

RAG Transposase Can Capture and Commit to Target DNA before or after Donor Cleavage

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The discovery that the V(D)J recombinase functions as a transposase in vitro suggests that transposition by this system might be a potent source of genomic instability. To gain insight into the mechanisms that regulate transposition, we investigated a phenomenon termed target commitment that reflects a functional association between the RAG transposase and the target DNA. We found that the V(D)J recombinase is quite promiscuous, forming productive complexes with target DNA both before and after donor cleavage, and our data indicate that the rate-limiting step for transposition occurs after target capture. Formation of stable target capture complexes depends upon the presence of active-site metal binding residues (the DDE motif), suggesting that active-site amino acids in RAG-1 are critical for target capture. The ability of the RAG transposase to commit to target prior to cleavage may result in a preference for transposition into nearby targets, such as immunoglobulin and T-cell receptor loci. This could bias transposition toward relatively “safe” regions of the genome. A preference for localized transposition may also have influenced the evolution of the antigen receptor loci.

Jawed vertebrates create a diverse repertoire of antigen receptors through a series of programmed DNA rearrangements during lymphocyte differentiation. This process, V(D)J recombination, is catalyzed by a multisubunit recombinase that contains two lymphoid-cell-specific proteins, RAG1 and RAG2. These proteins recognize recombination signal sequences (RSS) that flank all T- and B-cell antigen receptor gene segments (hereafter referred to as coding segments). Double-stranded DNA breaks are introduced at these sites via a two-step mechanism. First, a nick is introduced between the RSS and the adjacent coding segment; second, the newly generated 3'OH attacks the corresponding phosphodiester bond on the opposite strand, creating a covalently sealed (hairpin) coding end and a 5'-phosphorylated blunt signal end. The broken ends are repaired with the participation of several double-stranded break repair proteins, thus creating a diverse array of rearranged antigen receptor genes (reviewed in references 10 and 22).

There are many similarities between the mechanism of V(D)J recombination and the movements of transposable elements (reviewed in reference 32). In light of these similarities and the unusual organization of the RAG genes, it has been suggested that the V(D)J recombination system evolved from an ancient cut-and-paste-type transposon (29, 35, 39). This hypothesis is supported by the observation that RAG proteins, together with the nonspecific DNA-bending protein HMG1, can form an active transposase in vitro, capable of efficiently inserting DNA molecules terminating in signal ends into target DNA molecules without significant target site specificity (1, 14).

The discovery that the V(D)J recombinase can function as a

transposase in vitro raised the possibility that this system might be a potent source of genomic instability in developing lymphocytes that are actively undergoing V(D)J recombination (14). The presence of germ line antigen receptor rearrangements in sharks and skates suggests that RAG-mediated transposition could contribute to genome rearrangements in non-lymphoid cells as well (23, 31). It is, therefore, of great interest to understand the mechanism of transposition and its regulation.

Linear DNA molecules terminating in signal ends—the substrates for transposition—are quite abundant and apparently long-lived in developing lymphocytes (30, 33). These signal ends are thought to remain bound to the RAG proteins (2, 13). In view of the observation that such precleaved signal ends complexed to the RAG proteins efficiently undergo transposition in vitro, it would seem that V(D)J recombination in vivo is a rather risky enterprise. However, RAG-mediated transposition has not yet been documented in vivo, and it is likely that regulatory mechanisms are in place to limit the frequency of transposition in lymphocytes (1, 10, 14, 26, 32).

Selection of the target DNA molecule seems a reasonable step by which to regulate transposition specifically without affecting V(D)J recombination. Some transposons, such as Tn7, show a strong preference for specific target sites (8). In fact, cleavage at Tn7 ends does not proceed until a proper target site has been identified (6). Some mobile elements with much less stringent target sequence requirements, such as the bacteriophage Mu transposase, can interact with the target DNA both prior to and after donor cleavage (7, 27). In contrast, the Tn10 transposase, whose behavior closely parallels that of the V(D)J recombinase in other respects (17), does not stably interact with its target until after the transposon ends have been liberated from the flanking DNA (34). Cleavage may be required to expose the target DNA binding pocket of the transposase (34). Recent experiments have established that the RAG proteins contact the coding flank DNA (9), and it has

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been suggested that like *Tn10*, the RAG proteins may interact with the target DNA only after cleavage (1).

We investigated interactions between the RAG transposase and target DNA. In addition to identifying a RAG-donor-target complex (termed a target capture complex), we examined a phenomenon termed target commitment, a functional association between the RAG-RSS complex and target DNA that is resistant to addition of a competitor target. Target commitment can be distinct from target capture: in the case of *Tn10* only a subset of target capture complexes exhibit commitment, which is viewed as a more functionally significant interaction (34). We found that the RAG proteins indeed exhibit target commitment. Furthermore, our data demonstrate that, contrary to expectation, the V(D)J recombinase is a promiscuous transposase that can form productive complexes with target DNA both before and after RSS cleavage. The ability of the RAG transposase to commit to target prior to cleavage may result in a preference for transposition into nearby targets, such as immunoglobulin and T-cell receptor loci. Functional and evolutionary implications of this newly discovered property of the RAG proteins are discussed.

MATERIALS AND METHODS

DNA. Uncleaved 16-bp coding flank 12- and 23-RSS were created by annealing the DAR39/40 and DG61/62 (25) oligonucleotides, respectively. Uncleaved 41-bp coding flank 12- and 23-RSS oligonucleotides were created by annealing SK42 (5'-CTGCAGGTACAGACGAGTCTACAGATCTGGCTGTCTGC CACAGTGTACAGACTGGAACAAAAACCTGCAG) to its complement, SK43, and MBN21 (5'-CTGCAGGTACAGGACGAGTCTACAGATCTGGC CTGTCTGCCACAGTGGTAGTACTCCACTGTCTGGCTGTACAAAAAC CCTGCAG) to its complement, MBN22. Precleaved 12- and 23-RSS oligonucleotides were created by annealing DG10 and DG4 to their complements (25). The target used for physical detection of target capture complexes was the oligonucleotide mm30b (5'-ATCGAGGACGACGATTACGTTCCCGGAGA TC) annealed to its complement, mm30t. All oligonucleotides were purchased from GIBCO Life Technologies and were gel purified before use. pUC19 and pcDNA1/AMP were used as the short and long plasmid targets, respectively. The linearized target size standards were created by digesting the appropriate target plasmids with *Bam*HI and by 5' end labeling with T4 polynucleotide kinase.

