Cell Cycle-dependent Accumulation in Vivo of Transposition-competent Complexes between Recombination Signal Ends and Full-length RAG Proteins*

Received for publication, October 13, 2003, and in revised form, December 3, 2003
Published, JBC Papers in Press, December 4, 2003, DOI 10.1074/jbc.M311219200

Hao Jiang, Ashley E. Ross‡, and Stephen Desiderio§

From the Department of Molecular Biology and Genetics, Program in Immunology, Institute for Cell Engineering, and Howard Hughes Medical Institute, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

V(D)J recombination is initiated by a specialized transposase consisting of RAG-1 and RAG-2. Because full-length RAG proteins are insoluble under physiological conditions, most previous analyses of RAG activity in vitro have used truncated core RAG-1 and RAG-2 fragments. These studies identified an intermediate in V(D)J recombination, the signal end complex (SEC), in which core RAG proteins remain associated with recombination signal sequences at the cleaved signal ends. From transfected cells expressing affinity-tagged RAG proteins, we have isolated in vivo assembled SECs containing full-length RAG proteins and cleaved recombination substrates. SEC formation in vivo did not require the repair proteins DNA-dependent protein kinase, Ku80, or XRCC4. In the presence of full-length RAG-2, SEC formation in vivo was cell cycle-regulated and restricted to the G0/G1 phases. In contrast, complexes accumulated throughout cell cycle in cells expressing a RAG-2 CDR2 phosphorylation site mutant. Both core and full-length SECs supported transposition in vitro with similar efficiencies. Intracellular SECs, which are likely to persist in the absence of coding ends, represent potential donors whose transposition is not suppressed by the non-core regions of the RAG proteins.

Antigen receptor genes are encoded in separate DNA segments that are brought together during lymphoid development by V(D)J recombination, the only form of site-specific DNA recombination known in vertebrates and the principal means by which immunologic diversity is generated in mammals (1). RAG-1 and RAG-2, the sole lymphoid-specific components of the recombinase machinery (2, 3), initiate V(D)J recombination by cleaving participating gene segments at specific recombination signal sequences (RSSs).1 DNA cleavage produces two signal ends, terminating in flush double-stranded breaks, and two coding ends, terminating in hairpin structures (4). Recombination is then completed by components of the general cellular machinery for non-homologous DNA end joining (NHEJ) including Ku70, Ku80, Artemis, DNA-PKcs, XRCC4, and DNA ligase IV (1). The coding and signal ends generated by RAG-mediated DNA cleavage are resolved discordantly in vivo (5–7). Although coding ends are repaired quickly, signal ends are relatively long-lived. Whereas genetic approaches have proven powerful in identifying proteins that participate in the joining phase of V(D)J recombination, the precise molecular mechanisms remain obscure.

RAG-1 and RAG-2 are 1040 and 527 amino acid residues long, respectively. Core fragments comprising residues 384–1008 of RAG-1 and 1–387 of RAG-2 support recombination of extrachromosomal substrates and are more readily isolated than the full-length proteins (8, 9). For this reason, studies of V(D)J recombination in vitro have largely employed these forms (4, 10, 11). Nonetheless, the RAG-1 and RAG-2 non-core regions are conserved phylogenetically and regulatory functions have been ascribed to them. The non-core region of RAG-2, for example, couples V(D)J recombination to the cell cycle by supporting the periodic destruction of RAG-2 protein, which accumulates preferentially in G0/G1 cells and is degraded at the G1-S transition in dividing cells (12–14). The degradation signal overlaps an importin 5 (karyopherin β 3) binding site required for efficient nuclear uptake of RAG-2 in dividing cells (15). Removal of the non-core region has been reported to reduce the efficiency of recombination within extrachromosomal substrates (8, 9, 16–19) to increase the production of hybrid joints at the expense of coding and signal joints (20) and to impede V_{H} to DJ_{H} joining at endogenous loci (16, 21, 22), although the mechanisms underlying these effects are not well understood.

After DNA cleavage in vitro, the core RAG-1 and RAG-2 proteins remain associated stably with the cleaved RSSs (23, 24). In this signal end complex (SEC), the DNA ends are sequestered from the NHEJ apparatus (24). In vitro, the SEC is able to support the integration of signal ends into a recipient DNA molecule, but it remains an open question whether this is a significant source of genomic instability in vivo. At concentrations that mimic intracellular conditions, Mg2+ and GTP suppress RAG-mediated transposition in vitro (25, 26). Mg2+ promotes the reversal of the transposition reaction in a process termed disintegration (25). GTP selectively suppresses RAG-mediated transposition by inhibiting target capture while sparing DNA cleavage by the RAG complex (26). Either of these mechanisms may act to suppress RAG-mediated transposition in vivo. A third potential mechanism of suppression was suggested by the ability of the RAG non-core regions to inhibit hybrid joint formation in vivo (20), the chemistry of which resembles that of transposition. Correspondingly, two recent studies have suggested that the full-length RAG proteins carry

1 The abbreviations used are: RSS, recombination signal sequences; NHEJ, non-homologous DNA end joining; SEC, signal end complex; CHO, Chinese hamster ovary; MBP, maltose-binding protein; LM, ligase-mediated; MOPS, 4-morpholinepropanesulfonic acid; PK, protein kinase; HMG, high mobility group.
out transposition with reduced efficiency relative to the core proteins (26, 27).

In this communication, we describe the isolation of intracellular postcleavage complexes containing excised recombination signal ends and the full-length RAG proteins. Similar to SECs formed in vitro with core RAG proteins, the SECs obtained from transfected cells expressing full-length RAG proteins are stable and resistant to exonucleolytic attack. The formation of SECs in vivo is independent of an intact NHEJ machinery. Intracellular accumulation of SECs containing full-length RAG-1 and RAG-2 is coordinated with the cell cycle, in contrast to SECs containing a mutant RAG-2 defective in regulated degradation. We observed no significant difference in the efficiency of transposition supported by SECs formed in vitro with full-length or core RAG proteins. Failure of the RAG non-core regions to suppress transposition in this setting indicates that intracellular SECs are potential sources of genomic instability. If RAG-mediated transposition is inhibited in vivo, the presence of the non-core regions does not seem likely to account for this suppression.

