RAG-1 and RAG-2 initiate V(D)J recombination by introducing DNA breaks at recombination signal sequences flanking a pair of antigen receptor gene segments. Occasionally, the RAG proteins mediate two other alternative DNA rearrangements in vivo: the rejoining of signal and coding ends and the transposition of signal ends into unrelated DNA. In contrast, truncated, catalytically active “core” RAG proteins readily catalyze these reactions in vitro, suggesting that full-length RAG proteins directly or indirectly suppress these undesired reactions in vivo. To discriminate between direct and indirect suppression models, full-length RAG proteins were purified and characterized in vitro. From mammalian cells, full-length RAG-1 is readily purified with core RAG-2 but not full-length RAG-2 and vice versa. Despite differences in DNA binding activity, recombinase containing either core or full-length RAG-1 or RAG-2 possess comparable cleavage, rejoining, and end-processing activity, as well as similar usage preferences for canonical versus cryptic recombination signals. However, recombinase containing full-length RAG-2, but not full-length RAG-1, exhibits dramatically reduced transposition activity in vitro. These data suggest RAG-mediated transposition and rejoining are differentially regulated by the full-length RAG proteins in vivo (the former directly by RAG-2 and the latter indirectly through other factors) and argue that non-core portions of the RAG proteins have little or no direct influence over V(D)J recombinase site specificity.

The variable exons of antigen receptor genes are assembled by the site-specific rearrangement of component variable (V), diversity (D), and joining (J) gene coding segments by V(D)J recombination. This rearrangement process proceeds through two distinct phases (for reviews, see Refs. 1–3). In the first phase, two lymphoid cell-specific proteins, RAG-1 and RAG-2, collaborate to organize two discrete recombination signal sequences (RSS) abutting different receptor coding segments into a synaptic complex. Both RSSs in the synaptic complex contain conserved heptamer and nonamer elements, but the spacing between these elements in the two RSSs is typically different: in one RSS, the separation is 12 bp, whereas in the other, it is 23 bp. Within the synaptic complex, the RAG proteins coordinately introduce DNA double-strand breaks at both RSSs, positioned between the heptamer and adjacent coding sequence. The cleavage reaction generates two distinct DNA intermediates: blunt 5′-phosphorylated signal ends and coding ends terminating in covalently sealed DNA hairpin structures (4–6). Broken DNA intermediates are generated in two biochemical steps: first strand nicking at the 5′ end of the heptamer, followed by a direct transesterification reaction in which the 3′-OH exposed in the nicking step attacks the phosphodiester on the antiparallel DNA strand (7, 8). In the second phase of V(D)J recombination, signal ends are generally ligated to form precise signal joints, but coding ends are frequently processed and joined to create imprecise coding joints. Formation of both signal and coding joints requires components of the nonhomologous end-joining (NHEJ) machinery, including Ku70, Ku80, XRCC4, and DNA ligase IV (1). Coding joint formation additionally requires the activity of Artemis and DNA-PKcs, which collaborate in the resolution of hairpinned coding ends (9).

As full-length RAG proteins were found difficult to purify due to insolubility, cell-free assays of RAG activity were established by using truncated and catalytically active core regions of RAG-1 and RAG-2 (3, 7, 10). These core regions represent the minimal portions of RAG-1 and RAG-2 necessary to support rearrangement of exogenous and integrated substrates in cell culture assays of V(D)J recombination (11–15). Besides catalyzing the reactions that generate classical V(D)J recombinase intermediates, the core RAG proteins have been shown to catalyze other DNA strand cleavage and strand transfer reactions in vitro, including the nicking of DNA hairpins (16, 17), the cleavage of 3′-flap structures (18), the rejoining of coding and signal ends, as either open-shut joints (OSJ) or hybrid joints (HJ), in a reversal of the cleavage reaction (19), a mechanically related reaction in which signal ends are inserted into unrelated DNA via transposition (20, 21), and the resolution of transposition intermediates by disintegration or trans-esterification (22).

Since the core RAG proteins readily catalyze rejoining and transposition in vitro, one might expect that these events would frequently be observed in vivo. Interestingly, although evidence for their occurrence in vivo has been reported (23–25), they are nevertheless quite rare (especially transposition), raising the possibility that the regions of RAG-dispensable for V(D)J recombination play a central role in suppressing these
unwanted DNA rearrangements. Consistent with this hypothesis, full-length RAGs support significantly less HJ formation than core RAGs in V(D)J recombination assays performed in NHEJ-deficient cells (26), and accumulate signal ends to 10-fold lower levels than their core counterparts without affecting the level of coding ends produced (27). Taken together, these results provide evidence that full-length RAG post-cleavage complexes are rapidly disassembled in vivo, thereby imposing a limited window of opportunity for HJ formation and transposition to occur (27). Whether post-cleavage complex disassembly is the only mechanism to avoid these unwanted reactions, or whether the full-length RAG proteins provide an additional level of protection by suppressing them directly remains unclear.

In addition to accumulating HJs and signal ends in vivo, the core RAG proteins exhibit a modest reduction in recombination activity relative to full-length RAGs (11–15). Moreover, core RAG proteins exhibit defects in targeting specific antigen receptor loci relative to full-length RAG proteins (28–31). In principle, the recombination defects associated with core RAG proteins in vivo may be manifested at either the cleavage or joining phase of V(D)J recombination, and may be attributed to impaired or altered DNA binding or cleavage activity of the recombinase itself, loss of potential targets of post-translational modification, or modified association with protein factors involved in regulating V(D)J recombinase activity, its interaction with DNA targets, or the repair of its cleavage products.