Proteins. Recombinant truncated (core) RAG proteins (amino acids 384 to 1008 of RAG1 and 1 to 387 of RAG2) were purified from baculovirus-infected insect cells as previously described (3, 19, 25, 41). Both proteins contain a carboxy-terminal nine-histidine tag, three human *c-myc* epitope tags, and an amino-terminal maltose-binding protein fusion. The target capture assay and DDE mutant RAG experiments were performed using RAG-1 and RAG-2 glutathione *S*-transferase (GST) fusions copurified from Chinese hamster ovary (RMP41) cells as previously described (36–38). Both types of protein preparations were capable of target commitment; however, the GST epitope-tagged proteins were more active.

Target commitment. Unless otherwise noted, 10- μ l preincubation mixtures contained 32.7 mM K⁺-HEPES (pH 7.5), 2.6 mM dithiothreitol, 19.4 mM potassium glutamate, 5.1 mM CaCl₂, 6% glycerol, 60 μ g of bovine serum albumin/ μ l, 0.006% NP-40, 150 ng each of MR1 and MR2, 1.5 ng of HMG1/ μ l, and donor and target DNA as indicated. Following preincubation at the indicated time and temperature, reaction mixtures were spiked with mixtures of MgCl₂ or other Me²⁺ as indicated (3 mM, final concentration), polyethylene glycol 8000 (10%, final wt/vol ratio), and the indicated target or Tris-EDTA (TE) for a final reaction volume of 15 μ l and were incubated at 37°C for 30 min (except in the kinetic analyses). Fifteen microliters of stop buffer (100 mM Tris [pH 8.0], 10 mM EDTA, 0.2% sodium dodecyl sulfate [SDS], 0.35 mg of proteinase K/ml) was added, and incubation continued at 37°C for >1.5 h. All reactions were subjected to electrophoresis through 1% SeaKem GTG agarose (FMC BioProducts) 25-cm gels containing 0.2 μ g of ethidium bromide/ml at 180 V for ~200 min in 1 \times Tris-borate-EDTA. Dried gels were visualized with a PhosphorImager.

Target commitment assays. Uncleaved donor reactions were performed as indicated above with 0.02 pmol of 5' ³²P-end-labeled 12-RSS donor, 0.02 pmol of 23-RSS donor, and 0.04 pmol of pcDNA1/AMP and/or pUC19 target. Precleaved donor reactions were performed as indicated above, using 0.12 pmol of

5' ³²P-end-labeled 12-RSS donor, 0.12 pmol of 23-RSS donor, and 0.03 pmol of pcDNA1/AMP and/or pUC19. DDE mutant experiments were performed as indicated above with 0.02 pmol of 5' ³²P-end-labeled 12-RSS donor, 0.02 pmol of 23-RSS donor, and 0.04 pmol of pcDNA1/AMP and/or pUC19 target.

Target capture assays. Preincubations (6.5 μ l) were carried out at 37°C for 10 min, in mixtures containing 25 mM K-morpholinopropanesulfonic acid (MOPS) (pH 7.0), 4 mM dithiothreitol, 75 mM potassium glutamate, 5.4 mM CaCl₂, 100 μ g of bovine serum albumin/ml, 150 ng each of GST-R1 and GST-R2, 20 ng of HMG1, and 0.13 pmol each of uncleaved 12- and 23-RSS donor. The reaction mixtures were spiked with mixtures of MgCl (5 mM, final concentration), dimethyl sulfoxide (DMSO) (10%, final wt/vol ratio), and 0.65 pmol of annealed oligonucleotide target for a final reaction volume of 10 μ l and were incubated at 37°C for 15 min. These reactions were also performed without the addition of DMSO. Target capture was found to be completely independent of the presence of DMSO. Two microliters of 50% glycerol was added to all reaction mixtures except for the stop buffer-treated reaction mixture, which received 10 μ l of stop buffer and was incubated for 15 min at 37°C prior to the addition of 4 μ l of 50% glycerol. Entire reaction mixtures were then loaded directly onto a 4 to 20% gradient nondenaturing polyacrylamide gel and run at 120 V for 110 min in 1 \times Tris-borate-EDTA at 4°C. Dried gels were visualized by PhosphorImager analysis. Electrophoretic mobility shifts of labeled donor were conducted as previously described (21), except that glutaraldehyde was omitted.

RESULTS

Target commitment prior to donor cleavage. The ability of the *Tn10* transposase to interact with its target has been analyzed using staged reactions in which the transposase (along with appropriate transposon end sequences) is first incubated with target DNA in Ca²⁺, which promotes assembly of protein-DNA complexes but does not support transposition. This preincubation is followed by the addition of a second distinguishable target along with Mg²⁺, a divalent metal ion that allows transposition. A functionally significant interaction between the transposase-end complex and the target (commitment) is inferred if preferential integration into the first target is observed (34).

We adopted a similar strategy to study the interactions of the RAG transposase with target DNA. We performed staged reactions in which uncleaved 12- and 23-spacer RSS-containing oligonucleotides were preincubated with the RAG proteins and HMG1 under conditions that support assembly but not transposition (generally in Ca²⁺). Donor molecules containing either 16 or 41 bp of flanking DNA were tested, yielding similar results (Fig. 1B and C, respectively). Target plasmids of two distinct sizes (2.7 and 4.8 kb) were used (illustrated schematically in Fig. 1A). Target 1 was added in the preincubation, and target 2 was added, along with Mg²⁺, in the second incubation. If the RAG transposase captures the target prior to cleavage and remains committed to this target, then target 1 should be preferred over an equimolar amount of target 2. If, on the other hand, the RAG proteins do not commit to the first target prior to cleavage, then there should be no preference for transposition into either target.

