, 2000; Roth, 2000; Sakano et al., 1979; Thompson, 1995). This eminently reasonable conjecture leaves unanswered the question of how complex families of rearranging gene segments—e.g., containing up to several hundred V regions—might have arisen. There must have been some duplication and diversification events, after which the loci became integrated into several chromosomal locations to form three discrete immunoglobulin loci and four T cell receptor loci. How might this have occurred?

Several lines of evidence, including the discovery of "pre-rearranged" antigen receptor genes and the presence of RAG-1 and RAG-2 mRNA in germ cells, strongly suggest that the RAG proteins can cause rearrangements in the germline (discussed in Lee et al., 2000; Lewis and Wu, 2000; Roth, 2000). These data have led to speculations that the V(D)J recombinase may have played two distinct roles in shaping vertebrate evolution: catalyzing V(D)J rearrangements (to form pre-rearranged antigen receptor genes) and causing other rearrangements by transposition (Lee et al., 2000; Lewis and Wu, 2000; Roth, 2000). Our work suggests a third possibility: that RAG-initiated homologous recombination could lead to gene duplication events and perhaps other germline rearrangements. Thus, these remarkably versatile proteins might not only be central to the process of generating antigen receptor diversity in somatic cells, but may

also have played multiple roles in the evolution of antigen receptor loci themselves by catalyzing germline gene rearrangements.

Shepherds, Guardians, and DSB Repair

The discovery of a specific mechanism to guide DSBs into a particular repair pathway highlights the dynamic nature of the RAG postcleavage complex. Without the RAG proteins directing DNA ends to the proper, high-fidelity joining pathway, the NHEJ factors, "genome guardians" though they may be, would not be able to serve their protective function, at least not in the context of V(D)J recombination. The shepherding activities of the V(D)J recombinase could provide a paradigm for repair of DSB in other contexts. For example, chromatin proteins may serve to restrict access of broken ends to the alternative NHEJ pathway. It is tempting to speculate that similar shepherding complexes might be at work in other processes in which it would be advantageous to channel site-specific DNA strand breaks to particular repair pathways, such as meiotic recombination, yeast mating type switching, somatic hypermutation of immunoglobulin variable region genes, and immunoglobulin class switching. Further exploration of the processes that restrict the choice of repair pathways available to particular DSBs should provide important insights into mechanisms that maintain genomic stability.

Experimental Procedures

For details on Generation of Substrates, Flow Cytometry, Generation of Integrated Substrates, and Thermal Stability assay, please see Supplemental Experimental Procedures online at http://www.cell.com/cgi/content/full/117/2/171/DC1.

Cell Culture

RMP41 fibroblasts were grown in DMEM (Invitrogen) supplemented with fetal bovine serum (10%), nonessential amino acids, and penicillin-streptomycin. Cells were grown at 37°C in the presence of 5% CO_2. Cells containing integrated substrates were selected and grown in the presence of 1.5 μg/ml Geneticin (Gibco).

Protein Expression

Truncated RAG proteins were expressed in the pEBG-1N vector (RAG-1) (Spanopoulou et al., 1996) and the pMal-1 vector (RAG-2) (Landree et al., 1999). The I-SceI homing endonucleases, a generous gift of Dr. Maria Jasin, were expressed in the pCMV-ISceI vector (Rouet et al., 1994). Mutations in the core RAG genes producing a nick-only or joining-deficient phenotype are indicated by residue number and have been previously characterized (Huye et al., 2002; Qiu et al., 2001; Yarnall Schultz et al., 2001). The DDE mutant (D600A/D708A/E962A) is a catalytically dead RAG-1 mutant used as a control (Landree et al., 1999).

Transient Transfections

CHO (RMP41) fibroblasts were grown to 50% confluency in 96-well plates (Corning). Transfections were done in groups of 8 wells that were pooled upon harvesting. Each transfection contained 800 ng of each plasmid (substrate as indicated, and either I-SceI or RAG expression vectors as indicated), 240 μl of serum-free DMEM, and 4 μl of Fugene transfection reagent (Roche). When using cell lines containing integrated substrates, substrate plasmids were omitted, and Fugene was reduced to 3 μl. As measures of consistent transfection efficiency, we show the following: (1) error bars show the data to be highly reproducible; (2) Western blots show consistent expression of RAG mutant proteins (Supplemental Figure S3 on *Cell* website); (3) recovery of transfected substrate DNA is consistent (see Supplemental Figure S4).