EXPERIMENTAL PROCEDURES

Cell Lines—NIH3T3 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum. CHO-K1 cells or their NHEJ-deficient derivatives srs-5 (Ku80-deficient), XR-1 (XRCC4-deficient), and V3 (DNA-PKcs-deficient) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum.

DNA Constructs—The extrachromosomal recombination substrates pH200, pH290, and pDVG42 have been described previously (11, 28, 30). Plasmids pMR1 and pMR2 encoding maltose-binding protein (MBP) fusions to core RAG-1 (residues 384–1008) and core RAG-2 (residues 1–387), tagged at the carboxyl terminus with a Myc epitope and polyhistidine, have been described previously (30). Plasmids pMFR1 and pMFR2 encoding MBP fusions to full-length RAG-1 or RAG-2, respectively, were generated by PCR-based subcloning. First, an AscI site was introduced at the 3’ end of the MBP cassette and an FseI or EcoRI site was introduced at the 5’ end of the Myc epitope cassette by PCR amplification of plasmid pMR2. A PCR product containing full-length RAG-2 cDNA flanked by AscI and EcoRI sites was then ligated between cognate sites in the appropriate MBP fusion vector. Plasmid pcDNA-MBP expressing MBP alone was created by subcloning an MBP cDNA flanked by BamHI and EcoRI sites into pcDNA1. Plasmids pKI and pR2 expressing Myc epitope-tagged core RAG-1 or core RAG-2 were generated by PCR subcloning of the corresponding cDNAs from the appropriate MBP fusion vectors using primers directed to the 5’ ends of core RAG-1 or RAG-2 (generating a BamHI site) and to the 3’ end of the Myc epitope (generating a NotI site). The amplified products were subcloned into pcDNA1. All of the DNA constructs were confirmed by nucleotide sequence analysis.

Isolation of SECs from Transfected Cells—NIH3T3 cells or CHO-K1 cell lines were grown to 70% confluency and then cotransfected with plasmids encoding core MBP-RAG-1 and MBP-RAG-2 chimeras (pMR1 and pMR2), full-length MBP-RAG-1 and MBP-RAG-2 chimeras (pMFR1 and pMFR2) or combinations of full-length and core proteins as indicated. Cells were cotransfected with an extrachromosomal recombination substrate (pJH200 or pH290) and pcSVT. Transfections were performed using LipofectAMINE (Invitrogen) according to the manufacturer’s instructions.

At 36–48 h after transfection, cells were harvested by scraping and the cell pellets were resuspended to ~106 cells/ml in RSB (10 mM Tris, pH 7.4, 10 mM NaCl, 1 mM MgCl2, 0.5% Nonidet P-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml pepstatin A) containing 0.5% Nonidet P-40 and then with 20 column volumes of wash buffer without Nonidet P-40. SECs were eluted in wash buffer containing 10 mM maltose at a volume of 3 ml/106 lysed cells. Throughout the loading, wash, and elution steps, 1-ml fractions were collected.

To prepare fractions for assay of signal ends, 200-μl aliquots were deproteinized by incubating with 500 μg/ml proteinase K in 0.5% SDS, 20 mM Tris, and 10 μg EDTA for 1 h at 37 °C followed by phenol-chloroform extraction and precipitation with ethanol. The resuspended DNA was subjected to a ligation-mediated PCR (LM-PCR) assay for signal ends. For detection of RAG fusion proteins, 20-μl samples of appropriate fractions were boiled for 5 min in SDS-PAGE loading buffer and fractionated by electrophoresis through a 4–12% SDS-PAGE gel. Fusion proteins were detected by immunoblotting using the anti-Myc antibody 9e10.

Signal End Detection—LM-PCR assays for signal ends were modified from published methods (10) as follows. Signal ends were first ligated to a linker composed of annealed primers, FM25 (5’-CGCGTGGACGCCGGAGACTGAAATG-3’) and FM15 (5’-CCTCAGAGTC-3’). After activating DNA ligase by heating to 65 °C for 20 min, PCR was performed using primer FM25 and either OHJ004 (5’-GGCGGTTCTGCGTTCTGAAATG-3’) or pH200. Following denaturation for 5 min at 94 °C, 28 cycles of amplification were performed, each consisting of 20 s at 94 °C, 30 s at 60 °C, and 20 s at 72 °C. Finally, samples were subjected to extension for 5 min at 72 °C. Products were fractionated by electrophoresis through a 6% polyacrylamide gel and detected by staining with ethidium bromide.

Competition Assays—Blunt-ended, RSS-flanked competitor DNA was derived by PCR amplification from pDVG2 using primers OHJ042 (5’-CACAGTGCACAGCGGACAAACCC-3’) and OHJ043 (5’-CAGCGTGGATCCTACCTGTCGT-3’), which anneal to the hepatamers of the 12 and 23 RSSs, respectively. The PCR product was purified by gel electrophoresis and phosphatylated at its 5’ ends using polynucleotide kinase (New England Biolabs). Competitor was added to the lysis buffer before cell lysis. Lysates were then subjected to amnylose chromatography as described above.

Cell Cycle Analysis—NIH3T3 cells were transfected with plasmids pMFR1 and either pMFR2 or pMFR2(T490A). Cells were cotransfected with pH290. At 10 h after transfection, cells were subjected to serum starvation by incubation in Dulbecco’s modified Eagle’s medium containing 0.5% calf serum. After 48 h, the concentration of serum was restored to 20% and cells were collected at various times thereafter. For flow cytometric analysis of DNA content, aliquots of cells were fixed overnight at 4 °C in 50% ethanol and then stained at room temperature for 30 min in phosphate-buffered saline containing 100 μM propidium iodide and 10 μg/ml RNase A. Samples were analyzed using a Coulter EPICS 752 flow cytometer. The remaining cells (103) were assayed for SEC formation by amnylose affinity chromatography as described above.