Defining the molecular mechanisms by which the dispensable regions of the RAG proteins modulate the activity of the core recombinase would be greatly facilitated by obtaining full-length RAG proteins for study in vitro. We report here that, when fused to maltose-binding protein (MBP), full-length RAG-1 is readily purified when coexpressed in mammalian cells with core MBP-RAG-2 but not full-length MBP-RAG-2 and vice versa. All-core recombinase and recombinase containing either full-length RAG-1 or full-length RAG-2 exhibit some differences in DNA binding activity, yet when normalized for these differences, they possess comparable cleavage, rejoining, and end-processing activity, as well as similar usage preferences for canonical versus cryptic recombination signals. However, recombinase containing full-length RAG-2, but not full-length RAG-1, exhibits dramatically reduced transposition activity in vitro. These data suggest RAG-mediated transposition and rejoining are differentially regulated by the full-length RAG proteins for study in vitro.

**EXPERIMENTAL PROCEDURES**

**Expression Vectors**—Eukaryotic expression constructs encoding core RAG-1 or core RAG-2, each fused at the amino terminus to MBP without additional sequence tags (pcMR1 and pcMR2, respectively), have been described previously (32). Variations of these constructs encoding either full-length MBP-RAG-1 (pcMR1FL) or full-length MBP-RAG-2 (pcMR2FL) were assembled by PCR and/or subcloning by using pCRII (as noted in the text) and native or N-deacetylated oligonucleotide probe JH7265R (5'-GACATGGCCTACCTGCTCTGTTC-3'). These reactions were performed using the plasmid V(D)J recombination substrate pJH200 (40). Briefly, reactions (10 μl final volume) containing various amounts of cMR1/cMR2 (wild-type or D600A mutant), cMR1/FLMR2, or FLMR1/ cMR2 (as noted in the text) and 25 ng of pHJ200 (supercoiled or AatII linearized) were assembled under binding conditions used for EMSA, except that 1 mM MgCl2 was substituted with 1 mM MnCl2. Reactions were incubated for 1 h at 37 °C and then diluted 2-fold with TE (10 mM Tris (pH 8.0), 1 mM EDTA). An aliquot of the diluted sample (15 μl) was fractionated on a 7% native polyacrylamide gel (1× TBE, 19:1 acrylamide:bis(acrylamide), and reaction products were transferred to nylon membrane (Zeta Probe GT, Bio-Rad) in 0.5× TBE for 18 h at 20 V and at 4 °C. Membranes were subjected to Southern blotting using a 5′-labeled oligonucleotide probe JH7181, 5′-ACCTCAGAATCTCACTGATGTTGTTCACAGGCTCGGTTGC-3′. The membranes were incubated for 2 h at 42 °C in hybridization buffer (6× SSPE, 5× Denhardt’s reagent, 20% formamide, 100 μg/ml single-stranded salmon sperm DNA, 0.1% SDS, and 1.25× dextran sulfate), and then incubated with the probe for 18 h under the same conditions. Membranes were washed twice for 15 min at 60 °C in buffer WB (2× SSC, 0.1% SDS) and hybridization products visualized using a PhosphorImager (Amersham Biosciences).

**In-gel Cleavage and Transposition Assays**—Prepared RAG-RSS complexes were assayed for cleavage and transposition activity using in-gel enzyme assays as described previously (32, 39).

Plasmid Cleavage and Hybrid Joint Assays—These assays were performed using the plasmid V(D)J recombination substrate pHJ200 (40). Briefly, reactions (10 μl final volume) containing various amounts of cMR1/cMR2 (wild-type or D600A mutant), cMR1/FLMR2, or FLMR1/ cMR2 (as noted in the text) and 25 ng of pHJ200 (supercoiled or AatII linearized) were assembled under binding conditions used for EMSA, except that 1 mM CaCl2 was substituted with 1 mM MgCl2. Reactions were incubated for 1 h at 37 °C and then diluted 2-fold with TE (10 mM Tris (pH 8.0), 1 mM EDTA). An aliquot of the diluted sample (15 μl) was fractionated on a 7% native polyacrylamide gel (1× TBE, 19:1 acrylamide:bis(acrylamide), and reaction products were transferred to nylon membrane (Zeta Probe GT, Bio-Rad) in 0.5× TBE for 18 h at 20 V and at 4 °C. Membranes were subjected to Southern blotting using a 5′-labeled oligonucleotide probe JH7181, 5′-ACCTCAGAATCTCACTGATGTTGTTCACAGGCTCGGTTGC-3′. The membranes were incubated for 2 h at 42 °C in hybridization buffer (6× SSPE, 5× Denhardt’s reagent, 20% formamide, 100 μg/ml single-stranded salmon sperm DNA, 0.1% SDS, and 1.25× dextran sulfate), and then incubated with the probe for 18 h under the same conditions. Membranes were washed twice for 15 min at 60 °C in buffer WB (2× SSC, 0.1% SDS) and hybridization products visualized using a PhosphorImager.

For detection of hybrid joints, PCR (100 μl) were assembled containing an aliquot (2 μl) of the remaining diluted sample as a template, HJ primers A (JH7265R, 5′-GACATGGCCTACCTGCTCTGTTC-3′) and B (J- H7419R, 5′-CTACCACAGTCTACGTTTC-3′) (500 pmol each) and native or N-deacetylated oligonucleotide (2.5 units; Invitrogen) in PCR buffer (50 mM Tris (pH 8.4), 50 mM KCl, 200 μM each dNTP, 1.5 mM MgCl2). PCRs were subjected to initial denaturation (95 °C, 5 min), 25 cycles of amplification (95 °C for 15 s, 50 °C for 30 s, and 72 °C for 20 s), and a final extension (72 °C for 5 min) using a PTC-100 Thermal Cycler (MJ Research, Waltham, MA). As a positive control, a fragment of the chloramphenicol acetyltransferase gene on pJH200 was amplified from the same reaction sample volume by 15 cycles of PCR under the above conditions using gene-specific primers (CATFORF, 5′-ACCAGACCTTGT-CAGCTGGA-3′; CATREV, 5′-CTACACAGGAGTCTGTAGGCA-3′). PCR products were resolved on 2% agarose gels (1:1 agarose:Ampliprige- agarose (Bio-Rad)) and visualized by staining with ethidium bromide. PCR products noted, PCR products were gel-purified and cloned using the TOPO XL PCR cloning kit (Invitrogen). Randomly selected clones were sequenced.