Fluorescence Microscopy

Forty-eight hours after transfection, cells were trypsinized, resuspended, and placed in Lab-Tek II 4-chambered slides (Nalge Nunc International). Cells were visualized using a Leica DMIRB inverted fluorescence microscope. Pictures were taken using an Axiocam HR and Axiovision software (Zeiss).

Southern Blots

DNA was prepared from cells by the Hirt method we have used previously (Steen et al., 1996). DNA was digested using HpaI and NcoI for 2 hr, then run on a 2% native agarose gel. DNA was transferred to Genescreen membrane using a posiblotter (Stratagene). The DNA was then crosslinked to the membrane by UV irradiation. Probes were generated with a random priming DNA labeling kit (Gibco) using a gel-purified HpaI-NcoI fragment from the pECFP-int vector as a template. Probes were hybridized to blots overnight at 65°C in a solution of 10% Dextran Sulfate, 1 M NaCl and 1% SDS. Blots were visualized using a Phosphorimager and ImageQuant Software (Molecular Dynamics).

Acknowledgments

The authors are grateful to Mike Dustin, Fred Gimble, Ellen Hsu, Dan Littman, Richard Novick, John Petrini, Gerald Smith, Susan Smith (no relation), Steve West, John Wilson, and members of the Roth lab for stimulating discussions, and to our anonymous reviewers for insightful comments. We thank Jennifer Posey for preparing S723A protein, Anna Miller for helpful discussions and unwavering support, and Takeshi Egawa, John Hirst, Dorothy Lewis, and Jeff Scott for assistance with flow cytometry. Vicky Brandt shepherded the manuscript toward greater clarity through many drafts. This work was supported by the Irene Diamond Foundation; the initial generation of the RAG mutations studied here was supported by a grant from the NIH (AI36420).

Received: September 10, 2003
Revised: January 30, 2004
Accepted: February 12, 2004
Published Online: April 1, 2004

References

Agrawal, A., and Schatz, D.G. (1997). RAG1 and RAG2 form a stable postcleavage synaptic complex with DNA containing signal ends in V(D)J recombination. Cell *89*, 43–53.

Agrawal, A., Eastman, Q.M., and Schatz, D.G. (1998). Transposition mediated by RAG1 and RAG2 and its implications for the evolution of the immune system. Nature *394*, 744–751.

Arcangioli, B. (1998). A site- and strand-specific DNA break confers asymmetric switching potential in fission yeast. EMBO J. *17*, 4503–4510.

Arcangioli, B., and de Lahondes, R. (2000). Fission yeast switches mating type by a replication-recombination coupled process. EMBO J. *19*, 1389–1396.

Baumann, P., and West, S.C. (1998). DNA end-joining catalyzed by human cell-free extracts. Proc. Natl. Acad. Sci. USA *95*, 14066–14070.

Brandt, V.L., and Roth, D.B. (2002). A recombinase diversified: new functions of the RAG proteins. Curr. Opin. Immunol. *14*, 224–229.

Chen, H.T., Bhandoola, A., Difilippantonio, M.J., Zhu, J., Brown, M.J., Tai, X., Rogakou, E.P., Brotz, T.M., Bonner, W.M., Ried, T., and Nussenzweig, A. (2000). Response to RAG-mediated VDJ cleavage by NBS1 and gamma-H2AX. Science *290*, 1962–1965.

Cuomo, C.A., Mundy, C.L., and Oettinger, M.A. (1996). DNA sequence and structure requirements for cleavage of V(D)J recombination signal sequences. Mol. Cell. Biol. *16*, 5683–5690.

Fabre, F. (1978). Induced intragenic recombination in yeast can occur during the G1 mitotic phase. Nature *272*, 795–798.

Ferguson, D.O., and Alt, F.W. (2001). DNA double strand break repair and chromosomal translocation: lessons from animal models. Oncogene *20*, 5572–5579.

Ferguson, D.O., Sekiguchi, J.M., Chang, S., Frank, K.M., Gao, Y., DePinho, R.A., and Alt, F.W. (2000). The nonhomologous end-joining pathway of DNA repair is required for genomic stability and the suppression of translocations. Proc. Natl. Acad. Sci. USA *97*, 6630–6633.

Frank-Vaillant, M., and Marcand, S. (2002). Transient stability of DNA ends allows nonhomologous end joining to precede homologous recombination. Mol. Cell *10*, 1189–1199.

Goedecke, W., Eijpe, M., Offenberg, H.H., van Aalderen, M., and Heyting, C. (1999). Mre11 and Ku70 interact in somatic cells, but are differentially expressed in early meiosis. Nat. Genet. *23*, 194–198.