Transposition Assays—Eluates containing SECs were concentrated from an initial volume of 5 ml to 50 μl by Microcon YM-10 filtration (Millipore). Aliquots (5 μl) were frozen in liquid nitrogen and stored at −80 °C. Transposition reactions (final volume 20 μl) were carried out in a buffer containing 25 mM MOPS, pH 7.0, 100 ng/μl bovine serum albumin, 10% glycerol, 2 mM MgCl2, 60 mM potassium glutamate, and 2 mM dithiothreitol. To some reactions, HMG-1 (100 ng) was added. For each transposition reaction, 5 μl of thawed SEC was added to 4 μl of the recipient plasmid pBlueScript or pOT2A at 37 °C for 1 h. DNA was then depurinized by phenol-chloroform extraction, precipitated in ethanol, and resuspended in 30 μl of 10 μM Tris-Cl, pH 8.6. A 10-μl sample of eluted DNA was used as template in a PCR reaction using primers OHJ004 and OHJ006 (5’-CTCATGCTAGGAGGATCCCGAG-3’), which anneal within the pH290 donor. Reactions were supplemented with 0.5 U/ml AmpliTaq and 100 μM deoxynucleoside triphosphates. After denaturation for 5 min at 94 °C, 30 cycles of amplification were performed, each consisting of 30 s at 94 °C, 30 s at 60 °C, and 1 min at 72 °C. Samples were subjected to extension for 15 min at 72 °C after the last cycle. Products were purified using the QIAquick kit (Qiagen), fractionated by electrophoresis through a 4–20% nondenaturing polyacrylamide gel or a 1% agarose gel, and visualized using a PhosphorImager. To induce site duplications, transposition reactions were fractionated on a 1% agarose gel and transposition products were excised. DNA was purified using the QIAquick gel extraction kit and cloned into the pcR2.1 vector (Invitrogen). DNA sequence was determined using M13 forward and reverse primers.
Transposition of RAG Signal End Complexes

**RESULTS**

Isolation of SECs Containing Full-length RAG Proteins from Transfected Cells—Following DNA cleavage by the RAG proteins in vitro, a stable postcleavage complex between the RAG proteins and signal ends is formed (23, 24). We wished to detect the formation of such complexes in vivo and to isolate them for further study. Plasmids encoding full-length or core RAG proteins fused to MBP at the amino termini and to c-Myc epitope at the carboxyl termini were cotransfected with an extrachromosomal recombination substrate into NIH3T3 cells. At 48 h after transfection, cell lysates were fractionated by amylose affinity chromatography. Column fractions were assayed for the presence of RAG proteins by immunoblotting (anti-Myc, bottom panel), and deproteinized samples were assayed for the presence of 12-RSS signal ends (LM-PCR, middle panel). B, cells were transfected with plasmids encoding core RAG proteins, and the pJH290 extrachromosomal recombination substrate and SECs were isolated as in A with the exception that after the column wash step, MBP was removed from the RAG proteins by treatment with factor Xa protease. An assay for 12-RSS signal ends is shown. Lanes 1 and 2, flow-through; lanes 3 and 4, wash fractions 1, 5, 10, 15, and 20; lanes 10 and 11, elution. C, cells were transfected with pJH290 and combinations of plasmids encoding MBP-tagged and untagged RAG constructs as indicated. MBP, unfused MBP; cr1 and cr2, core RAG-1 and RAG-2, respectively; MBP-flR1 and MBP-flR2, MBP fused to full-length RAG-1 or RAG-2, respectively; MBP-cR1 and MBP-cR2, MBP fused to core RAG-1 or RAG-2, respectively. Chromatography was carried out as in A. Deproteinized samples from the last flow-through fraction (lanes 1, 4, 7, 10, and 13), the last wash fraction (lanes 2, 5, 8, 11, and 14), and the first eluate fraction (lanes 3, 6, 9, 12, and 15) were assayed for 12-RSS signal ends.

The presence of cleaved 12-spacer signal ends (Fig. 1A, middle panel), in contrast to that of uncombined plasmid (Fig. 1A, upper panel) or signal joints (data not shown), was strongly correlated with the amount of RAG-1 and RAG-2 in each fraction (Fig. 1A, lower panel, and data not shown), which decreased during the wash phase and increased sharply upon elution with maltose. This behavior was consistent with the persistent association of signal ends with the RAG proteins following DNA cleavage. Complexes were detected in lysates of cells expressing any pairwise combination of full-length and core RAG-MBP fusion proteins (Fig. 1C, lanes 6, 9, 12, and 15). The differences in relative yields of SECs were not consistent, and this may reflect the variability inherent in the assay, which couples isolation of small quantities of intermediates with amplification by the PCR. Similar results were obtained using an LM-PCR assay specific for 23-spacer signal ends (data not shown). The recombination substrates pJH200 and pJH290 contain identical RSSs and behaved equivalently in the assay for complex formation. Subsequent experiments therefore employed either pJH200 or pJH290 as indicated.

RAG proteins and signal ends could also be released from the column by proteolytic separation of the MBP moiety from the RAG fusion proteins after the binding and wash steps (Fig. 1B). To more rigorously exclude a contribution of the MBP moiety to the binding of cleaved signal ends, MBP was coexpressed with the unfused core RAG proteins in the presence of the extrachromosomal substrate. In this instance, signal ends were detectable in the flow-through fraction but not in the eluate (Fig. 1C, lanes 1–3).

It has been suggested (23) that SECs formed in vitro in the presence of cell-free extracts may be associated with components of the NHEJ machinery. Although immunoblotting failed to detect Ku80 or XRCC4 in SECs isolated from wild-type CHO-K1 cells (data not shown), we asked whether intact NHEJ might have been required at some previous stage for the formation of the complexes we detected in lysates of transfected cells. To this end, we assayed the formation of SECs in wild-type CHO-K1 cells and in their derivative, the xrs-5 cell line, in which NHEJ is defective because of a lack of functional Ku80 (31). SECs were detected in lysates of the wild-type and mutant cell lines (Fig. 2A, lanes 3 and 6). SECs were also recovered from mutant CHO cells deficient in DNA-PKcs and its wild-type progenitor (Fig. 2A, lanes 9 and 12) as well as from cells deficient in the NHEJ component XRCC4 (Fig. 2A, lane 15). Although these results do not rule out an effect of NHEJ deficiency on the absolute yield of SECs, they do show that NHEJ is not essential for SEC formation.