[1] L. Wang and P. C. Swanson, unpublished data.
Disintegration Assay—This assay was performed using the branched substrates and reaction conditions described by Melek and Gellert (22). Briefly, cMR1/cMR2 (wild-type or D600A mutant), cMR1/FLMR2, or FLMR1/cMR2 (∼50 ng each) was incubated with a radiolabeled branched substrate (∼0.02 pmol) in the presence of CaCl₂ under binding conditions as used for EMSA (10-μl reaction volume) for 10 min at 25 °C. Subsequently, MgCl₂ was added to a final concentration of 5 mM, and samples were incubated for 1 h at 37 °C. Reactions were terminated by adding 2 volumes of loading buffer (95% formamide, 10 mM EDTA) and heated for 2 min at 75 °C. Reaction products were fractionated on 15% polyacrylamide-urea gels and visualized using a PhosphorImager.

RESULTS

Purification of Coexpressed Core and Full-length RAG Proteins—Early attempts to purify full-length RAG proteins were largely unsuccessful due to poor protein solubility. Thus, all biochemical assays of RAG activity have been conducted with more readily purified truncated, catalytically active core forms of RAG-1 and RAG-2. We were interested in determining whether full-length RAG-1 and RAG-2 are more amenable to purification when expressed as MBP fusion proteins. Based on our previous experience indicating that core MBP-RAG-1/2 proteins are recovered in higher yield and with greater activity when the two proteins are coexpressed, rather than individually expressed, in mammalian cells, and evidence suggesting that RAG-1 and RAG-2 form a complex in the absence of DNA (41–44), we speculated that coexpression of core MBP-RAG-1 with full-length MBP-RAG-2 might promote the solubility of full-length MBP-RAG-2 via RAG-1-RAG-2 interactions and vice versa. To explore this possibility, we cotransfected 293 cells with expression constructs encoding either the core or full-length forms of MBP-RAG-1 (cMR1 and FLMR1, respectively) and MBP-RAG-2 (cMR2 and FLMR2, respectively) in pairwise combinations (Fig. 1A). The coexpressed RAG proteins were purified by amylose affinity chromatography and analyzed by staining SDS-polyacrylamide gels with silver (Fig. 1B).

We find that FLMR2 is readily purified when coexpressed with cMR1, but its recovery is poor when coexpressed with FLMR1. Similarly, FLMR1 is purified when coexpressed with cMR2 but not FLMR2. The yields of both RAG proteins in the cMR1/FLMR2 and FLMR1/cMR2 preparations were about 2-fold less than their counterparts in the cMR1/cMR2 preparation. The observed reduction in the yield of cMR1/FLMR2 relative to cMR1/cMR2 is not attributable to differences in RAG-2 expression, as levels of cMR2 and FLMR2 in whole cell lysates were comparable as assessed by immunoblotting using antib-MBP antibodies (data not shown). In contrast, FLMR1 is consistently present at ∼2-fold lower levels than cMR1 in whole cell lysates, providing a partial explanation for the poorer recovery of FLMR1/cMR2 compared with cMR1/cMR2. The reason why full-length RAG-1 is expressed at lower levels than core RAG-1 in 293 cells remains unclear. The biochemical basis of the poor recovery of coexpressed full-length RAG-1 and full-length RAG-2 is also unknown but, based on earlier reports (3), is probably attributed to the poor solubility of the full-length RAG-1-RAG-2 complex.

Recombinase Incorporating FLMR1 or FLMR2 Displays Altered RSS Binding Activity Compared with All-core Recombinase—To assess how the inclusion of the dispensable portion of RAG-1 and/or RAG-2 into the V(D)J recombinase affects the DNA binding activity of the protein complex, the four purified RAG protein preparations were tested by EMSA for their ability to form single or paired RSS complexes with intact, prenickled, or pre-cleaved RSS substrates (Fig. 1C). Core RAG-1 and RAG-2 assemble two distinct protein-DNA complexes on a single RSS, termed SC1 and SC2 (Fig. 2, lane 1). Both of these complexes were shown previously to contain a RAG-1 dimer, and either one (SC1) or two (SC2) subunits of RAG-2 (32).

Compared with cMR1/cMR2, cMR1/FLMR2 forms complexes of similar mobility (Fig. 2, compare lane 4 to lane 5), but their abundance relative to their all-core counterparts is about 4-fold less, as judged by comparison to 2-fold serial dilutions of cMR1/cMR2. Interestingly, the ratio of SC1 to SC2 is ∼5:1 in the cMR1/cMR2 samples, whereas this ratio is ∼1:1 in the cMR1/FLMR2 sample, suggesting that the carboxyl-terminal portion of RAG-2 helps promote SC2 formation. In contrast to cMR1/cMR2 and cMR1/FLMR2, discrete protein-DNA complexes were not observed by EMSA for FLMR1/cMR2 under these conditions, although a broad band is detected whose mobility lags comparable complexes containing core RAG-1 (Fig. 2, compare lane 4 to lane 6). For FLMR1/FLMR2, only faint binding could be detected, reflecting the low abundance of protein in the sample. Hence, further studies compared the activities of only the first three RAG protein preparations.