As shown in Fig. 1B, incubation of the RAG proteins with either target 1 or 2 alone yielded predominantly double-ended transposition events, resulting in linearization of the target plasmid (Fig. 1B, lanes 3 and 4; compare with linearized plasmid standards in lanes 1 and 2), in agreement with previous studies (1, 14). Consistent with earlier reports (1, 14), approximately 1% of the radiolabeled donor was integrated into the plasmid targets. As shown in Fig. 1, the staged reactions reveal a clear preference for the first target, regardless of whether the smaller or larger plasmid served as target 1 (Fig. 1B, lanes 7

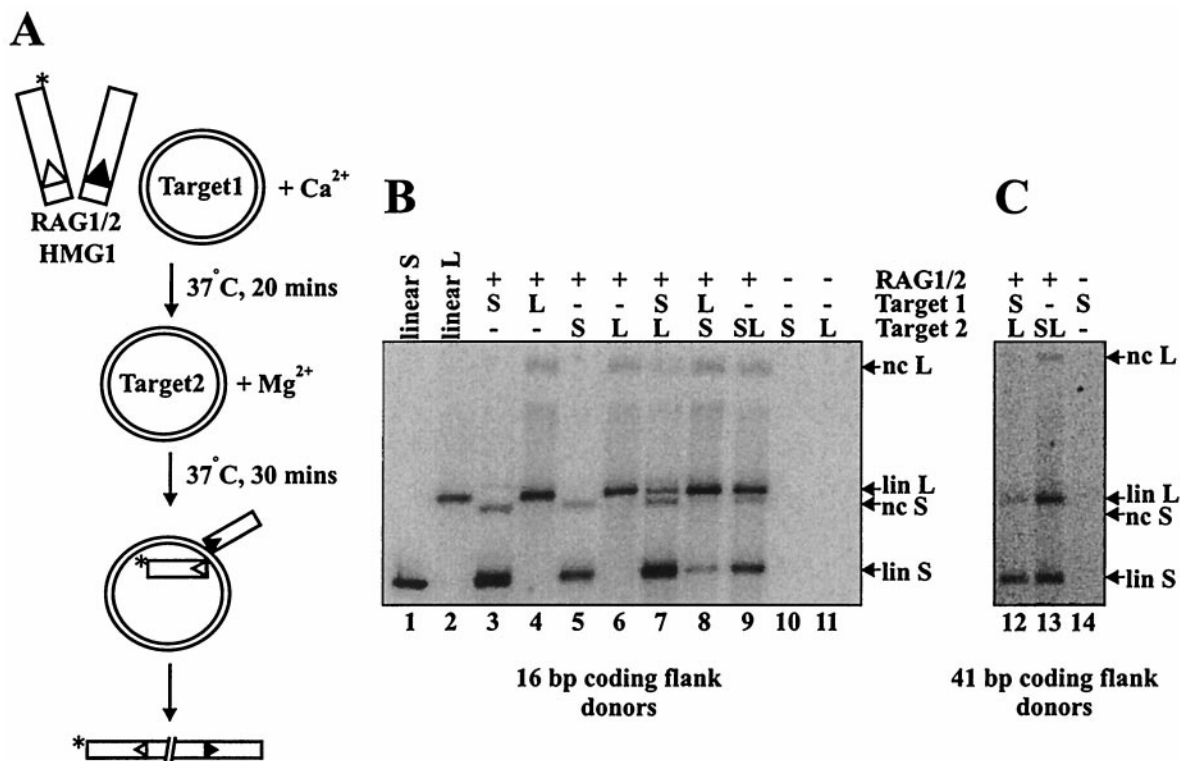


FIG. 1. The RAG transposase can commit to target prior to donor cleavage. (A) Schematic of target commitment assay with uncleaved donor. The asterisk indicates the location of radiolabel. (B) Transposition products are either linearized (lin) or nicked circular (nc) target species resulting from concerted or single integration of donor molecules, respectively (single events are not pictured in the schematic). Linear products of concerted transposition events migrate slightly more slowly than the linearized standards due to the presence of the integrated donor molecules. – indicates that dialysis buffer was substituted for RAG protein or that TE was substituted for DNA in indicated reactions. S and L refer to short and long targets, respectively.

and 8, and C, lane 12). The preference for target 1 was at the expense of target 2 utilization (compare Fig. 1B, lanes 7 and 8, with lanes 6 and 5, respectively). As controls, both plasmids were added together during the preincubation (data not shown) or at the time of Mg^{2+} addition (Fig. 1B, lane 9, and C, lane 13); as expected, no target preference was observed. Thus, the RAG proteins, unlike the *Tn10* transposase, interact with and commit to the target prior to cleavage.

Target commitment after donor cleavage. We next examined the behavior of precleaved donor DNA molecules. Staged reactions were performed as described above, except that the preincubation step was performed at $0^{\circ}C$ to prevent transposition during the preincubation (Fig. 2A), because precleaved donors are active for transposition in Ca^{2+} (14). To verify that transposition did not occur at $0^{\circ}C$, additional reactions with target present during the Ca^{2+} preincubation phase were performed in parallel without a Mg^{2+} addition step; no transposition was observed in multiple experiments (for example, see Fig. 2B, lanes 7 and 8). No preferential use of either target was observed when both targets were present in the preincubation (Fig. 2B, lane 6) or in the second, Mg^{2+} -containing incubation (data not shown). The staged reactions, however, revealed a preferential use of target 1 (Fig. 2B, lane 5; see also Fig. 6B, lane 6), indicating that precleaved complexes support target commitment.

Preincubation with target does not accelerate the rate of strand transfer. Target commitment could simply reflect accelerated kinetics of transposition, as the formation of a target capture complex could be a rate-limiting step. Indeed, in the case of *Tn10*, preincubation of target with transposition complexes containing precleaved donor DNA molecules substantially accelerates the rate of strand transfer in staged reactions (34). We therefore examined the effects of preincubation with uncleaved donor DNA on the kinetics of strand transfer, following the protocols schematized in Fig. 3A and B. We added target either simultaneously with Mg^{2+} (Fig. 3A) or during the preincubation (Fig. 3B). The rate of strand transfer was not significantly accelerated by preincubating with target (Fig. 3C). As expected, preincubation with target did not affect the rate of donor cleavage (data not shown). Transposition decreased after approximately 40 to 60 min, perhaps as a result of disintegration (26).