Grawunder, U., and Lieber, M.R. (1997). A complex of RAG-1 and RAG-2 proteins persists on DNA after single-strand cleavage at V(D)J recombination signal sequences. Nucleic Acids Res. *25*, 1375–1382.

Guidos, C.J., Williams, C.J., Grandal, I., Knowles, G., Huang, M.T.F., and Danska, J.S. (1996). V(D)J recombination activates a p53-dependent DNA damage checkpoint in scid lymphocyte precursors. Genes Dev. *10*, 2038–2054.

Haber, J.E. (1999). DNA repair. Gatekeepers of recombination. Nature *398*, 665–667.

Han, J.-O., Steen, S.B., and Roth, D.B. (1997). Ku86 is not required for protection of signal ends or for formation of nonstandard V(D)J recombination products. Mol. Cell. Biol. *17*, 2226–2234.

Han, J.-O., Erskine, L.A., Purugganan, M.M., Stamato, T.D., and Roth, D.B. (1998). V(D)J recombination intermediates and non-standard products in XRCC4-deficient cells. Nucleic Acids Res. *26*, 3769–3775.

Hiom, K., and Gellert, M. (1998). Assembly of a 12/23 paired signal complex: A critical control point in V(D)J recombination. Mol. Cell *1*, 1011–1019.

Hiom, K., Melek, M., and Gellert, M. (1998). DNA transposition by the RAG1 and RAG2 proteins: a possible source of oncogenic translocations. Cell *94*, 463–470.

Holliday, R. (1964). A mechanism for gene conversion in fungi. Genet. Res. Camb. *5*, 282–304.

Huye, L.E., Purugganan, M.M., Jiang, M.M., and Roth, D.B. (2002). Mutational analysis of all conserved basic amino acids in RAG-1 reveals catalytic, step arrest, and joining-deficient mutants in the V(D)J recombinase. Mol. Cell. Biol. *22*, 3460–3473.

Jasin, M. (1996). Genetic manipulation of genomes with rare-cutting endonucleases. Trends Genet. *12*, 224–228.

Jones, J.M., and Gellert, M. (2001). Intermediates in V(D)J recombination: a stable RAG1/2 complex sequesters cleaved RSS ends. Proc. Natl. Acad. Sci. USA *98*, 12926–12931.

Kabotyanski, E.B., Gomelsky, L., Han, J.-O., Stamato, T.D., and Roth, D.B. (1998). Double-strand break repair in Ku86- and XRCC4-deficient cells. Nucleic Acids Res. *26*, 5333–5342.

Landree, M.A., Wibbenmeyer, J.A., and Roth, D.B. (1999). Mutational analysis of RAG-1 and RAG-2 identifies three active site amino acids in RAG-1 critical for both cleavage steps of V(D)J recombination. Genes Dev. *13*, 3059–3069.

Lee, S.S., Fitch, D., Flajnik, M.F., and Hsu, E. (2000). Rearrangement of immunoglobulin genes in shark germ cells. J. Exp. Med. *191*, 1637–1648.

Leu, T.M., Eastman, Q.M., and Schatz, D.G. (1997). Coding joint formation in a cell-free V(D)J recombination system. Immunity *7*, 303–314.

Lewis, S.M. (1994). The mechanism of V(D)J joining: Lessons from molecular, immunological and comparative analyses. Adv. Immunol. *56*, 27–150.

Lewis, S.M., and Wu, G.E. (2000). The old and the restless. J. Exp. Med. *191*, 1631–1636.

Meselson, M.S., and Radding, C.M. (1975). A general model for genetic recombination. Proc. Natl. Acad. Sci. USA *72*, 358–361.

Moynahan, M.E., Pierce, A.J., and Jasin, M. (2001). BRCA2 is required for homology-directed repair of chromosomal breaks. Mol. Cell *7*, 263–272.

Nacht, M., Strasser, A., Chan, Y.R., Harris, A.W., Schlissel, M., Bronson, R.T., and Jacks, T. (1996). Mutations in the p53 and SCID genes cooperate in tumorigenesis. Genes Dev. *10*, 2055–2066.

Pâques, F., and Haber, J.E. (1999). Multiple pathways of recombination induced by double-strand breaks in Saccharomyces cerevisiae. Microbiol. Mol. Biol. Rev. *63*, 349–404.

