

# Understanding how the V(D)J recombinase catalyzes transesterification: distinctions between DNA cleavage and transposition

Catherine P. Lu<sup>1,2</sup>, Jennifer E. Posey<sup>1,2,3</sup> and David B. Roth<sup>1,2,\*</sup>

<sup>1</sup>Program in Molecular Pathogenesis, Helen L. and Martin S. Kimmel Center for Biology and Medicine at the Skirball Institute for Biomolecular Medicine, <sup>2</sup>Department of Pathology, New York University School of Medicine, 540 First Avenue, New York, NY 10016 and <sup>3</sup>Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030, USA

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## ABSTRACT

The Rag1 and Rag2 proteins initiate V(D)J recombination by introducing site-specific DNA double-strand breaks. Cleavage occurs by nicking one DNA strand, followed by a one-step transesterification reaction that forms a DNA hairpin structure. A similar reaction allows Rag transposition, in which the 3'-OH groups produced by Rag cleavage are joined to target DNA. The Rag1 active site DDE triad clearly plays a catalytic role in both cleavage and transposition, but no other residues in Rag1 responsible for transesterification have been identified. Furthermore, although Rag2 is essential for both cleavage and transposition, the nature of its involvement is unknown. Here, we identify basic amino acids in the catalytic core of Rag1 specifically important for transesterification. We also show that some Rag1 mutants with severe defects in hairpin formation nonetheless catalyze substantial levels of transposition. Lastly, we show that a catalytically defective Rag2 mutant is impaired in target capture and displays a novel form of coding flank sensitivity. These findings provide the first identification of components of Rag1 that are specifically required for transesterification and suggest an unexpected role for Rag2 in DNA cleavage and transposition.

## INTRODUCTION

Developing lymphocytes rearrange variable (V), diversity (D) and joining (J) gene segments in a combinatorial

fashion to generate a diverse repertoire of immunoglobulins and T-cell receptors. This process, called V(D)J recombination (1,2), is initiated when the protein products of the recombination activating genes (Rag)-1 and -2 bind to specific recognition signal sequences (RSS) and introduce double-strand breaks (DSB) between these and the adjacent coding segments. The resulting postcleavage complex then directs the ends to the classical nonhomologous end joining (NHEJ) machinery for repair (3,4). *In vitro*, these ends can undergo an alternative fate: they can capture an unrelated target DNA and undergo Rag-mediated transposition (5,6). *In vivo*, however, the Rag proteins are strongly biased towards recombination, with only rare transposition events detected to date (7–11). Deciphering the mechanisms by which the Rag complex carries out these two transesterification reactions should help provide insight into how transposition is suppressed in developing lymphocytes.

The Rag complex generates DSB in two steps, first introducing a nick between the RSS and the adjacent coding segment (the coding flank) and then using the resulting 3'-hydroxyl group to attack the phosphodiester backbone of the opposite DNA strand. This direct transesterification reaction forms a covalently sealed hairpin structure at the coding flank and a flush DSB at the signal end (Figure 1A) (12). The Rag proteins employ this same chemical mechanism to perform transposition. In this case, after cleavage, the Rag complex uses direct transesterification to insert a segment of DNA terminating in blunt signal ends into a new target location (5,6) (Figure 1B).

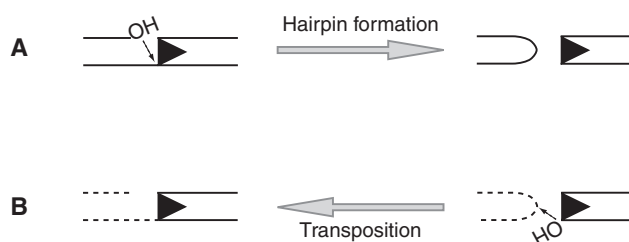
Hairpin formation requires local distortion of the coding flank DNA, in order to allow the attacking nucleophile to align precisely with the phosphodiester bond of the opposite strand. Substrates with unpaired