We next studied the kinetic effects of target preincubation on transposition by precleaved donor molecules, as depicted schematically in Fig. 4A and B. Target was added at the time of Mg^{2+} addition (Fig. 4A) or during the preincubation (Fig. 4B). As with the uncleaved donor, the time course of transposition of precleaved donor molecules was not significantly affected by preincubation with target (Fig. 4C). These data demonstrate that preincubation with target DNA molecules does

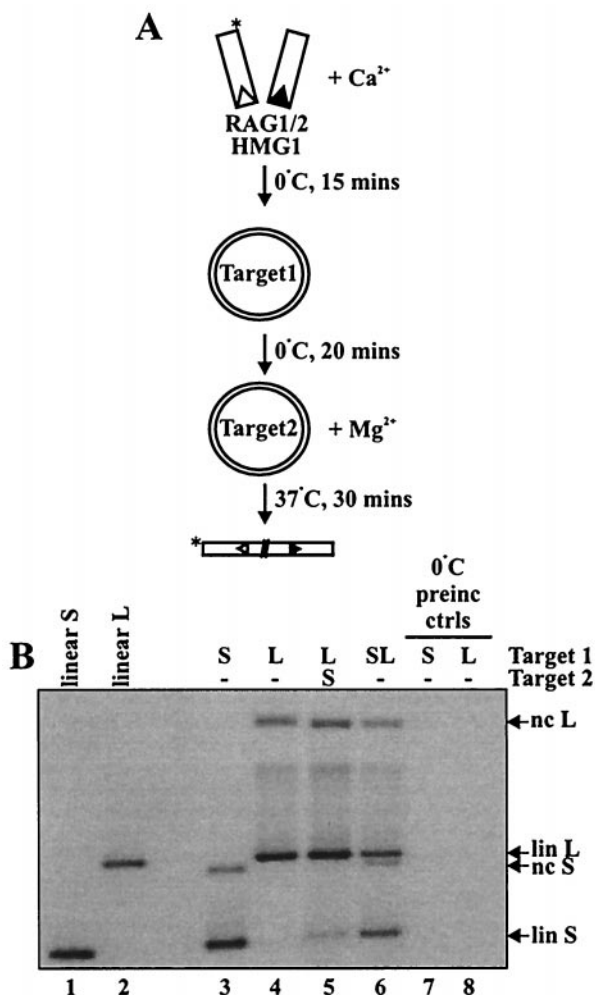


FIG. 2. The RAG transposase can commit to target after donor cleavage. (A) Schematic of RAG transposase target commitment assay with precleaved donor end molecules. Asterisks indicate the position of radiolabel. (B) Agarose gel of transposition products. -, no addition of target; preinc, preincubation; ctrls, controls; lin; linear; nc, nicked circular; S, short; L, long.

not significantly increase the rate of transposition, suggesting that a reaction step subsequent to target capture is rate limiting. In fact, varying the time of target 1 preincubation revealed that target commitment occurred very quickly, in less than 1 min (the earliest time point; data not shown).

Transposase-target interactions are reversible in Ca²⁺. The target commitment experiments described above demonstrate that transposase-target associations formed during preincubation in Ca²⁺ are resistant to competitor target added at the time of Mg²⁺ addition. Since Ca²⁺ supports binding, but not catalysis, we wondered whether the target-transposase association would also be resistant to competitor added in Ca²⁺. To examine this question, samples preincubated with uncleaved donor and target 1 in Ca²⁺ were subjected to a second 20-min preincubation in Ca²⁺ with various amounts of target 2 as competitor (illustrated schematically in Fig. 5A). Even an equimolar amount of target 2 abolished the preference for target 1 (Fig. 5B, lane 4); 3- and 10-fold molar excesses of

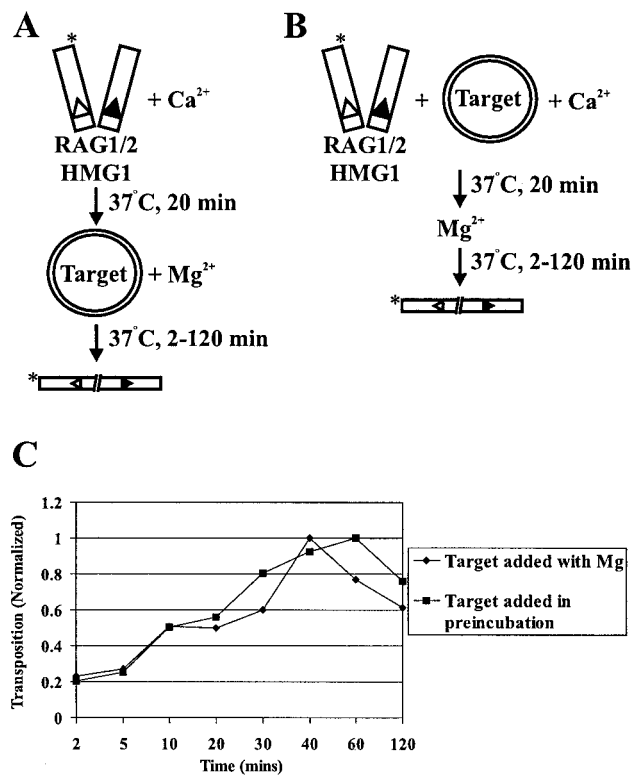


FIG. 3. Preincubation with target DNA does not accelerate the kinetics of strand transfer of uncleaved donor ends. Panels A and B show assays utilizing uncleaved donor molecules in which target was added either with Mg²⁺ or during preincubation. Asterisks indicate the position of radiolabel. (C) The amount of transposition is plotted as a function of the incubation time with Mg²⁺ at 37°C. Target was added either with Mg²⁺ or during preincubation.

target 2 progressively diminished the use of target 1 (Fig. 5B, lanes 5 and 6). Note that when high concentrations of competitor target were added in the Ca²⁺ preincubation, the efficiency of transposition was somewhat diminished (Fig. 5B, compare lanes 3 and 6). This is because the large amounts of plasmid DNA act as a nonspecific competitor, inhibiting synaptic complex formation (12; M. B. Neiditch and D. B. Roth, unpublished data).