Certain architectural DNA binding factors of the HMG box family, of which HMG-1 is a prototypical member, are known to promote the assembly of RSS complexes containing core RAG-1 and RAG-2 and stimulate RAG-mediated cleavage within these complexes in vitro (36, 45–47). Consistent with previous re-
Pre-assembled RAG-RSS Complexes Containing Core or Full-length RAG-1 or RAG-2 Possess Comparable Cleavage Activities in Vitro—The distinct DNA binding properties of core and full-length RAG-1 and RAG-2 pose difficulties in interpreting standard cleavage assays performed without prior fractionation of protein-DNA complexes, because such assays cannot distinguish whether observable differences in cleavage activity are due to differences in DNA binding activity or altered catalytic activity. To overcome this problem, we have used an in-gel cleavage assay, described previously (39), to directly compare the catalytic activities of pre-formed RSS complexes assembled with cMR1/cMR2, cMR1/FLMR2, and FLMR1/cMR2. The three RAG protein preparations were incubated under binding conditions (in the presence of Ca\(^{2+}\)) with intact or pre-nicked 12- or 23-RSS substrates in the absence or presence of HMG-1 and/or cold partner DNA in combinations comparable with those described in Fig. 2B. As a negative control, a binding reaction containing cMR2 coexpressed with a form of cMR1 bearing a single active site mutation (D600A; hereafter MT-cMR1) was assembled under conditions to form a PC. Samples were fractionated by EMSA, and the gel was soaked in buffer containing Mg\(^{2+}\) for 1 h at 37 °C to initiate DNA cleavage within the protein-DNA complexes. DNA derived from the SC, HSC, and PC species was recovered, normalized, and fractionated by denaturing gel electrophoresis (Fig. 3). As expected from previous results (32), cMR1-cMR2 complexes incorporating HMG-1 possess greater nicking and transesterification activity than their counterparts lacking HMG-1, particularly on 23-RSS substrates. Moreover, the addition of appropriate cold partner RSS stimulates about a 4-fold increase in transesterification in the PC relative to the HSC2 species. Interestingly, we find that, despite differences in DNA binding activity, the catalytic activity of SC, HSC, and PC complexes assembled with cMR1/cMR2 and FLMR1/cMR2 are quite similar, regardless of the type of RSS substrate bound. In contrast, when compared with cMR1/cMR2, cMR1/FLMR2 exhibits a modest impairment in the catalysis of both nicking (−2-fold) and transesterification (−3-fold) in complexes assembled on 23-RSS substrates in the presence of HMG-1 (i.e. HSC1, HSC2, and PC), but this effect is less apparent when 12-RSS complexes are similarly analyzed. Interestingly, unlike oligonucleotide substrates, cMR1/FLMR2 cleaves a plasmid V(D)J recombination substrate as well as cMR1/cMR2 (see Fig. 5C). This observation raises the possibility that efficient RSS binding and cleavage by cMR1/FLMR2 requires protein-DNA interactions ranging beyond the RSS that are not supported by oligonucleotide substrates.

Pre-assembled Signal End Complexes Containing Full-length RAG-2, but Not Full-length RAG-1, Exhibit Impaired

![Diagram of DNA binding properties](image-url)
Discrete RAG-RSS complexes containing core or full-length RAG-1 or RAG-2 possess comparable catalytic activity. A, cMR1/cMR2, cMR1/FLMR2, or FLMR1/cMR2 was incubated with intact 12-RSS (left) or 23-RSS (right) substrates with or without HMG-1 and/or cold partner DNA (12-RSS or 23-RSS) under binding conditions in the same combinations as in Fig. 2B and subjected to an in-gel cleavage assay (see "Experimental Procedures"). As a negative control, paired complexes were also assembled with a form of cMR1/cMR2 that is catalytically inactive (RAG-1 D600A), and analyzed for cleavage in parallel (lane 1). Reaction products recovered from complexes indicated above the gel were normalized and fractionated by denaturing gel electrophoresis. Positions of nicked and hairpin products are shown at left and right. The hairpin species shown on gels of 12- and 23-RSS substrates have been shown previously to co-migrate. The percentage of nicked (%N) and hairpin (%HP) products in each lane is quantified below the gel and accounts for slight variations in the amount of DNA actually loaded. B, in-gel cleavage
Regulation of RAG-mediated Transposition and Rejoining

Fig. 4. Full-length RAG-2 but not full-length RAG-1 suppresses transposition in vitro. A, RAG-mediated transposition of radiolabeled signal ends into a cold target plasmid covalently links the signal ends to the target DNA and linearizes the plasmid. B, wild-type or active site mutant (D600A) cMR1 coexpressed with cMR2 (WT-cMR1/cMR2 or MT-cMR1/cMR2, respectively), cMR1/FLMR2, or FLMR1/cMR2 was incubated with a radiolabeled 23-SE with HMG-1 and cold partner DNA (12-SE), under binding conditions as indicated above the gel, and then subjected to an in-gel transposition assay (see “Experimental Procedures”). An autoradiograph of the DEAE-cellulose paper to which the DNA was transferred is shown here, with the position of the signal end complex (SEC) shown at right. C, reaction products were isolated from the SECs using the autoradiograph in B and analyzed on a native linear 4–20% gradient gel. Linearized 5’-end-labeled pDNA1 and pre-cleaved substrate (lanes 1 and 2; indicated at right) serve as markers. The percentage of recovered plasmid DNA that is linearized is quantified below the gel (%TP). Similar results were obtained with radiolabeled 12-SE substrates (data not shown).

Transposition Activity Compared with Their Core Counterparts—Core RAG proteins possess the ability to integrate cleaved signal ends into nonhomologous target DNA via transposition in vitro (20, 21). This observation contrasts with the rarity of such events in vivo, raising the possibility that the dispensable regions of RAG-1 or RAG-2 play a direct role in suppressing transposition. To examine this possibility, we used an in-gel transposition assay to directly compare transposition activity in pre-formed signal end complexes (SEC) assembled with cMR1/cMR2, cMR1/FLMR2, and FLMR1/cMR2. In this assay, described previously (32), plasmid DNA embedded in the native polyacrylamide gel serves as the target for transposition of signal ends by the RAG proteins in the fractionated SEC. Double-ended insertion of signal ends linearizes the plasmid, resulting in the covalent linkage of a radiolabeled signal end (Fig. 4A). An autoradiograph of the bound DNA following electrophoretic transfer to DEAE-cellulose shows that cMR1/cMR2 (both wild-type and mutant forms) and cMR1/FLMR2 form comparable levels of the SEC, but cFLMR1/cMR2 does not (Fig. 4B). The observation that full-length RAG-2 forms the SEC as well as core RAG-2, but not the SC, HSC, or PC, suggests that the presence of coding sequence interferes with the stability of complexes containing full-length RAG-2. However, despite the relatively facile assembly of the SEC by cMR1/FLMR2, transposition activity within the SEC is dramatically reduced (~10-fold) compared with cMR1/cMR2 (Fig. 4C). In contrast, even though SEC formation by FLMR1/cMR2 is relatively poor, complexes that do form support signal end transposition almost as well as the all-core recombinase. Thus, full-length RAG-2, but not full-length RAG-1, significantly suppresses transposition in vitro.