\*To whom correspondence should be addressed. Tel: +212 263 0945; Fax: +212 263 5711; Email: roth@saturn.med.nyu.edu

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

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**Figure 1.** Schematic representation of Rag-mediated transesterification reactions. **(A)** Hairpin formation is an intramolecular transesterification reaction in which the 3'-hydroxyl liberated by Rag nicking is used to attack the opposite strand of DNA at the heptamer border, resulting in a blunt signal end and a covalently sealed hairpin coding end. The RSS is indicated by a triangle. **(B)** Transposition is an intermolecular transesterification reaction in which the 3'-hydroxyl of a signal end is used to attack the phosphodiester backbone of the target DNA. Hairpin DNA structures facilitate Rag transposition. Both types of transesterification reaction require the catalytic triad (D600, D708 and E962) as well as the divalent cation  $Mg^{2+}$ .

bases at the coding flank facilitate this reaction (13,14). Similarly, transposition is facilitated by certain target DNA structures, including unpaired DNA, triplex DNA and some G-C rich sequences, suggesting that distortion is also required for transposition (5,6,15). The best targets appear to be hairpin tips (16,17), which are thought to adopt a variety of unusual DNA structures determined by the sequence of the terminal nucleotides (18). Indeed, certain hairpin sequences stimulate surprisingly efficient Rag transposition that approaches levels of cleavage (17).

Although there is as yet no structural information about the active site of the Rag complex, Rag1 is considered the catalytic subunit because it contains a number of amino acids required for cleavage (19–22), including a DDE triad responsible for coordinating catalytic metal ions (23–25). Rag2 plays various regulatory roles (3,26–34), but has not been firmly implicated in catalysis. By analogy with other hairpin-forming transposases (35,36), we and others searched for aromatic amino acids in Rag1 that could stabilize an extrahelical, flipped-out base. Through mutational and biochemical analysis, we found an aromatic residue in Rag1, W893, that may be involved in base flipping (20); others have proposed a second candidate, W956 (21). Several other amino acids in Rag1 are critical for hairpin formation and likely play key roles in generating or stabilizing a distorted DNA intermediate (20). One class of hairpin-deficient mutants is unusual in that the defect is conditional, depending on the coding flank sequence: some coding flank sequences prohibit hairpinning, whereas other flanks allow wild-type levels of hairpin formation (37–39). Because unpaired bases in the coding flank rescue this type of hairpinning defect, we and others have suggested that these conditional mutants may have difficulty generating sufficient distortion of the coding flank DNA to allow hairpin formation (13,14,39).

Although these earlier studies provided important clues about the DNA distortion phase of hairpin formation, the transesterification step was not examined. Here, we identify a mutant with a specific defect in transesterification, implicating basic amino acids in the

catalytic core of Rag1 in this process. We also show that some Rag1 mutants with severe defects in hairpin formation nonetheless catalyze substantial levels of transposition, and, in several cases, are stimulated by hairpin targets. Lastly, we analyze a catalytically defective Rag2 mutant (the only such mutant identified) and find that it shows a defect in target capture and a novel form of coding flank sensitivity, suggesting an unexpected role for Rag2 in DNA cleavage and transposition.