These data demonstrate that the transposase-target interactions leading to commitment are reversible in Ca²⁺. Identical results were obtained with precleaved donor molecules (data not shown), indicating that the target capture complex is not stabilized by the absence of flanking donor DNA. In contrast, commitment to target 1 was resistant to even a 10-fold molar excess of target 2 added during the Mg²⁺ addition step (Fig. 5B, lane 9). This competitor resistance was also observed when the 2.7-kb target was used as target 1 and when the 4.8-kb target was utilized as target 2 (data not shown). The relative inability of target 2 to compete with target 1 in Mg²⁺ implies that the RAG-RSS-target complexes are fairly stable in Mg²⁺ (see below for further discussion).

Effects of divalent metal ions on target commitment. The lack of stable target commitment in Ca²⁺ indicates that the transposase-target interactions responsible for commitment are readily reversible under these conditions. Because target

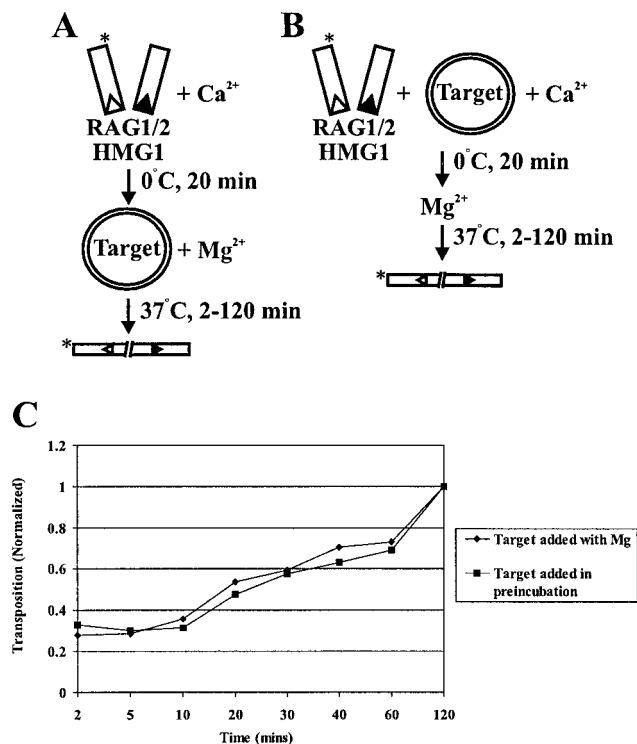


FIG. 4. Preincubation with target DNA does not accelerate the kinetics of strand transfer of precleaved donor ends. Panels A and B picture assays utilizing precleaved donor molecules in which target was added either with Mg^{2+} or during the preincubation. Asterisks indicate position of radiolabel. (C) The amount of transposition observed is plotted as a function of the incubation time with Mg^{2+} at $37^{\circ}C$. Target was added either with Mg^{2+} or during preincubation.

commitment is observed upon addition of Mg^{2+} , we wondered whether Mg^{2+} might increase the stability of transposase-target interactions, locking the transposase onto the target. Alternatively, the establishment of conditions that support catalysis (rather than the presence of Mg^{2+} per se) might promote an alteration of the transposase-donor-target interaction that induces target commitment.

To test these hypotheses, we made use of the fact that precleaved donor DNA molecules are competent for transposition in both Ca^{2+} and Mg^{2+} . We performed staged reactions in which the second step was carried out in Ca^{2+} at $37^{\circ}C$, allowing catalysis to be initiated in the absence of Mg^{2+} (Fig. 6A). Reactions in which both incubations were carried out in Ca^{2+} exhibited target commitment that was indistinguishable from reactions performed in the standard fashion, in which Ca^{2+} is followed by Mg^{2+} (a representative experiment is shown in Fig. 6B; compare lanes 4 and 6). Target commitment was also observed when target 1 was preincubated in Mg^{2+} and target 2 was added with Ca^{2+} (Fig. 6B, lane 8) and when target 1 was preincubated in Mg^{2+} and target 2 was added with (Fig. 6B, lanes 11 and 12) or without (Fig. 6B, lanes 9 and 10) additional Mg^{2+} . Thus, it is the switch to catalytic conditions, rather than the presence of a specific metal ion, that induces target commitment.

Physical analysis of target capture complexes. The ability of the RAG transposase to display target commitment implies a