In vitro, the core RAG proteins form a stable postcleavage complex in which signal ends are protected from exonuclease digestion (24). Signal ends are similarly sequestered in the full-length SECs that we isolated from transfected cells. Treatment of these complexes with λ exonuclease did not reduce the amount of signal ends detectable by LM-PCR (Fig. 2B, compare lanes 1 and 2). In contrast, heat denaturation of the complex by incubation for 15 min at 70 °C prior to exonuclease treatment resulted in the complete disappearance of signal ends (Fig. 2B, compare lanes 3 and 4). The resistance of the unadenated SECs to λ exonuclease treatment indicates that the amount of free signal ends present in fractions containing purified SECs must be small.

**SECs Isolated from Transfected Cells Originate in Vivo—** Whereas these results demonstrated an association between RAG proteins and signal ends, it remained possible that some or all of these complexes were formed by the binding of RAG proteins to signal ends after cell lysis rather than within the cell. Additionally, the RAG proteins might have cleaved unrecombined plasmid and become bound to its signal ends after
cell lysis. To exclude these possibilities, we performed two competition assays.

To rule out binding of RAGs to free signal ends, a linear competitor was derived from the extrachromosomal substrate pDVG42, terminating in 5′-phosphorylated signal ends identical to those of pJH200 or pJH290. We had previously determined using the same experimental system as described above for pJH200 and pJH290 that complexes between the RAG proteins and cleaved pDVG42 can be detected in lysates of transfected cells (data not shown). To assay formation of new complexes from uncomplexed substrate, intact pDVG42 was used as a competitor. Cells were transfected with vectors encoding the MBP-RAG fusion proteins and with pJH290. At 48 h after transfection, immediately before lysis, the pDVG42-derived competitor fragment (Fig. 3, A and B) or intact pDVG42 plasmid (Fig. 3, C and D) was added at increasing concentrations. The amounts of linear competitor added were estimated by a preliminary experiment (data not shown) to represent a 10-, 100-, or 1000-fold excess over pJH290 signal ends. The amounts of circular competitor added were set similarly, relative to uncomplexed pJH290. Lysates were fractionated by amylose affinity chromatography, and pJH290 signal ends were detected in serially diluted fractions by LM-PCR. The recovery of pJH290 signal ends in the eluate was not significantly diminished, even at a 1000-fold excess of competitor (Fig. 3, A and C, bottom panels, compare lanes 1–3 with lanes 10–12). Moreover, pDVG42 signal ends were not retained on the amylose column in the presence of either competitor (Fig. 3, B and D).

Thus, neither the exchange of RAG proteins among signal ends nor the de novo formation of stable RAG-signal end complexes is detectable in vitro under the conditions employed here. These observations strongly support the interpretation that the complexes detected in lysates of transfected cells were formed in vivo. We cannot formally rule out the possibility that these SECs are formed after cell lysis by coupled cleavage within a preexisting paired complex, but this possibility seems unlikely given our observation that the RAG proteins do not maintain stable interactions with intact plasmid DNA under the experimental conditions employed here.

SEC Accumulation in the G0 and G1 Cell Cycle Phases—RAG-2 protein accumulates periodically in dividing cells, accumulating in the G1 cell cycle phase and undergoing rapid degradation at the G1-to-S transition (12–14). Accumulation of signal ends shows a similar periodicity (12, 32, 33). Mutation of a CDK2 phosphorylation site at threonine 490 of RAG-2 abolishes the periodic destruction of RAG-2 and permits signal end accumulation throughout the cell cycle (12, 14, 34). Although it has been suggested that the programmed degradation of RAG-2 might serve to release sequestered signal ends from the postcleavage complex (24), it has remained an open question whether a RAG-2 mutant defective in cell cycle-dependent degradation would, in fact, support persistence of SECs throughout the cell cycle.

To assess this possibility, RAG-1 and either wild-type RAG-2 or the RAG-2(T490A) mutant was expressed in NIH3T3 cells that had been co-transfected with an extrachromosomal recombination substrate. At 10 h after transfection, the cells were induced to enter quiescence by withdrawal of serum for 48 h. Entry into G1 and cell cycle progression were then initiated by the addition of serum. Cells were collected at the time of release (0 h) or at subsequent times thereafter (Fig. 4A), and cell lysates were assayed for the presence of SECs as noted above. The cell cycle distribution was determined in parallel by fluorescence cytometry (Fig. 4B). In cells expressing wild-type RAG-2, SECs were present at 0 and 13 h when a substantial proportion of cells in G0 or G1 (Fig. 4C, upper panel, lanes 3 and 6) were undetectable at 18 h, at which time essentially all of the cells were in the S or G2/M cell cycle phases (Fig. 4C, upper panel, lane 9) and reappeared by 24 h after cells had re-entered the G1 phase (Fig. 4C, upper panel, lane 12). In contrast, RAG-2(T490A) supported intracellular accumulation of SECs throughout the cell cycle (Fig. 4C, lower panel, lanes 3, 6, 9, and 12). We interpret these results to suggest that the presence of SECs is ordinarily constrained to the G0 and G1 cell cycle phases and that, when periodic destruction of the recombinase is blocked, RAG proteins remain associated with signal ends throughout the cell cycle.