Core and Full-length RAG-2 Exhibit Comparable RAG-mediated Rejoining Activity, but Full-length RAG-1 Displays Reduced Cleavage Activity at Some Cryptic RSSs and Improves the Fidelity of Disintegration Reactions Compared with Core RAG-1—Hybrid joints form when signal ends are rejoined to different coding ends through a reversal of the RAG-mediated cleavage reaction (1). The “dispensable” regions of RAG-1 and RAG-2 have been implicated in the suppression of HJ formation in cell culture models of V(D)J recombination (26). To test whether this effect is directly or indirectly mediated by the non-core portion of RAG-1 or RAG-2, we used PCR to amplify hybrid joints formed on a plasmid V(D)J recombination substrate, called pJH200 (40), following incubation with cMR1/cMR2, cMR1/FLMR2, or FLMR1/cMR2 (Fig. 5B, top and middle panels). A schematic representation of the assay is depicted in Fig. 5A. Amplification of a chloramphenicol acetyltransferase gene fragment was also performed under the same PCR conditions as a positive control for the presence of the pJH200 template (Fig. 5B, lower panel). In experiments in which supercoiled pJH200 was incubated with WT-cMR1/cMR2, no amplicons were detected with primers A and B. However, when this substrate was incubated with cMR1/cMR2, cMR1/FLMR2, or FLMR1/cMR2, we not only detected an amplicon of the expected size (~190 bp), but we also observed two other unanticipated products of ~320 (major) and ~400 bp (minor). The abundance and distribution of the PCR products were quite comparable between the three RAG protein preparations, although at the highest protein concentrations, about 2-fold more of the 190- and 400-bp PCR products were detected in reactions containing FLMR1/cMR2 than in reactions containing cMR1/cMR2 and cMR1/FLMR2.

To evaluate the composition of the PCR products, the three amplicons were cloned and analyzed by sequencing (Fig. 5C). As expected, the 190-bp product detects rejoining of the canonical 23-RSS and the coding end abutting the canonical 12-RSS. Notably, all sequences of “canonical” HJs derived from samples containing cMR1/cMR2, cMR1/FLMR2, or FLMR1/cMR2 were precise. Interestingly, we find that the 320-bp product detects joining of the 23-RSS to a site located 130-bp upstream of the canonical 12-RSS. Three lines of evidence suggest that this joining event represents a bona fide HJ. First, 11 of 13 sequences (representing clones derived from samples containing cMR1/cMR2, cMR1/FLMR2, or FLMR1/cMR1) possessed a single “T” nucleotide inserted between the cryptic coding end and the heptamer of the 23-RSS, likely derived from asymmetric hairpin opening to form a palindromic (P) nucleotide (see Fig. 5C; the other two clones were precise). Second, as discussed below, a broken DNA intermediate of the appropriate size (~330 bp) is observed after Southern hybridization of the cleaved pJH200 plasmid DNA (see Fig. 5D). Third, this site has been mapped previously as a rearrangement hotspot, termed 6131, in a similar plasmid V(D)J recombination substrate and experiments using pre-nicked 12-RSS (left) or 23-RSS (right) substrates were performed as described above and presented in the same order. Quantitative analysis of hairpin formation is shown below the gel. Note that the distribution of protein-DNA complexes formed with intact and pre-nicked RSS substrates resembled those shown in Fig. 2, and all reaction products shown in each panel of A and B are derived from a single native gel subjected to the in-gel cleavage reaction. The abundance and distribution of the cleavage products observed are representative of independent experiments.
identified as a cryptic 12-RSS (48, 49).

The ~400-bp PCR product represents a wider array of joining events occurring between the 23-RSS and positions located between 50 and 90 bp upstream of the 6131 cryptic 12-RSS. Identifying these events was problematic due to the abundance of the 350-bp PCR product which gave rise to many contaminating clones, resulting in too few sequences to draw comparisons between the different RAG preparations. Nevertheless, we identified three unique integration sites, all of which contained a CAC element (Fig. 5C). In each case, however, the orientation of this sequence and/or the location where the 23-RSS is linked is inconsistent with rejoining occurring after standard RAG-mediated cleavage at the 5′ end of the CAC sequence. Moreover, no P nucleotides are evident at the sequence junctions. Thus, either these joints arose through aberrant cleavage followed by precise rejoining, or they originated by a different mechanism. Interestingly, adjacent to the CAC elements present in two of the three sites lie GC-rich sequences, which are preferred sites for RAG-mediated transposition (20, 21). If transposition underlies these DNA rearrangements, it did not involve cleavage at either the canonical or the 6131 cryptic 12-RSS, as both are intact (which is not surprising given the position of the primer A). The most probable explanation, given the large number of cryptic sites in pJH200 (48, 49), is that cleavage occurred at the 23-RSS and a cryptic RSS located downstream of primer B, followed by insertion of the 23-signal end into a site upstream of the 6131 cryptic 12-RSS.

We considered the possibility that substrate topology influ-
ences the composition of PCR products observed in this assay. Therefore, the assay was repeated using linearized pJH200 (Fig. 5B, middle panel). We find that both the size and distribution of the major PCR products are largely unchanged, but the spectrum of products is more focused toward the major amplicons when linear DNA is used.