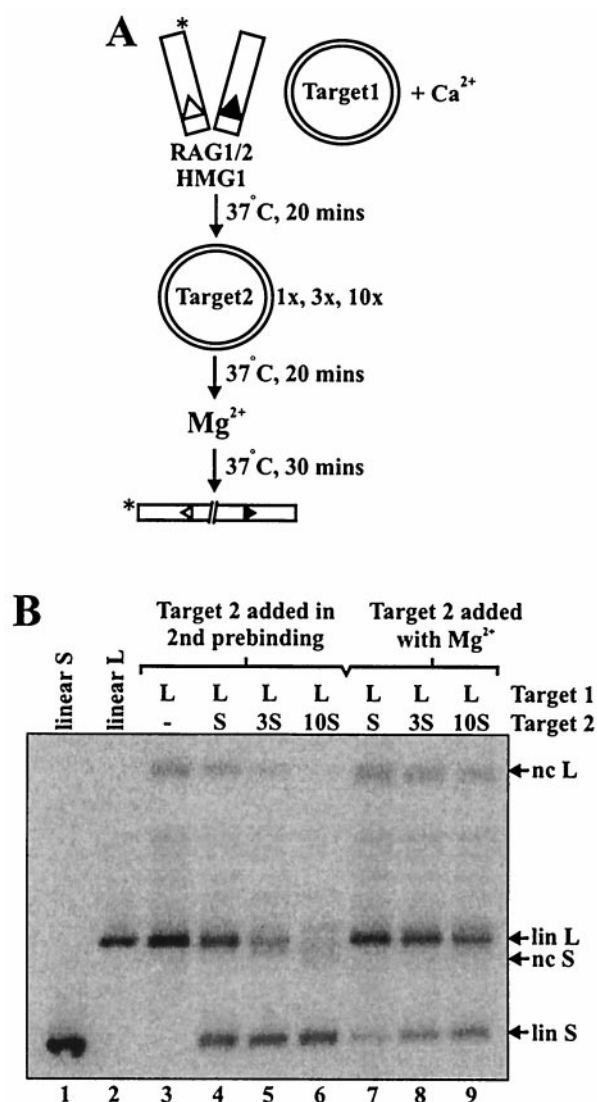


FIG. 5. Transposase-target interactions leading to commitment are reversible in Ca^{2+} . (A) Schematic of assay utilized to determine the stability of target commitment under preincubation and catalytic conditions. The asterisk indicates the position of radiolabel. (B) Preincubations were performed for 20 min, with 0.02 pmol of 12- and 23-spacer RSS donors and $1 \times$ (0.04 pmol) pcDNA1/AMP (target 1). $1 \times$, $3 \times$, or $10 \times$ molar amounts of pUC19 (target 2) were added as indicated (lanes 4 to 6), and preincubations were continued for 20 min. Reaction mixtures were supplemented with $MgCl_2$ and polyethylene glycol, bringing the final reaction conditions to 3 mM $MgCl_2$ and 10% polyethylene glycol 8000 in 15 μ l. These reactions were compared to similar reactions in which $1 \times$, $3 \times$, or $10 \times$ target 2 was added at the time of Mg^{2+} addition (lanes 7 to 9). nc, nicked circular; lin, linear; S, short; L, long; -, no addition of target 2.

stable association between the RAG-RSS complex and the target. To detect such target capture complexes directly, we performed electrophoretic mobility shift assays using a radiolabeled oligonucleotide. No protein cross-linking reagents (such as glutaraldehyde) were used. We observed target capture complexes (Fig. 7B, lane 2) that were dependent on the presence of both RAG proteins (Fig. 7B, lane 1) and donor RSS (Fig. 7B, lane 5). As expected, efficient target capture

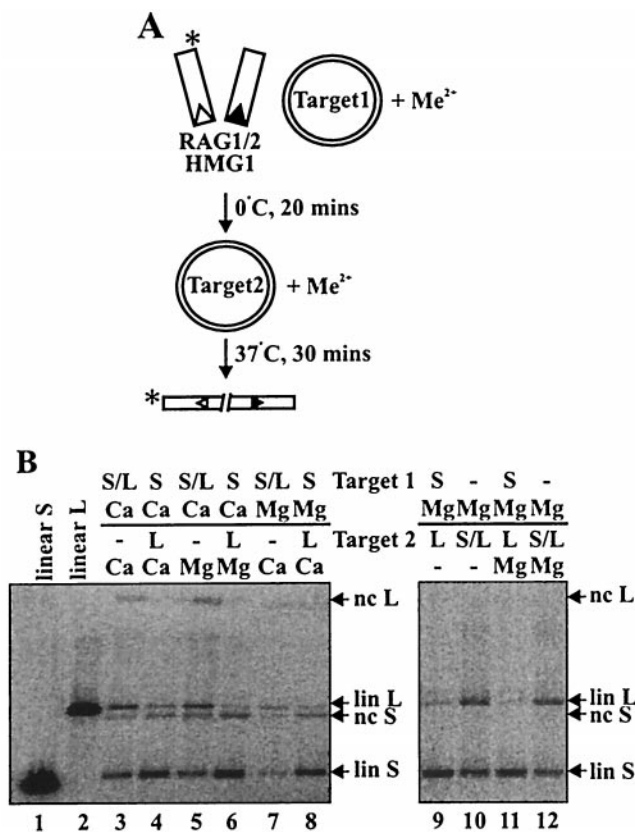


FIG. 6. The RAG transposase commits to target in the presence of both Ca^{2+} and Mg^{2+} . (A) Schematic of assay employed to test the contributions of Ca^{2+} and Mg^{2+} to target commitment. Preincubation mixtures contained 5.1 mM concentrations of the indicated divalent metal ion; the indicated divalent metal ion was added with target 2 or TE to a final concentration of 3 mM. Asterisks indicate the position of radiolabel. (B) Truncated RAG proteins (lacking the N-terminal maltose-binding protein fusion) were used in the experiment shown. Commitment to target 1 was observed in $\text{Ca}^{2+}/\text{Ca}^{2+}$, $\text{Ca}^{2+}/\text{Mg}^{2+}$, $\text{Mg}^{2+}/\text{Ca}^{2+}$, and $\text{Mg}^{2+}/\text{Mg}^{2+}$ reactions. lin, linear; nc, nicked circular; S, short; L, long; -, no addition of target.

complex formation required the presence of both a 12- and a 23-RSS (Fig. 7B, lane 6, and data not shown). Ca^{2+} alone supported inefficient but detectable target capture (Fig. 7B, lanes 4 and 8). Some target capture complex formation occurred in the absence of HMG1, yielding a complex with faster electrophoretic mobility (Fig. 7B, lane 7) that was not observed in standard reactions.

To determine whether the DNA-protein complexes contained transposition products, reaction mixtures were deproteinized by treatment with SDS and proteinase K and loaded directly to the gel without extraction or precipitation. The target capture complex band was completely ablated upon SDS-proteinase K treatment, and a new band of altered mobility was detected, representing products resistant to SDS-proteinase K treatment. This indicates a covalent association between donor and target DNA, which was further verified by denaturing gel electrophoresis (data not shown). In multiple experiments, however, we noted that only approximately 50% of the target capture complexes were resistant to SDS-proteinase K treatment, indicating that a substantial fraction of target

capture complexes had not undergone strand transfer. This observation is consistent with the ability of target capture complexes to form in Ca^{2+} , a condition that does not support strand transfer of uncleaved donor RSS. These data demonstrate the presence of RAG-donor-target complexes (some containing substrates and some containing completed transposition products) that are sufficiently stable to withstand gel electrophoresis.