Perkins, E.J., Nair, A., Cowley, D.O., Van Dyke, T., Chang, Y., and Ramsden, D.A. (2002). Sensing of intermediates in V(D)J recombination by ATM. Genes Dev. *16*, 159–164.

Perrin, A., Buckle, M., and Dujon, B. (1993). Asymmetrical recognition and activity of the I-SceI endonuclease on its site and on intron-exon junctions. EMBO J. *12*, 2939–2947.

Pierce, A.J., Johnson, R.D., Thompson, L.H., and Jasin, M. (1999). XRCC3 promotes homology-directed repair of DNA damage in mammalian cells. Genes Dev. *13*, 2633–2638.

Pierce, A.J., Hu, P., Han, M., Ellis, N., and Jasin, M. (2001). Ku DNA end-binding protein modulates homologous repair of double-strand breaks in mammalian cells. Genes Dev. *15*, 3237–3242.

Qiu, J.X., Kale, S.B., Yarnell Schultz, H., and Roth, D.B. (2001). Separation-of-function mutants reveal critical roles for RAG2 in both the cleavage and joining steps of V(D)J recombination. Mol. Cell *7*, 77–87.

Raghavan, S.C., Kirsch, I.R., and Lieber, M.R. (2001). Analysis of the V(D)J recombination efficiency at lymphoid chromosomal translocation breakpoints. J. Biol. Chem. *276*, 29126–29133.

Ramsden, D.A., McBlane, J.F., van Gent, D.C., and Gellert, M. (1996). Distinct DNA sequence and structure requirements for the two steps of V(D)J recombination signal cleavage. EMBO J. *15*, 3197–3206.

Ramsden, D.A., Paull, T.T., and Gellert, M. (1997). Cell-free V(D)J recombination. Nature *388*, 488–491.

Richardson, C., and Jasin, M. (2000). Coupled homologous and nonhomologous repair of a double-strand break preserves genomic integrity in mammalian cells. Mol. Cell. Biol. *20*, 9068–9075.

Ristic, D., Modesti, M., Kanaar, R., and Wyman, C. (2003). Rad52 and Ku bind to different DNA structures produced early in double-strand break repair. Nucleic Acids Res. *31*, 5229–5237.

Roth, D.B. (2000). From lymphocytes to sharks: V(D)J recombinase moves to the germline. Genome Biol. *1*, 1011–1014.

Roth, D.B. (2002). Amplifying mechanisms of lymphomagenesis. Mol. Cell *10*, 1–2.

Roth, D.B. (2003). Restraining the V(D)J recombinase. Nat. Rev. Immunol. *3*, 656–666.

Roth, D.B., and Wilson, J.H. (1985). Relative rates of homologous and nonhomologous recombination in transfected DNA. Proc. Natl. Acad. Sci. USA *82*, 3355–3359.

Roth, D.B., and Wilson, J.H. (1986). Nonhomologous recombination in mammalian cells: role for short sequence homologies in the joining reaction. Mol. Cell. Biol. *6*, 4295–4304.

Rouet, P., Smith, F., and Jasin, M. (1994). Introduction of double-strand breaks into the genome of mouse cells by expression of a rare-cutting endonuclease. Mol. Cell. Biol. *14*, 8096–8106.

Sadofsky, M.J., Hesse, J.E., and Gellert, M. (1994a). Definition of a core region of RAG-2 that is functional in V(D)J recombination. Nucleic Acids Res. *22*, 1805–1809.

Sadofsky, M.J., Hesse, J.E., McBlane, J.F., and Gellert, M. (1994b). Expression and V(D)J recombination activity of mutated RAG-1 proteins. Nucleic Acids Res. *22*, 550.

Sakano, H., Huppi, K., Heinrich, G., and Tonegawa, S. (1979). Sequences at the somatic recombination sites of immunoglobulin light-chain genes. Nature *280*, 288–294.

Spanopoulou, E., Zaitseva, F., Wang, F.-H., Santagata, S., Baltimore, D., and Panayotou, G. (1996). The homeodomain region of Rag-1 reveals the parallel mechanisms of bacterial and V(D)J recombination. Cell *87*, 263–276.

Steen, S.B., Gomelsky, L., and Roth, D.B. (1996). The 12/23 rule is enforced at the cleavage step of V(D)J recombination in vivo. Genes Cells *1*, 543–553.

Steen, S.B., Gomelsky, L., Speidel, S.L., and Roth, D.B. (1997). Initia-

tion of V(D)J recombination in vivo: role of recombination signal sequences in formation of single and paired double-strand breaks. EMBO J. *16*, 2656–2664.