The similar abundance of PCR products observed in samples containing cMR1/cMR2, cMR1/FLMR2, or FLMR1/cMR2 suggests that the three protein preparations have comparable cleavage activities when assayed on plasmid substrates. To test this possibility, a portion of each cleavage reaction used for the PCR shown in Fig. 5B was fractionated on a native polyacrylamide gel, and the reaction products were analyzed by Southern hybridization using an oligonucleotide probe specific for the coding sequence abutting the canonical 12-RSS (Fig. 5D).

Three major cleavage products are detected by this probe for reactions containing supercoiled pJH200 and either cMR1/cMR2 or cMR1/FLMR2 (designated “a–c”, Fig. 5D). These products are comparable in both their abundance and distribution, with an approximate ratio of 4:95:1 (“a–c”). In contrast, in the presence of similar amounts of FLMR1/cMR2, products “a” and “b” are produced at 5–7-fold lower levels, but product “c” formation is selectively suppressed 15–20-fold relative to cMR1/cMR2. The reduced cleavage activity associated with FLMR1/cMR2 at canonical and cryptic sites is likely attributed, at least in part, to reduced DNA binding activity as shown in Fig. 2, but we cannot fully explain the selective reduction of cleavage at the site yielding the “c” product. Similar results were obtained when linear pJH200 is used as a cleavage substrate.

Products “a” and “b” likely arise from cleavage at the 23-RSS and either the canonical (“a”) or 6131 cryptic (“b”) 12-RSS, based on their predicted size, relative distribution, and involvement in HJ formation. Product “c”, estimated at ~80–100 bp, most likely arises from cleavage at the canonical 12-RSS and cleavage downstream of the probe sequence. We have identified a heptamer-like sequence (5′-CAACCAAT-3′) that lies 80 bp downstream of the canonical 12-RSS heptamer and is positioned in the same orientation. In this orientation, HJ formation would create a deletion in the plasmid and a small excision circle, neither of which would be detectable in this assay because they occur upstream of primer A. To provide further evidence for the composition of “a–c”, cleavage products were subjected to digestion with HindIII (Fig. 5E). Digestion of uncleaved pJH200 with HindIII yields a 349-bp fragment detectable using the oligonucleotide probe. Product “a” migrates slightly faster than the HindIII fragment and is digested with HindIII. However, the resulting product cannot be uniquely visualized, as it comigrates with the HindIII digestion product arising after RAG-mediated single site cleavage at the 23-RSS. In contrast, HindIII does not digest products “b” and “c”, consistent with the predicted locations of RAG cleavage sites on these fragments.

Mechanistic similarities between HJ formation and retroviral disintegration have been drawn previously by others (19). Based on this similarity, cMR1/cMR2 and cMR1/FLMR2 might be expected to comparably catalyze disintegration of transposition intermediates since the two protein preparations support nearly equivalent levels of HJ formation as assessed by PCR-based assays. To test this hypothesis, we incubated cMR1/cMR2, cMR1/FLMR2, and FLMR1/cMR2 with a preassembled disintegration substrate containing a 12-RSS described by Melek and Gellert (22) (Fig. 6). We find that WT-cMR1/cMR2 and cMR1/FLMR2 rejoined the top strand of the transposition target with similar efficiency, but FLMR1/cMR2 catalyzed this disintegration reaction to lower levels, probably attributed in part to poorer substrate binding (data not shown). Interestingly, cMR1/cMR2 and cMR1/FLMR2 exhibited a similar pattern and distribution of alternative reaction products, but FLMR1/cMR2 shows a more restricted pattern of products when normalized for band intensity, suggesting that full-length RAG-1 may play a role in improving the fidelity of disintegration in the resolution of transposition intermediates.

Taken together, these data show that cMR1/FLMR2, when compared with cMR1/cMR2, displays significantly less transposition activity but catalyzes similar levels of DNA cleavage, HJ formation, and disintegration. On the other hand, when normalized for reduced DNA binding activity, FLMR1/cMR2 possesses catalytic activity that is reasonably comparable in all respects to cMR1/cMR2. However, when compared with cMR1/cMR2, FLMR1/cMR2, but not cMR1/FLMR2, demonstrates selective suppression of cleavage at one of two predominant cryptic RSSs in pJH200 and reduces alternative reaction products resulting from resolution of transposition intermediates.
DISCUSSION

Differential Modulation of Hybrid Joint Formation and Transposition Activity by Full-length RAG-1 and RAG-2—During V(D)J recombination, RAG-mediated cleavage at RSS pairs generate four DNA ends, two signal ends and two coding ends, whose subsequent repair typically yields one signal joint and one coding joint. However, two alternative outcomes to these standard V(D)J rearrangement products are supported by the V(D)J recombinase as follows: the rejoining of signal ends and coding ends (as either OSJ or HJ) and the insertion of signal ends into nonhomologous target DNA via transposition (1). Both types of outcomes have been detected in vitro, although the latter reaction has been convincingly documented in only one report (24). Nevertheless, such alternative reaction outcomes may contribute to the formation of potentially oncogenic chromosomal translocations, particularly if recombination intermediates arising from RAG-mediated cleavage at cryptic RSSs outside antigen receptor loci are also considered.