SECs Formed Intracellularly with Full-length or Core RAG Proteins Support Transposition in Vitro with Similar Efficiencies—RAG-1 and RAG-2 support DNA transposition in vitro, and this activity has been ascribed to the SECs produced following DNA cleavage. We asked whether the SECs that we had isolated from transfected cells could also mediate transposition in vitro. If so, this would provide an opportunity to assess the relative efficiencies with which SECs containing full-length or core RAG proteins support transposition. Initially, SECs were isolated from cells expressing the core RAG proteins and incubated with a recipient plasmid, either pBluescript or pOT2A, for 1 h under reaction conditions that support transposition. After transposition, the 3′ ends of the donor DNA are joined to the targets but the 5′ ends are not, leaving gaps between the 5′ ends of the donor DNA and the 3′ ends of the target DNA molecules. To detect these events, we employed a PCR-based

**Fig. 2. NHEJ independence and exonuclease resistance of SECs formed in vivo.** A, the following cell lines were transfected with pJH290 and plasmids encoding full-length RAGs, CHO-K1 and the Ku80-deficient derivative xsr-5, AA8, and the DNA-PKcs-deficient derivative V3, and the XRCC4-deficient cell line XR-1. At 48 h after transfection, cell lysates were fractionated by amylose affinity chromatography. Signal ends were assayed in deproteinized samples from the last flow-through fraction (F, lanes 1, 4, 7, 10, and 13), the last wash fraction (W, lanes 2, 5, 8, 11, and 14), and the first eluate fraction (E, lanes 3, 6, 9, 12, and 15). B, purified SECs containing full-length RAG proteins were treated with λ exonuclease (lanes 1 and 3) or buffer alone (lanes 2 and 4) with (lanes 3 and 4) or without (lanes 1 and 2) prior heating for 15 min at 70 °C. Signal ends were assayed as above.
In the first round of PCR amplification, the gaps were filled and extended across the donor sequence. In subsequent rounds of the PCR, primers directed outwards from the donor fragment amplified DNA across the RSSs and through the target plasmid (Fig. 5, A and B). Amplification was performed in the presence of $[^{32}P]$dCTP.

Transposition into pBluescript and pOT2A was predicted to yield radiolabeled PCR products of 3.1 and 1.8 kb, respectively. Products of the appropriate sizes were detected in complete transposition reactions incubated for 1 h (Fig. 5, A, lanes 1, 4, and 6, and B, lanes 2 and 3). Products were absent if the reaction was stopped at zero time (Fig. 5A, lane 2), if divalent cation was chelated with EDTA (Fig. 5, A, lane 3, and B, lane 1), if donor SECs were preincubated at 70 °C (Fig. 5A, lanes 5 and 8), or if the donor SECs were omitted (Fig. 5A, lane 7). The addition of HMG-1 was not essential for the formation of product (Fig. 5A, lane 1 and 4).

Transpositional junctions were amplified by PCR using a forward oligonucleotide primer specific for the donor fragment and a reverse primer corresponding to a fixed point within the recipient DNA (Fig. 5C). PCR products were subcloned. Excision of each insert yielded a single dominant DNA fragment of distinctive length, consistent with random integration with respect to the recipient plasmid (Fig. 5C). The nucleotide se-
Transposition of RAG Signal End Complexes

Fig. 4. Intracellular accumulation of SECs is cell cycle-dependent. A, experimental scheme. NIH3T3 cells were transfected with pJH290 together with plasmids encoding full-length RAG-1 and wild-type (wt) RAG-2 or RAG-2(T490A). At 10 h after transfection (txfn), serum (CS) was reduced to 0.5%. After 48 h of serum starvation, cells were released into medium containing 20% serum and collected 0, 13, 18, and 24 h thereafter. B, cell cycle distribution at indicated times after release of synchronized cells as assessed by fluorescence-activated cell sorter. Cell number is plotted as a function of DNA content. C, SECs were isolated by affinity chromatography from cells transfected with wild-type RAG-2 (upper panel) or RAG-2(T490A) (lower panel) collected at the times indicated above. Deproteinized samples from the last flow-through fraction (L, lanes 1, 4, 7, and 10), the last wash fraction (W, lanes 2, 5, 8, and 11), and the first eluate fraction (E, lanes 3, 6, 9, and 12) were assayed for 12-RSS signal ends.

Fig. 5. Transposition activity of SECs formed in vivo. A and B, SECs containing core RAG proteins purified by affinity chromatography from transfected cells were combined with supercoiled plasmids pBS (A, lanes 1–5; B, lanes 1 and 2) or pOT2A (A, lanes 6 and 7; B, lane 3) for 1 h at 37 °C. After deproteinization, transposition products were detected by PCR as diagrammed at left side in the presence of [α-32P]dCTP. Products were fractionated by electrophoresis through polyacrylamide (A) or agarose (B) and detected with a PhosphorImager. The expected sizes of PCR products generated by transposition into pBS and pOT2A are indicated by the filled and open arrows, respectively. The presence (+) or absence (−) of SEC, heat-killed SEC (pretreatment at 70 °C for 20 min), HMG-1 (100 ng/20 μl), and EDTA (5 mM) are indicated above. In the lower panel, the sizes of radiolabeled DNA markers (M) (in kb) are indicated at the left side. C, products of transposition reactions containing core SEC and pBS were subjected to junctional PCR as diagrammed at left side. Amplified fragments were cloned into pCR2.1, excised, and fractionated by agarose gel electrophoresis. Size markers are run at left side.

In support of this idea has recently been presented (26, 27). From the exonuclease protection experiment presented in Fig. 5, we knew that few free signal ends were present in our SEC preparations. Therefore, we were able to normalize transposition reactions containing core or full-length SECs with respect to RAG-associated signal ends. Core and full-length SECs were prepared from XRCC4-deficient XR-1 cells so as to eliminate contamination from signal joints that might interfere with subsequent assays. These SEC preparations were adjusted to equal concentrations of signal ends, and transposition reactions were performed using serial dilutions of the adjusted preparations. The equivalence of input donor signal ends was verified by LM-PCR for two separate preparations of core and full-length SECs (Fig. 6, A and B, upper panels). Moreover, immunoblotting confirmed that similar amounts of RAG-1 and RAG-2 were present in core and full-length SEC preparations containing equivalent numbers of signal ends (Fig. 6, C and D, and data not shown). In contrast to our expectation, we found that the core and full-length SECs supported similar levels of transposition, either using pBS (Fig. 6A, lower panel) or pOT2A (Fig. 6B, lower panel) as a target plasmid. Similar results were obtained in the presence (Fig. 6A) or absence (Fig. 6B) of HMG-1 protein. It remained possible that intracellular signal end complexes containing either full-length or core RAG proteins might display differences in target site preference and target site duplication length. To examine this possibility, products of transposition between core or full-length SEC donors and pOT2A were cloned. From the nucleotide sequences of the transpositional junctions, we deduced the sites of integration within pOT2A and the associated target site duplication. In all cases, the 3’ end of the RSS heptamer was joined directly to the target plasmid (data not shown).