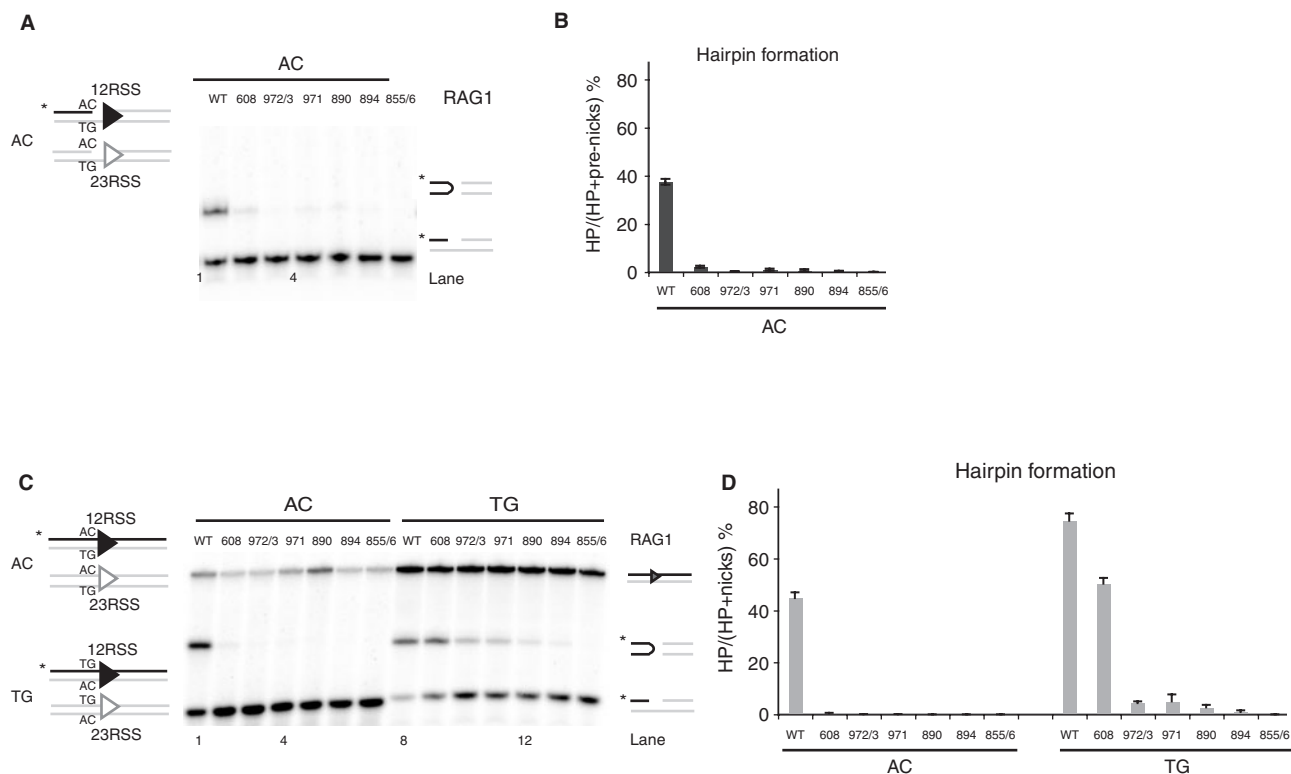
## MATERIALS AND METHODS

### Rag proteins

Experiments were performed with recombinant glutathione S-transferase (GST)-tagged mouse core Rag1 (residues 384–1008) and core Rag2 (residues 1–387). 240  $\mu$ g of Rag1 and 280  $\mu$ g of Rag2 in the pEBG vector (40) were transiently co-transfected into Chinese hamster ovary (RMP41) cells using 780  $\mu$ l of Fugene-6 transfection reagent (Roche, Indianapolis, Indiana). Proteins were co-purified as described (39), using GST affinity resin beads (GE, Piscataway, NJ). Multiple protein preparations were made, and the most active preparations were used. Mutant and wild-type Rag proteins shown in this article were prepared at the same time to eliminate variations between different preparations as a confounding factor in interpreting results, and were diluted such that equal amounts of each preparation ( $\sim$ 70 ng of Rag1 and 30 ng of Rag2) were added to reactions, as shown in Supplementary Figure 1.