Previous studies suggest nonstandard RAG-mediated reactions can be controlled in two general ways: by reversing the reaction outcome (22) or by suppressing reaction initiation (26). These control mechanisms may be directly mediated by the RAG proteins themselves or may be contributed by factors and forces collaborating with or acting beyond the RAG proteins. Establishing a direct role for the RAG proteins in controlling unwanted DNA rearrangements has been problematic due to the difficulty in purifying full-length RAG-1 and RAG-2. In this study, we have purified full-length RAG-1 and RAG-2 as MBP fusion proteins, and we compared their activity to their core counterparts with respect to DNA binding, RSS substrate cleavage, transposition, and HJ formation using a combination of mobility shift and in-gel and in-tube assays. The results presented here extend previous studies by demonstrating the following: (i) full-length RAG-1 is readily purified with core RAG-2, but not full-length RAG-2, and vice versa; (ii) full-length RAG-1 exhibits catalytic activities comparable with core RAG-1, despite its relative inability to assemble RAG-RSS complexes in vitro; (iii) transposition and two different rejoining reactions (HJ formation and disintegration), although mechanistically similar, are nevertheless distinct reactions, with the former reaction being specifically suppressed by full-length RAG-2 but not full-length RAG-1; and (iv) recombinase incorporating full-length RAG-2 cleaves cryptic RSSs with a frequency comparable with all-core recombinase, whereas recombinase containing full-length RAG-1 exhibits reduced cleavage of a cryptic RSS whose intersignal distance is less than 100 bp; and (v) the non-core regions of the RAGs do not appear to alter the profile of RAG-mediated P nucleotide insertion associated with HJ formation.

While this work was in progress, others presented evidence that full-length RAG-2 suppresses transposition by interfering with target site capture (50, 51). Data presented here are consistent with this conclusion. However, whether full-length RAG-1 similarly suppresses transposition could not be unambiguously determined from the study that examined the activity of full-length RAG-1 (51). By using in-tube assays, the authors of that study showed that recombinase containing full-length RAG-1 exhibits a 25-fold lower level of cleavage activity and at least a similar reduction in transposition activity than its counterpart containing core RAG-1. However, since no DNA binding data were presented in that study, one cannot determine whether recombinase containing full-length RAG-1 supports less cleavage and transposition than its counterpart containing core RAG-1 due to an intrinsic impediment to RAG-RSS complex assembly, a low fraction of active protein, or a defect in catalysis. We show here for the first time that when preformed RAG-RSS complexes containing full-length RAG-1 are analyzed using in-gel assays, they possess cleavage and transposition activity comparable with those containing core RAG-1. This conclusion is consistent with the results of a previous study demonstrating that core and full-length RAG-1 support the formation of coding end intermediates to similar levels in V(D)J recombination assays performed in cell lines (27). In principle, the generally lower DNA binding activity of full-length RAG-1 (and to a lesser extent RAG-2, particularly on intact RSS substrates) could be attributed to a reduction in the fraction of active protein recovered after purification. Although speculative, we consider it equally plausible that full-length RAG proteins require longer range interactions with DNA or association with other proteins to facilitate RAG-RSS complex assembly, which are otherwise unnecessary for efficient core RAG-RSS complex formation. Identifying these requirements may reveal factors and forces that impose additional regulation on the initiation of V(D)J recombination.

There is also a discrepancy between the two previous studies regarding whether full-length RAG-2 suppresses HJ formation. In one study, full-length and core RAG-2 were shown to support HJ formation to comparable levels when incubated with core RAG-1 (each protein was individually expressed) and assayed using a plasmid V(D)J recombination substrate (51). In the other study, full-length RAG-2 coexpressed with core RAG-1 (each with a different arrangement of tags from the other study) was demonstrated to support considerably less HJ formation (albeit detectable) than the all-core recombinase when assayed on a body-labeled linear DNA substrate (50). Our findings are consistent with the former study, despite differences in fusion partners, expression systems, and HJ substrates. Substrate topology can also be ruled out as an explanation for the apparent discrepancy, as we show that HJ formation is essentially equivalent using either supercoiled or linearized pHJ200. We also extend the previous results by documenting that disintegration is catalyzed to similar levels with recombinase containing either core or full-length RAG-2. Therefore, these results cause us to conclude that, despite some mechanistic similarities, HJ formation and transposition are separable reactions whose outcomes are differentially regulated in the cell.

In addition to more fully describing how the full-length RAG proteins modulate recombinase activity in DNA binding, transposition, and HJ assays, these data significantly extend the previous studies by demonstrating that recombinase site specificity and the profile of RAG-mediated P nucleotide insertion associated with HJ formation is largely unaffected by the inclusion of non-core regions of RAG-1 or RAG-2 into the V(D)J recombinase. Moreover, new evidence is presented that suggests full-length RAG-1 improves the fidelity of disintegration reactions that resolve transposition intermediates. The significance of these latter findings is discussed in more detail below.

Implications for the Regulation of Alternative Outcomes of V(D)J Recombination—The findings presented here that full-length RAG proteins support HJ formation to levels comparable with (RAG-2), or perhaps slightly exceeding (RAG-1), their core counterparts in vitro contrasts with an earlier report (26) suggesting that full-length RAG proteins support lower levels of hybrid joint formation in NHEJ-deficient cell lines than their core counterparts. However, how the non-core portions of the RAG proteins function to suppress HJ formation could not be determined from that study. Since the data presented here argue that the non-core regions do not directly inhibit HJ formation in vitro, this evidence raises the possibility that other factors associating with or modifying the RAG-dispensable regions may act to inhibit HJ formation in vitro. In principle, such factors might suppress HJ formation by any of
several mechanisms as follows: (i) sequestering the coding ends or sterically impairing joining; (ii) inducing conformational changes in the RAG proteins after RSS cleavage that inhibit joining or that facilitate the transfer of coding ends from the four-ended post-cleavage complex to components of the NHEJ machinery; or (iii) catalyzing a post-translational modification that promotes the inactivation, disassembly, or degradation of the RAG complexes themselves. At first approximation, mechanisms requiring stable association of factors with the RAGs seem unlikely, given the difficulty in identifying RAG-interacting proteins, although some potential candidates have not yet been completely investigated for their role in V(D)J recombination (52, 53). However, association of such factors might require prior RAG-RSS complex formation, and/or conformational changes in the RAGs after RSS cleavage, to expose an interface necessary for stable interaction with the RAG-RSS complex. Mechanisms involving post-translational modifications may be more plausible, as a role for phosphorylation in the regulation of V(D)J levels in the cell cycle has been established (54, 55), and the amino-terminal non-core portion of RAG-1 functions as an E3 ubiquitin ligase in vitro (56), although the physiological target remains unknown.