It has been proposed that the non-core regions of the RAG proteins function to suppress transposition (20), and evidence in support of this idea has recently been presented (26, 27). From the exonuclease protection experiment presented in Fig. 5, we knew that few free signal ends were present in our SEC preparations. Therefore, we were able to normalize transposition reactions containing core or full-length SECs with respect to RAG-associated signal ends. Core and full-length SECs were prepared from XRCC4-deficient XR-1 cells so as to eliminate contamination from signal joints that might interfere with subsequent assays. These SEC preparations were adjusted to equal concentrations of signal ends, and transposition reactions were performed using serial dilutions of the adjusted preparations. The equivalence of input donor signal ends was verified by LM-PCR for two separate preparations of core and full-length SECs (Fig. 6, A and B, upper panels). Moreover, immunoblotting confirmed that similar amounts of RAG-1 and RAG-2 were present in core and full-length SEC preparations containing equivalent numbers of signal ends (Fig. 6, C and D, and data not shown). In contrast to our expectation, we found that the core and full-length SECs supported similar levels of transposition, either using pBS (Fig. 6A, lower panel) or pOT2A (Fig. 6B, lower panel) as a target plasmid. Similar results were obtained in the presence (Fig. 6A) or absence (Fig. 6B) of HMG-1 protein. It remained possible that intracellular signal end complexes containing either full-length or core RAG proteins might display differences in target site preference and target site duplication length. To examine this possibility, products of transposition between core or full-length SEC donors and pOT2A were cloned. From the nucleotide sequences of the transpositional junctions, we deduced the sites of integration within pOT2A and the associated target site duplications. The lengths of target site duplications created by core or full-length RAG proteins were similar (Fig. 6E). No differences in target site preference were discerned (Fig. 6, E and F, and data not shown). We conclude that, at least in SECs formed intracellularly, the presence of the non-core regions of RAG-1 and RAG-2 have no significant effect on transposition efficiency, target site selection, or target site duplication.
DISCUSSION

The SECs that are formed in vitro between RSS signal ends and the core RAG proteins following DNA cleavage support DNA transposition. Signal ends are readily detected in vivo where they are relatively long-lived, and RAG proteins have been shown to be associated with these signal end DNAs after fixation (35). Nonetheless, catalytically active, stable SECs have not hitherto been isolated from living cells. Such structures might represent a potential source of genomic instability. Here we demonstrate intracellular accumulation of stable SECs containing RAG-1 and RAG-2. Although the full-length RAG proteins have been difficult to purify in active form, we were able to efficiently isolate full-length and core signal end complexes using identical affinity purification schemes. The success of this approach may reflect increased solubility or stability of the full-length RAG proteins when associated with DNA in general or with cleaved signal ends in particular. The formation of SECs in vivo was supported by full-length or core RAG proteins in any combination and was independent of Ku80 or other components of the NHEJ machinery. In SECs isolated from transfected cells, as in SECs formed in vitro, signal ends are protected from exonucleolytic attack. The intracellular accumulation of SECs, similar to the accumulation of signal ends themselves, is regulated in the cell cycle and restricted to the G0 and G1 cell cycle phases. If the periodic degradation of RAG-2 is abolished by mutation, intracellular SECs persist throughout the cell cycle. Unexpectedly, SECs assembled intracellularly from full-length or core RAG proteins supported transposition in vitro with similar efficiencies.

Properties of SECs Generated in Vivo—The relatively slow repair of signal ends in vivo suggested that they remain protected from exonucleolytic attack. The intracellular accumulation of SECs, similar to the accumulation of signal ends themselves, is regulated in the cell cycle and restricted to the G0 and G1 cell cycle phases. If the periodic degradation of RAG-2 is abolished by mutation, intracellular SECs persist throughout the cell cycle. Unexpectedly, SECs assembled intracellularly from full-length or core RAG proteins supported transposition in vitro with similar efficiencies.

Properties of SECs Generated in Vivo—The relatively slow repair of signal ends in vivo suggested that they remain protected from exonucleolytic attack. The intracellular accumulation of SECs, similar to the accumulation of signal ends themselves, is regulated in the cell cycle and restricted to the G0 and G1 cell cycle phases. If the periodic degradation of RAG-2 is abolished by mutation, intracellular SECs persist throughout the cell cycle. Unexpectedly, SECs assembled intracellularly from full-length or core RAG proteins supported transposition in vitro with similar efficiencies.

Properties of SECs Generated in Vivo—The relatively slow repair of signal ends in vivo suggested that they remain protected from exonucleolytic attack. The intracellular accumulation of SECs, similar to the accumulation of signal ends themselves, is regulated in the cell cycle and restricted to the G0 and G1 cell cycle phases. If the periodic degradation of RAG-2 is abolished by mutation, intracellular SECs persist throughout the cell cycle. Unexpectedly, SECs assembled intracellularly from full-length or core RAG proteins supported transposition in vitro with similar efficiencies.

Fig. 6. SEC generated intracellularly from full-length or core RAG proteins undergo transposition in vitro with similar frequencies. A and B, the concentrations of core and full-length SEC preparations purified by affinity chromatography were normalized to equivalent signal ends. These normalized preparations were diluted serially 5-fold. An aliquot of each dilution was deproteinized, and signal ends in core (left panels) or full-length (right panels) SECs were assayed by LM-PCR (top panels). A and B represent two separate SEC purifications. Serially diluted SECs were then combined with pBS (A) or pOT2A (B) and incubated at 37 °C for 1 h. Transposition products were detected by PCR as in Fig. 5A. HMG-1 (100 ng/20 μl) was included in transposition reactions containing pOT2A. C and D, detection of core (lanes 1) or full-length (lanes 2) RAG-1 (filled arrows) and RAG-2 (open arrows) in SEC preparations normalized to equivalent signal ends. Preparations used in transposition reactions displayed in A and B are assayed in C and D, respectively. Protein was detected by immunoblotting with an anti-Myc antibody. E, target site duplications associated with transposition of core (left) or full-length (right) SECs into pOT2A. The precursor target sequence and 5-bp flanking sequence on either side are given. Sequences duplicated in the transposition products are displayed in boldface. Numbers at left side indicate coordinates (in bp) of the duplicated site relative to the pOT2A sequence. F, distribution of integration sites in pOT2A. Integration of SECs containing core or full-length RAG proteins are indicated by black and gray arrowheads, respectively.
bled intracellularly.\textsuperscript{2} Moreover, we observed accumulation of SECs in cells deficient in Ku80, XRCC4, or DNA-PKcs. Although these components of the NHEJ machinery are apparently not required for SEC formation in \textit{vivo}, it remains possible that one or more of these proteins interacted with SECs in \textit{vivo} but were lost in the course of purification.