Analysis of catalytically deficient RAG mutants. The Tn10 transposase active site has been implicated in transposase-target interactions (16). To gain insight into the mode of target capture employed by the RAG transposase, we examined catalytically deficient RAG mutants bearing point mutations in acidic amino acids important for coordination of divalent metal ions (the so-called DDE motif). DDE mutants are defective for both the hydrolysis and transesterification steps of RSS cleavage and fail to carry out transposition of either uncleaved or precleaved donor RSS in Mg^{2+} (11, 18, 21).

We used the target capture assay to examine the ability of D600N and E962Q to capture target in Mg^{2+} . Both mutants (as well as wild-type RAG-1) bound RSS in the presence of wild-type RAG-2, yielding a RAG-RSS complex that has a somewhat faster mobility than the RAG-RSS-target complex (Fig. 7B, lanes 14 to 16). The mutants, however, failed to form stable target capture complexes (Fig. 7B, lanes 11 and 12). These data indicate that target capture involves active-site residues in RAG-1.

The D600 and E962 mutants are capable of catalyzing inefficient but detectable transposition of precleaved donor molecules in the presence of Mn^{2+} (18; Neiditch and Roth, unpublished data). We took advantage of this fact to examine the effects of the D600N and E962Q mutants on target commitment using precleaved donor RSS. Although transposition was quite inefficient, both mutants exhibited target commitment under these conditions (data not shown). These data suggest that under these conditions (in the presence of Mn^{2+} , which bypasses the effects of the DDE mutants on the strand transfer step), the mutant proteins can bind to target DNA. It is difficult to draw firm conclusions about the roles of D600 and E962 in target commitment from these data, however, because it is not clear why Mn^{2+} rescues the strand transfer activity of the mutant proteins.

DISCUSSION

The interaction between the transposase and target DNA is a critical step in RAG-mediated transposition. Here we show that the RAG-RSS complex stably associates with target DNA. To examine the functional significance of these complexes, we employed staged reactions in which a RAG-RSS complex was preincubated with target DNA under conditions that allow binding but not strand transfer and was then incubated under catalytic conditions. Transposition into the first target was favored even when a large excess of a second target was added in the second incubation. Target commitment was observed with both uncleaved and precleaved donor DNA molecules, contrary to expectation (1). In this respect, the RAG proteins resemble the Mu transposase, which can capture target both before and after donor cleavage (27), and are quite distinct from Tn7 and Tn10, which capture target DNA only before

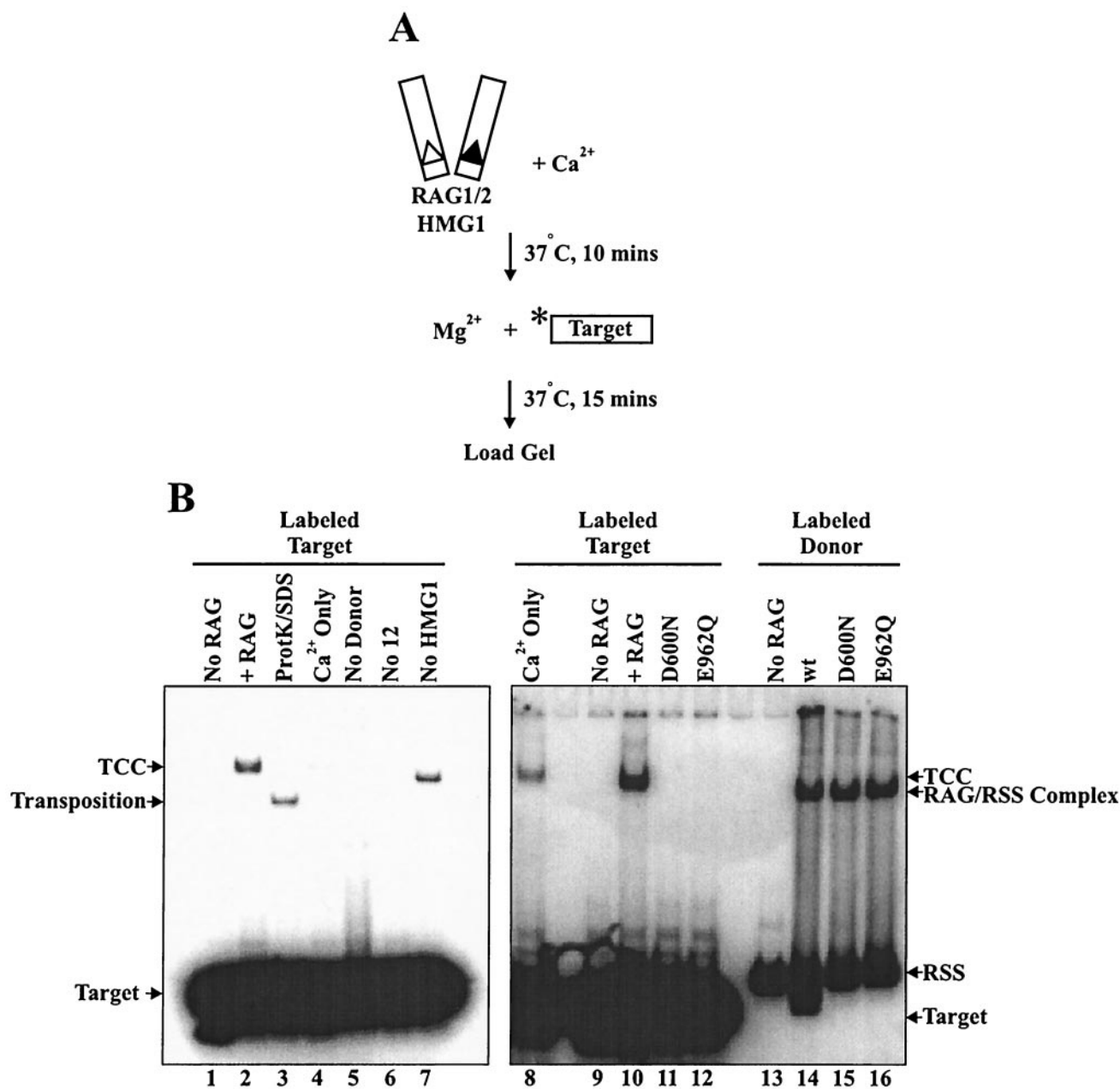


FIG. 7. Physical detection of RAG target capture complexes. (A) Schematic of assay employed to detect noncovalently associated RAG-donor-target complexes. The asterisk indicates the position of radiolabel. (B) Lanes marked + RAG contain RAG proteins, HMG1, 12- and 23-donor RSS, target, and Mg²⁺. TCC denotes the position of target capture complexes. All reaction mixtures were loaded directly to gel without extraction. ProtK, proteinase K; wt, wild type.

and after cleavage, respectively (5, 6, 34). The RAG transposase is also different from Tn10 in that it does not show an increased rate of strand transfer if preincubated with target. This suggests that the rate-limiting step for RAG-mediated transposition occurs after target capture.