### HMG protein

Recombinant human HMGB1 was transformed into BL21(DE3) *Escherichia coli* (41). A culture (450 ml) of transformed cells was grown to an  $OD_{600}$  of 0.5; isopropyl-beta-D-thiogalactopyranoside was then added to a final concentration of 1 mM, followed by shaking at 37°C for 4 h. Cells were then resuspended in 20 ml of resuspension buffer (100 mM Tris pH 8.0; 1 mM EDTA; 10% glycerol, w/v) and centrifuged at 10 000 r.p.m. for 15 min at 4°C. Pellet was resuspended in 3 ml of resuspension buffer per gram of pellet. 2-Mercaptoethanol was added to a final concentration of 2 mM, followed by 0.2 vol of 5 mg/ml lysozyme. The mixture was kept on ice for 30 min. Next, Tween-20 was added to a final concentration of 0.1% and NaCl was added to a final concentration of 1 M. The mixture was incubated on ice for 10 min and then centrifuged at 30 000 r.p.m. for 30 min at 4°C. An equal volume of 10 mM imidazole buffer was added to supernatant (0.5 M NaCl; 20% glycerol; 20 mM Tris-Cl, pH 7.9 at 4°C; 10 mM imidazole, pH 8.0; 2 mM 2-mercaptoethanol). Chelating Sepharose (GE, Piscataway, NJ) was prepared with nickel sulphate; slurry was then washed with water, followed by 10 mM imidazole buffer. Slurry was added to lysate, and bound in batch overnight at 4°C. After binding, slurry was washed twice with 10 mM imidazole. HMG was then eluted in five fractions using 60 mM imidazole buffer, dialyzed in dialysis buffer (25 mM Tris, pH 8.0; 1 mM EDTA; 1 mM DTT; 150 mM KCl; 10% glycerol). Finally, HMG was heat-inactivated at 65°C for 30 min.



**Figure 2.** Rag1 mutants exhibit defective hairpin formation. (A) *In vitro* cleavage assay using 12- and 23-RSS oligonucleotide substrates that mimic a nick on an AC flank, as depicted in schematic representation on the left. (B) Quantitation of gel in A, showing the percentage of pre-nicks converted to hairpins, was performed using ImageQuant software. Error bars represent SEM of three experiments. (C) *In vitro* cleavage assay using oligonucleotide substrates with either an AC or TG coding flank on both 12- and 23-RSS, as depicted on the left. Only the strands drawn as black lines were 5'-radiolabeled (\*), and thus detectable after denaturing gel electrophoresis. The reaction was performed for 30 min, in the presence of paired 12- and 23-RSS oligonucleotides, purified Rag1 (wild-type or mutants) and Rag2 proteins, HMG and  $Mg^{2+}$ . Reaction products were separated on a 12% sequencing gel and visualized by autoradiography. Reaction products are shown on the right (from top to bottom): uncleaved substrates, hairpin and nicks. (D) Quantitation of gel in C, showing the percentage of nicks converted to hairpins, was performed using ImageQuant software. Error bars represent SEM of three experiments.

### Oligonucleotide DNA substrates

Oligonucleotides used for cleavage assays were designed according to plasmid pJH290 (20), except that the sequence of the designated two base pairs at the coding flank was varied. For each reaction, both the 12 and 23-RSS are either uncleaved or pre-nicked and share the same coding flank sequence, whether matched or mismatched. Oligonucleotide-encoded hairpins used for transposition assays were described in (17) and contain the sequence 5'-CTGGGGCTGCAGG<sub>xx-xx</sub>CCTGCAGCC CCAG, where 'xx-xx' represents the tip of the hairpin. For a TG hairpin, 'xx-xx' represents TG-CA; for an AC hairpin, 'xx-xx' represents AC-GT; for a GC hairpin, 'xx-xx' represents GC-GC, etc.

### Plasmid DNA substrates

Plasmid targets used for transposition assays were pUC8 and F14C, a pUC8 plasmid with a 106 bp inverted repeat cloned into the EcoRI site (42).