In contrast to HJ formation, which is likely mediated within a post-cleavage RAG complex containing both signal and coding ends, the RAG proteins may catalyze transposition in a complex containing only signal ends. Thus, the dissociation of coding ends from a four-ended post-cleavage complex eliminates the possibility of the former reaction but not the latter. Therefore, there may be greater need to directly inhibit transposition. The finding here that full-length RAG-2, but not full-length RAG-1, specifically suppresses transposition provides a simple, direct means to reduce the likelihood of this reaction. However, on the rare occasions where transposition does occur, the RAG proteins may resolve the intermediates by catalyzing a disintegration reaction (22). The data shown here that recombinase containing core or full-length RAG-2 possesses similar activity in disintegration assays, and that full-length RAG-1 reduces the prevalence of alternative reaction outcomes, suggest full-length RAGs retain the capacity to catalyze disintegration and enhance reaction fidelity as a defense against rare transposition events.

Implications for V(D)J Recombinase Target Site Selection, Post-cleavage Sequence Specificity, and End Processing—Recombinase containing core RAG-2 does not support efficient V-to-DJ rearrangement in cell lines and animals (28–30), but this deficiency is complemented by coexpression of the non-core portion of RAG-2 with core RAG-2 (29). The defect, although general, is more severe for 3' V_{H} and 3' V_{P} segments than for V_{H}/V_{N}/V_{J}/V_{β} segments (29). This observation raises the possibility that the carboxyl-terminal portion of RAG-2 directly or indirectly enables the recombinase to discriminate between RSSs of different composition. Although we do not compare different V gene segments for their suitability as substrates for RAG-mediated cleavage, our finding that recombinase containing either core or full-length RAG-2 cleaves a well defined cryptic RSS within pJH200 with similar frequency in vitro suggests that recombinase incorporating full-length RAG-2 has no better capacity to discriminate between RSSs of different composition than does core RAG-2. Thus, rather than influencing V segment usage directly, or indirectly through RAG-1 or HMG-1, these data suggest that the dispensable portion of RAG-2 acts indirectly through other factors to guide the patterning of V gene segment usage.

Our finding that FLMR1/cMR2 does not cleave a proximal cryptic RSS ~80 bp downstream of the canonical 12-RSS as well as cMR1/cMR2 is interesting, as it demonstrates full-length RAG-1 may influence the site specificity of the V(D)J recombinase. However, these results raise a question as to why cleavage is suppressed at this site and not at the 6131 cryptic 12-RSS. One possible explanation is that the size of FLMR1 sterically precludes synopsis or cleavage by the recombinase at this site due to the short intersignal distance between this cryptic RSS and either the 12- or 23-RSS. Alternatively, or in addition, the recombinase may recognize this cryptic RSS as a 12-RSS, in which case the cleavage event represents 12/12 cleavage that is specifically suppressed by full-length RAG-1. These possibilities are currently being investigated.

The data presented here show that recombinase containing either core or full-length RAG-2 cleaves the 6131 cryptic 12-RSS rather than the canonical 12-RSS about 5% of the time when both signals are present on the same substrate, but this value may underestimate the true frequency of cryptic site usage if single-site cleavage of the canonical 12-RSS occurs after cleavage of the cryptic 12-RSS. Interestingly, the frequency of cleavage at the cryptic site is close to, but slightly higher than, the observed recombination frequency between these sites (~1% (49)). As discussed previously by others (57), this difference might reflect a level of sequence specificity that is imposed by the RAGs on the recombination intermediates to reduce the likelihood of inappropriate joining events, thereby providing a post-cleavage checkpoint against aberrant recombination.

Substantial diversity is introduced into antigen receptor genes during their assembly as a result of processing events that modify the nucleotide sequence of coding ends prior to their joining. One source of junctional diversity arises through the asymmetric opening of hairpinned coding end intermediates, resulting in a protruding 3' or 5' single strand whose sequence is palindromic up to the site where the hairpin is nicked. Most P nucleotides appearing in coding joints are likely generated by hairpin opening mediated by Artemis in association with the catalytic subunit of DNA-dependent protein kinase (9), but a substantial number of P nucleotides present in HJ and OSJ, particularly those generated under circumstances in which NHEJ is defective, are probably derived from RAG-mediated rejoining of coding and signal ends (19, 58). Where P nucleotides have been analyzed in this situation, the RSSs involved in HJ formation have been canonical. In general, the majority of RAG-mediated HJs formed with canonical RSSs lack P nucleotides. For example, using core RAGs in vitro, Melek et al. (19) showed that only 10 of 31 HJs formed at a canonical 23-RSS contained P nucleotides. Similarly, we find that RAG-mediated HJs formed between a canonical 23-RSS and the coding end abutting a canonical 12-RSS are precise. We extend that study by showing that the RAG proteins also mediate HJ formation with cryptic RSS signals. Moreover, we show that recombinase containing core or full-length RAG proteins display similar patterns of P nucleotide addition. Interestingly, in contrast to canonical HJ, the great majority of recovered "non-canonical" HJ formed between a canonical 23-RSS and the coding segment abutting the 6131 cryptic 12-RSS contain a single P nucleotide insertion. This outcome may be attributed to structural differences between post-cleavage complexes containing canonical RSSs versus non-canonical RSSs that influence which phosphodiester bond on the coding end is attacked by the 3'-OH group on the signal end. Alternatively, coding end sequence effects may underlie preferences in which coding and signal ends are rejoined, as they are also thought to influence how coding end intermediates are processed during normal V(D)J recombination (59–61). Additional work is needed to determine whether the high frequency of P nucleotides in the non-canonical HJ reported here is representative of such HJ in general.
Note Added in Proof—In-gel transposition assays in which MgCl₂ substitutes for CaCl₂ in the transposition buffer yield results comparable to those presented in Fig. 4C, despite an overall reduction in transposition activity.

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