Steen, S.B., Han, J.-O., Mundy, C., Oettinger, M.A., and Roth, D.B. (1999). Roles of the "dispensable" portions of RAG-1 and RAG-2 in V(D)J recombination. Mol. Cell. Biol. *19*, 3010–3017.

Strathern, J.N., Weinstock, K.G., Higgins, D.R., and McGill, C.B. (1991). A novel recombinator in yeast based on gene II protein from bacteriophage f1. Genetics *127*, 61–73.

Taccioli, G.E., Rathbun, G., Oltz, E., Stamato, T., Jeggo, P.A., and Alt, F.W. (1993). Impairment of V(D)J recombination in double-strand break repair mutants. Science *260*, 207–210.

Takata, M., Sasaki, M.S., Sonoda, E., Morrison, C., Hashimoto, M., Utsumi, H., Yamaguchi-Iwai, Y., Shinohara, A., and Takeda, S. (1998). Homologous recombination and non-homologous end-joining pathways of DNA double-strand break repair have overlapping roles in the maintenance of chromosomal integrity in vertebrate cells. EMBO J. *17*, 5497–5508.

Thompson, C.B. (1995). New insights into V(D)J recombination and its role in the evolution of the immune system. Immunity *3*, 531–539.

Tsai, C.L., Drejer, A.H., and Schatz, D.G. (2002). Evidence of a critical architectural function for the RAG proteins in end processing, protection, and joining in V(D)J recombination. Genes Dev. *16*, 1934–1949.

Tycko, B., and Sklar, J. (1990). Chromosomal translocations in lymphoid neoplasia: a reappraisal of the recombinase model. Cancer Cells *2*, 1–8.

Van Dyck, E., Stasiak, A.Z., Stasiak, A., and West, S.C. (1999). Binding of double-strand breaks in DNA by human Rad52 protein. Nature *398*, 728–731.

Vanasse, G.J., Concannon, P., and Willerford, D.M. (1999). Regulated genomic instability and neoplasia in the lymphoid lineage. Blood *94*, 3997–4010.

Verkaik, N.S., Esveldt-van Lange, R.E., van Heemst, D., Bruggenwirth, H.T., Hoeijmakers, J.H., Zdzienicka, M.Z., and van Gent, D.C. (2002). Different types of V(D)J recombination and end-joining defects in DNA double-strand break repair mutant mammalian cells. Eur. J. Immunol. *32*, 701–709.

Villa, A., Sobacchi, C., Notarangelo, L.D., Bozzi, F., Abinun, M., Abrahamsen, T.G., Arkwright, P.D., Baniyash, M., Brooks, E.G., Conley, M.E., et al. (2001). V(D)J recombination defects in lymphocytes due to RAG mutations: severe immunodeficiency with a spectrum of clinical presentations. Blood *97*, 81–88.

Wake, C.T., Gudewicz, T., Porter, T., White, A., and Wilson, J.H. (1984). How damaged is the biologically active subpopulation of transfected DNA? Mol. Cell. Biol. *4*, 387–398.

Yarnall Schultz, H., Landree, M.A., Qiu, J.X., Kale, S.B., and Roth, D.B. (2001). Joining-deficient RAG1 mutants block V(D)J recombination in vivo and hairpin opening in vitro. Mol. Cell *7*, 65–75.

Zhu, C., Bogue, M.A., Lim, D.-S., Hasty, P., and Roth, D.B. (1996). Ku86-deficient mice exhibit severe combined immunodeficiency and defective processing of V(D)J recombination intermediates. Cell *86*, 379–389.

Zhu, C., Mills, K.D., Ferguson, D.O., Lee, C., Manis, J., Fleming, J., Gao, Y., Morton, C.C., and Alt, F.W. (2002). Unrepaired DNA breaks in p53-deficient cells lead to oncogenic gene amplification subsequent to translocations. Cell *109*, 811–821.

Note Added in Proof

Raghavan et al. have just published data indicating that RAG recognition of an altered DNA structure might be the basis for some chromosomal translocations (Nature, 2004, *428*, 88–93). The authors examined a common breakpoint sequence and found that RAGs can nick one strand in vitro. In light of our current work showing that RAG-induced nicks can, in fact, initiate recombination in vivo and our previous work showing that RAGs do indeed prefer certain distorted DNA structures (Lee et al., 2002, Mol. Cell. Biol.), the notion that RAG nicking might contribute to oncogenic chromosome translocations is certainly worthy of further investigation.