### *In vitro* cleavage and transposition assays

Cleavage reactions and transposition reactions using oligonucleotide substrates were performed as described

in (17,20), respectively, except that 70 ng of Rag1 and 30 ng of Rag2 were added to each reaction. Reaction products were detected and quantified using PhosphorImager and ImageQuant software.

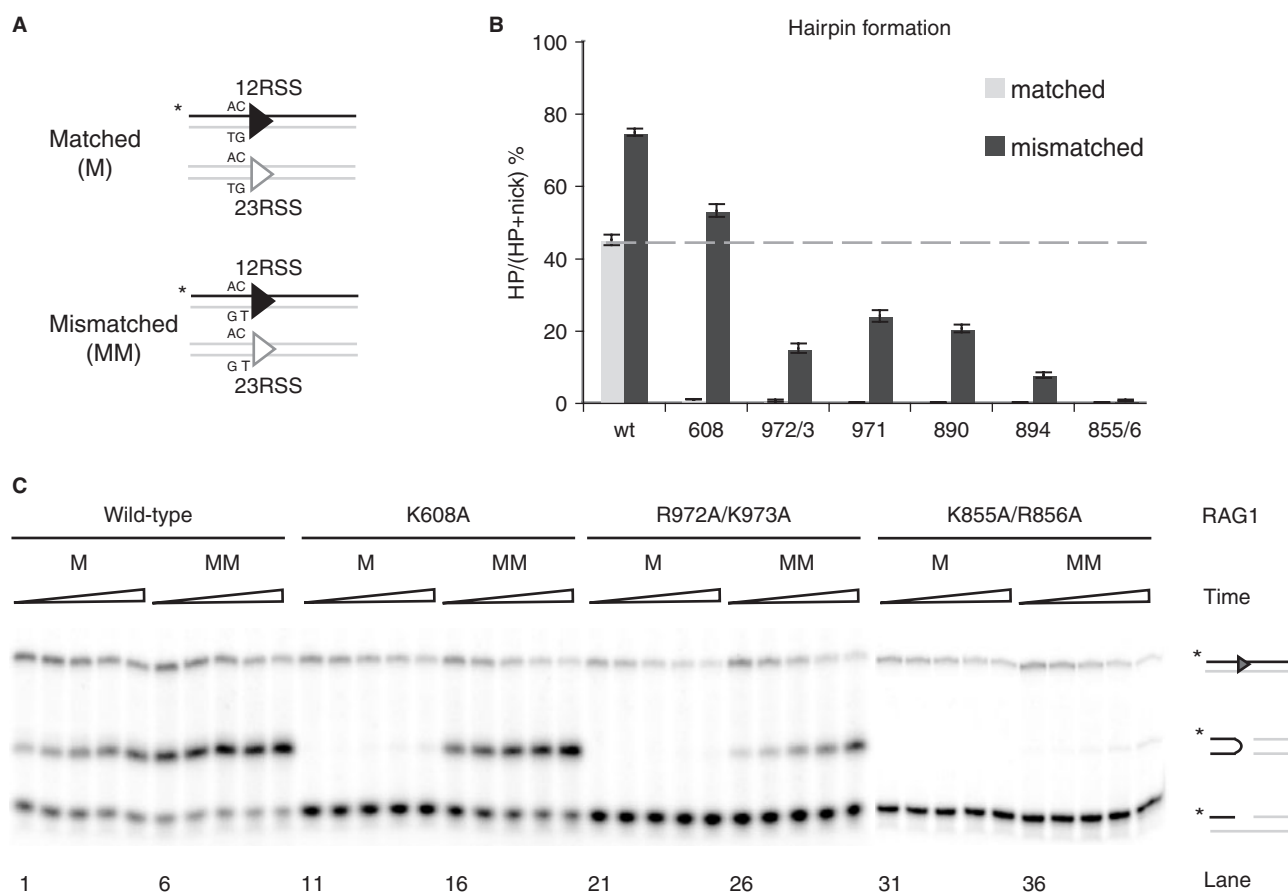
### Physical analysis of target capture

Target capture assays using radiolabeled hairpin target were performed as described (17), except that 70 ng of Rag1 and 30 ng of Rag2 were added to each reaction. Reactions were performed for 2 h before separation on a native 5% acrylamide gel at 100V, 4°C.