Regulated Accumulation of SECs in Dividing Cells—DNA cleavage at recombination signal sequences and accumulation of signal ends is restricted to the G\textsubscript{1} and G\textsubscript{2} cell cycle phases by a mechanism involving the periodic degradation of RAG-2 (12). The ability to identify and isolate intracellular SECs containing core or full-length RAG proteins permitted us to examine SEC accumulation in the cell cycle. In synchronously dividing cells expressing full-length RAG proteins, the yield of SECs was positively correlated with the proportion of cells in the G\textsubscript{0}/G\textsubscript{1} fraction, as expected. More importantly, RAG-2 mutants that escape cell cycle-regulated degradation, namely RAG-2(T490A) or core RAG-2, supported accumulation of SECs throughout the cell cycle.

Formation of signal joints must require disassembly or rearrangement of the SEC, so as to allow access to the NHEJ machinery. It has been proposed that degradation of RAG-2 at the G\textsubscript{1}-to-S transition functions in this process (24). Two observations argue against this possibility. Although the accumulation of SECs in cells expressing wild-type RAG-2 decreases sharply upon entry into S phase, we detected no corresponding increase in the yield of signal joints at the G\textsubscript{1}-S transition or throughout the S and G\textsubscript{2}/M phases. Moreover, the frequency of signal joint formation in cells expressing the degradation-deficient mutant RAG-2(T490A) was unaltered relative to the frequency supported by wild-type RAG-2.\textsuperscript{2} Together with our observation that the RAG degradation mutant retains signal ends throughout the cell cycle, it seems likely that degradation of RAG-2 at the G\textsubscript{1}-S transition is not required for the NHEJ machinery to gain access to signal ends, at least in extrachromosomal substrates. Rather, some other mechanism, as yet undefined, must account for the disassembly of SECs prior to signal end joining.

Transposition of SECs Containing Full-length or Core RAG Proteins—RAG-mediated transposition may have contributed beneficially in the segmentation of antigen receptor genes during evolution (35, 37), but it may also in principle exert deleterious effects on genomic integrity. Although such events have not yet been observed directly, evidence consistent with mutagenic transposition of cleaved signal ends has been described previously (38). Because of the potential danger posed by RAG-mediated transposition, it has been suggested that this process is disfavored in the intracellular environment (25, 26). Indeed, RAG-mediated transposition is inhibited \textit{in vitro} by Mg\textsuperscript{2+} (25) and GTP (26) at physiologic concentrations. Similar suppressive effects may be exerted \textit{in vivo}.

We observed that SECs isolated from cells expressing full-length or core RAG proteins underwent transposition with similar efficiency and with no discernable differences in target site selection or duplication. Transposition appears to favor long cruciform structures as targets when these are present in acceptor DNA (39). The pOT2A target plasmid, however, contains no palindromic sequence longer than 12 bp, and of the target sites sequenced for full-length or core RAG donors, none was positioned near a palindromic sequence greater than 8 bp in length. Consistent with previously published observations (40), we observed a bias toward GC-rich target sites for SECs containing full-length or core RAG proteins.

While our work was in preparation, there appeared two reports that transposition \textit{in vitro} by full-length RAG proteins relative to core proteins was substantially reduced when uncleaved RSS substrates were used as donors (26, 27). There are several ways in which our results may be reconciled with these observations. First, the SECs we isolated from transfected cells may carry biochemical modifications or copurifying species that stimulate transposition by full-length RAG proteins. Second, the activities of full-length RAG proteins purified in association with SECs may differ from those purified separately. Third, the conformation of SECs formed intracellularly may differ from those formed wholly \textit{in vitro}. Fourth, transposition of uncleaved donors by the full-length RAG proteins may be impeded at a step prior to the formation of SECs. This last possibility is consistent with the observation that purified full-length RAG proteins were able to support transposition of precleaved substrates, although under those conditions, quantitative comparison of full-length and core proteins was not possible as full-length and core RAG proteins differed with respect to the efficiency of signal end binding (27).

In a third report (41), intact RAG proteins, when assayed in transfected yeast, supported recombination and transposition at a level 10-fold lower than that supported by the core proteins. Although it is difficult to interpret results obtained in a heterologous setting, these differences are consistent with the possibility that the full-length RAG proteins perform DNA cleavage less efficiently than the core proteins as DNA cleavage is common to both recombination and transposition. The isolation of intracellular SECs containing full-length and core proteins allowed us to compare their transposition efficiencies directly in reactions containing equivalent numbers of signal ends, thereby bypassing any differences in cleavage activity. Under these conditions, the amounts of RAG proteins were also similar. Our results failed to support the notion that transposition \textit{per se} is inhibited directly by the non-core regions. Rather, the work raises the possibility that transposition \textit{in vivo} may be suppressed by one or more inhibitory factors that are absent from our \textit{in vitro} reactions. The \textit{in vitro} system described here might be used to isolate such factors.

Although it has been suggested that interaction of full-length RAGs with cleaved coding flanks may suppress transposition \textit{in vitro} by impeding target capture (27), it is not clear whether this mechanism would suffice to prevent transposition \textit{in vivo}. Signal ends are substantially longer lived than coding ends (5–7), and our results suggest that the majority of these intracellular signal ends are stably associated with SECs. Thus, we argue that intracellular SECs are likely to persist in the absence of coding ends and that these SECs represent potential donors whose transposition is not suppressed by the non-core regions of the RAG proteins.