What is the basis of target commitment? Preincubation in Ca²⁺ presumably favors the formation of productive RAG-RSS-target complexes. Our data indicate that these complexes are rapidly exchangeable under the preincubation conditions but rapidly become committed upon shift to catalytic condi-

tions, resisting even large molar excesses of a second target. How do catalytic conditions facilitate target commitment? We considered the possibility that commitment could simply reflect efficient strand transfer upon shift to conditions that support catalysis, but two lines of evidence argue against this model. First, strand transfer is slow relative to the rate of target capture complex dissociation: whereas formation of significant amounts of strand transfer products takes at least 10 min (Fig. 3 and 4), under preincubation conditions target capture complexes are readily dissociated by addition of a second target

within 2 min (Neiditch and Roth, unpublished observations). Second, target commitment can be established within 20 min at 0°C in the absence of detectable strand transfer (Fig. 2). These data demonstrate that target commitment and strand transfer are distinct steps. Furthermore, the observation that target commitment precedes strand transfer suggests that the RAG proteins may not be irreversibly committed to transposition after capture of the target. In the *in vivo* situation, signal joint formation may compete with transposition even after the transposase has captured (and possibly committed to) a target DNA molecule. Further experiments are required to examine this possibility.

Others have shown that target capture by *Tn10* requires removal of IHF, a DNA-bending protein that is important for assembly of the paired-end complex (34). Preincubation with plasmid DNA titrates IHF away from the *Tn10*-donor complex, causing a conformational change in the transposase that facilitates target capture. We have observed two distinct forms of the RAG target capture complex, with different electrophoretic mobilities, formed with and without HMG1. These data suggest that target capture can occur in the absence of HMG1 (although the presence of low levels of HMG1 in our RAG protein preparations has not been rigorously excluded). These two forms of the target capture complex may have different properties with respect to target binding and target commitment. Ongoing experiments are examining this possibility.

We hypothesize that commitment reflects a change in the mode of target binding, perhaps resulting from a conformational change in the active site of the transposase so that the target is bound more stably. Precedents for this model are provided by DNA binding proteins that search nonspecifically for target sequences using a readily reversible binding mode and then lock on using a different binding mode upon identifying specific recognition sequences (for example, see reference 15). A similar two-step binding model has been suggested for target capture by the *Tn10* transposase (34).

How do the RAG proteins bind to target DNA? The regions of the RAG transposase responsible for binding to target DNA remain unknown. The observation that the *Tn10* transposase exhibits target commitment only with precleaved donor DNA molecules suggests that the same binding site that contacts the flanking donor DNA also binds to the target (34). Our data do not support a similar model for the RAG transposase: we observed target commitment with both uncleaved and precleaved donor DNA molecules. Nevertheless, the stoichiometry of the RAG-RSS-target complex is not known, and the possibility remains that RAG monomers not involved in donor cleavage mediate important interactions with target DNA.

Taking into account studies of *Tn10* that indicate that the active-site amino acids responsible for coordinating catalytic metal ions play a role in target binding (16), we hypothesized that the more stable binding mode observed under catalytic conditions involves the acidic active-site residues recently discovered in RAG1 (11, 18, 21). Indeed, our finding that the D600N and E962Q mutants are severely defective for target capture in Mg^{2+} suggests that these active-site amino acids (which may function to coordinate catalytic divalent metal ions) play an important role in target binding. This is consistent with our finding that stable target commitment is observed only

under catalytic conditions. Exactly how metal coordinating residues are involved in target capture remains unclear. These amino acids may be directly involved in target DNA binding; alternatively, the mutations may affect target binding indirectly by altering the conformation of the active site (16).

Biological considerations. Once produced, signal ends have two possible fates: joining to form a signal joint (the normal pathway) or transposition. RAG-mediated transposition *in vivo* is likely to have deleterious consequences, for both the cell and the organism, including oncogenic chromosome translocations (14, 32). The fact that transposition has not yet been detected *in vivo* suggests that this reaction may indeed be carefully controlled (10). The ability of the RAG transposase to select a target DNA molecule prior to cleavage may have important biological ramifications. Target commitment may represent a critical decision point, and our data suggest that the decision to transpose actually precedes RSS cleavage.

The ability to commit to a target prior to cleavage may influence the choice of target sites. Donor cleavage removes a physical constraint and may allow the transposon to diffuse freely throughout the nucleus. If the target is chosen before the transposon is excised, transposition might preferentially occur into targets that are physically close to the relevant antigen receptor loci. Recent evidence demonstrates that genetic rearrangements preferentially involve DNA segments that are spatially juxtaposed (20, 28).

Thus, RAG-mediated transposition may favor targets that lie close to the donor sites, such as the antigen receptor loci themselves (which are in an accessible chromatin configuration at the time of rearrangement). Indeed, both the *Ac* plant transposable element and *Drosophila* P elements demonstrate a proclivity for transposition to local target sites (4, 24, 40), suggesting that these transposases also capture target before donor cleavage. A bias toward local transposition by the RAG proteins could serve to channel transposition events into relatively safe regions of the genome. Moreover, localized RAG-mediated transposition may have influenced the evolution of the antigen receptor loci. Evidence suggests that these loci have evolved via repeated rounds of local transposition into the germ line by an ancient transposon (39); this process could have been assisted by the ability of the RAG proteins to commit to target prior to cleavage.

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