## RESULTS

### Mutations distinguishing hairpin formation and transposition

We began by examining a set of Rag1 mutants (K608A, R972A/K973A, F971A, K890A, R894A and R855A/K856A) previously shown to have defects in hairpin formation, as confirmed using pre-nicked substrates (Figure 2A and B). It was not known whether these defects affect an early step (generation or stabilization of a distorted DNA intermediate) or a later phase of the reaction (transesterification). To determine the reaction step affected by these mutants, we first tested whether the



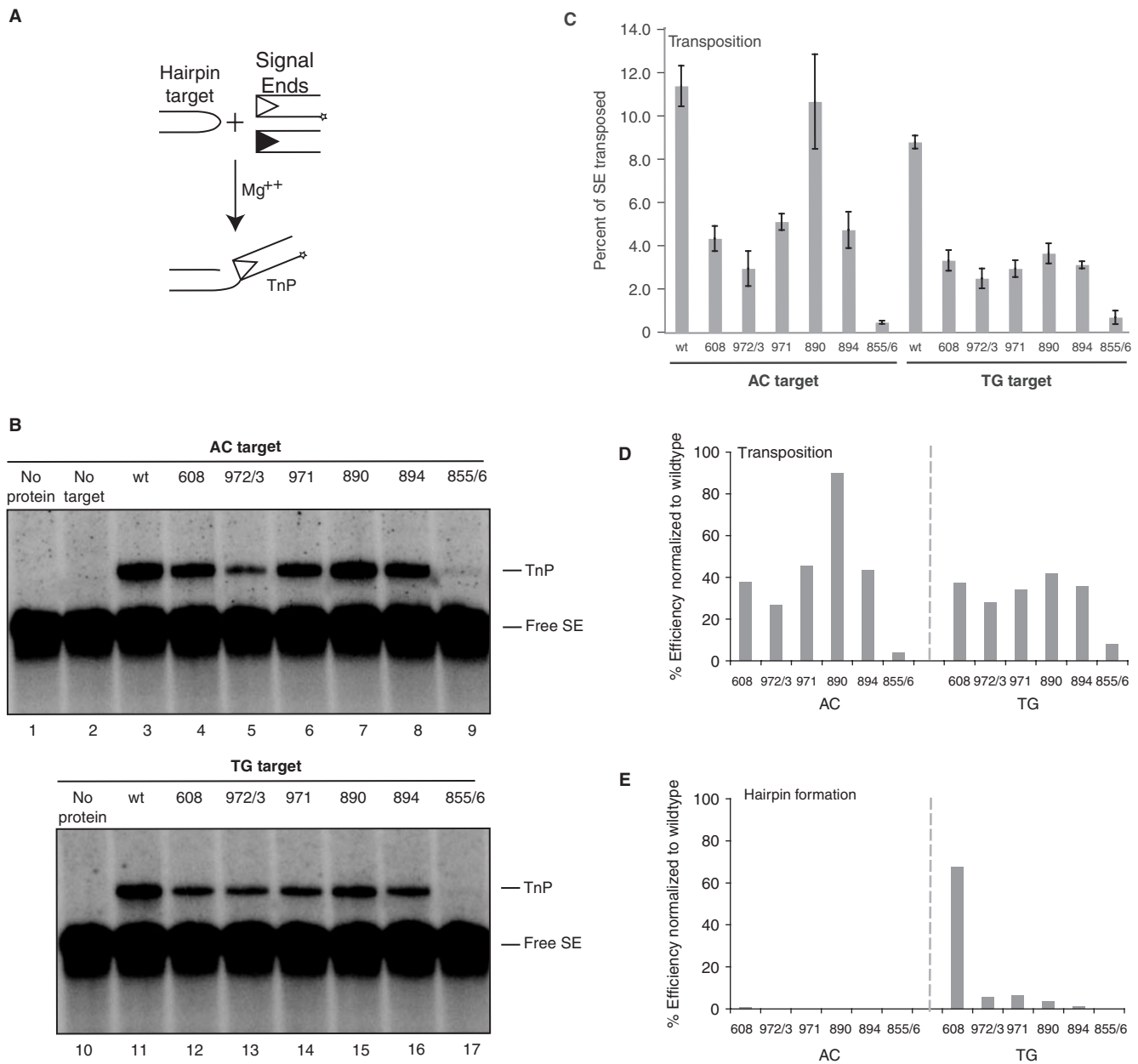
**Figure 3.** Rag1 mutants exhibit varying levels of hairpin formation when provided a mismatched substrate. (A) Schematic representation showing oligonucleotide substrates containing matched (M) or mismatched (MM) base pairs at the coding flank on both 12- and 23-RSS. Only the strands drawn as a black line were 5'-radiolabeled (\*), and thus detectable after denaturing gel electrophoresis. (B) Quantitation of *in vitro* hairpin formation after 30 min incubation time, showing the percentage of nicks converted to hairpins. Reaction products were separated on 12% sequencing gel, visualized by autoradiography and quantified using ImageQuant software. Dashed line represents the wild-type level of hairpin formation at a matched substrate. Error bars show SEM of three experiments. (C) *In vitro* hairpin formation assay. Representative results from wild-type Rag1, K608A, R972A/K973A and K855A/R856A are shown. Reaction products were separated on a 12% sequencing gel and visualized by autoradiography. Triangles represent increasing incubation times: 5, 10, 20, 40 and 80 min. Reaction products are depicted on the right (from top to bottom): uncleaved substrates, hairpins and nicks.