Acknowledgments—We thank Joanne Sekiguchi and Fred Alt for providing wild-type and NHEJ-deficient CHO cell lines, Patrick Swanson for providing HMG-1 protein, and our colleagues in the Department of Molecular Biology and Genetics for stimulating discussions.

REFERENCES

1. Gellert, M. (2002) \textit{Annu. Rev. Biochem.} 71, 101–132
2. Oettinger, M. A., Schatz, D. G., Gorka, C., and Baltimore, D. (1990) \textit{Science} 248, 1517–1523
3. Schatz, D. G., Oettinger, M. A., and Baltimore, D. (1989) \textit{Cell} 59, 1035–1048
4. McBlane, J. F., van Gent, D. C., Ramsden, D. A., Romeo, C., Cuomo, C. A., Gellert, M., and Oettinger, M. A. (1995) \textit{Cell} 83, 387–395
5. Roth, D. B., Nakajima, P. B., Menetski, J. P., Bosma, M. J., and Gellert, M. (1992) \textit{Cell} 70, 111–118
6. Ramsden, D. A., and Gellert, M. (1995) \textit{Genes Dev.} 9, 2409–2420
7. Livak, F., and Schatz, D. G. (1996) \textit{Mol. Cell. Biol.} 16, 609–618
8. Sadowski, M. J., Hesse, J. E., and Gellert, M. (1994) \textit{Nucleic Acids Res.} 22, 1805–1809
9. Cuomo, C. A., and Oettinger, M. A. (1994) \textit{Nucleic Acids Res.} 22, 1810–1814
10. van Gent, D. C., McBlane, J. F., Ramsden, D. A., Sadowski, M. J., Hesse, J. E., and Gellert, M. (1995) \textit{Cell} 81, 925–934
11. van Gent, D. C., Ramsden, D. A., and Gellert, M. (1996) \textit{Cell} 85, 107–113
12. Li, Z., Dordai, D. I., Lee, J., and Desiderio, S. (1996) \textit{Immunol.} 5, 575–589

\textsuperscript{2} H. Jiang and S. Desiderio, unpublished results.
Transposition of RAG Signal End Complexes

13. Lin, W. C., and Desiderio, S. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 2733–2737
14. Lin, W. C., and Desiderio, S. (1995) Science 260, 953–959
15. Ross, A. E., Vuica, M., and Desiderio, S. (2003) Mol. Cell. Biol. 23, 5308–5319
16. Kirch, S. A., Rathbun, G. A., and Oettinger, M. A. (1996) EMBO J. 17, 4881–4886
17. McMahon, C. J., Filippantonio, M. J., Rao, N., Spanopoulou, E., and Schatz, D. G. (1997) Mol. Cell. Biol. 17, 4544–4552
18. Sadofsky, M. J., Hesse, J. E., McBlane, J. F., and Gellert, M. (1993) Nucleic Acids Res. 21, 5644–5650
19. Steen, S. B., Han, J. O., Mundy, C., Oettinger, M. A., and Roth, D. B. (1999) Mol. Cell. Biol. 19, 3010–3017
20. Sekiguchi, J. A., Whitlow, S., and Alt, F. W. (2001) Mol. Cell 8, 1383–1390
21. Akamatsu, Y., Monroe, R., Dudley, D. D., Elkin, S. K., Gartner, F., Talukder, S. R., Takahama, Y., Alt, F. W., Bassing, C. H., and Oettinger, M. A. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 1209–1214
22. Liang, H. E., Hsu, L. Y., Cado, D., Cowell, L. G., Kelsoe, G., and Schlissel, M. S. (2002) Immunity 17, 639–651
23. Agrawal, A., and Schatz, D. G. (1997) Cell 89, 43–53
24. Jones, J. M., and Gellert, M. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 12926–12931
25. Melek, M., and Gellert, M. (2000) Cell 101, 625–633
26. Tsai, C. L., and Schatz, D. G. (2003) EMBO J. 22, 1922–1930
27. Elkin, S. K., Matthews, A. G., and Oettinger, M. A. (2003) EMBO J. 22, 1931–1938
28. Hesse, J. E., Lieber, M. R., Gellert, M., and Mizuuchi, K. (1987) Cell 49, 775–783
29. Lieber, M. R., Hesse, J. E., Lewis, S., Bosma, G. C., Rosenberg, N., Mizuuchi, K., Bosma, M. J., and Gellert, M. (1988) Cell 55, 7–16
30. Li, W., Chang, F. C., and Desiderio, S. (2001) Mol. Cell. Biol. 21, 3935–3946
31. Kemp, I. M., Sedgwick, S. G., and Jeggo, P. A. (1984) Mutat. Res. 132, 189–196
32. Schlissel, M., Constantinescu, A., Morrow, T., Baxter, M., and Peng, A. (1999) Genes Dev. 7, 2520–2532
33. Desiderio, S., Lin, W.-C., and Li, Z. (1996) Curr. Top. Microbiol. Immunol. 217, 45–59
34. Lee, J., and Desiderio, S. (1999) Immunity 11, 771–781
35. Perkins, E. J., Nair, A., Cowley, D. O., Van Dyke, T., Chang, Y., and Ramsden, D. A. (2002) Genes Dev. 16, 159–164
36. Agrawal, A., Eastman, Q. M., and Schatz, D. G. (1998) Nature 394, 744–751
37. Hsi, K., Melek, M., and Gellert, M. (1998) Cell 94, 463–470
38. Messier, T. L., O’Neill, J. P., Hou, S. M., Nicklas, J. A., and Finette, B. A. (2003) EMBO J. 22, 1381–1388
39. Lee, G. S., Neiditch, M. B., Sinden, R. R., and Roth, D. B. (2002) Mol. Cell. Biol. 22, 2068–2077
40. Tsai, C. L., Chatterji, M., and Schatz, D. G. (2003) Nucleic Acids Res. 31, 6180–6190
41. Clatworthy, A. E., Valencia, M. A., Haber, J. E., and Oettinger, M. A. (2003) Mol. Cell 12, 489–499