defect was due to coding flank sensitivity. Rag1 mutants mapping to amino acids 606–611 can nick but are unable to form hairpins on substrates with an AC coding flank, although they are capable of hairpinning on a TG coding flank (19,37,39). Only Rag1 K608A showed significant coding flank sensitivity, as evidenced by differential hairpin formation with substrates bearing AC or TG coding flanks (Figure 2C and D). Notably, R855A/K856A showed robust nicking but no detectable hairpinning activity with either TG or AC coding flanks (Figure 2C, lanes 7 and 14) or with substrates bearing a variety of other coding flank sequences (data not shown). Similar results were obtained with the single mutant, R855A (data not shown).

We next investigated the effects of mismatched coding flanks, which can rescue hairpin formation by certain Rag1 mutants [presumably by facilitating DNA distortion at the cleavage site (20,39)] (Figure 3A). In agreement with previous work (13,14,20,39), mismatches enhanced hairpin formation by the wild-type Rag complex and fully rescued hairpin formation by K608A (Figure 3B).

Mismatched substrates partially rescued hairpin formation by R972A/K973A, F971A, K890A and R894A (Figure 3B). K855A/R856A shows traces of hairpin products, which could only be detected at long incubation times (Figure 3C), suggesting a specific defect in transesterification.

To assess the effects of these mutations on the transesterification step, we tested their ability to catalyze transposition. We used a standard *in vitro* assay with precleaved signal ends (to bypass hairpin formation) and oligonucleotide hairpin targets chosen to correspond to the substrates with AC or TG flanks shown in Figure 2 (Figure 4A). Several mutants were much more proficient for transposition than hairpin formation (Figure 4B): Rag1 R972A/K973A, F971A, K890A and R894A demonstrated transposition efficiencies of 25–95% of wild-type on an AC hairpin target and 30–40% of wild-type on a TG target (Figure 4C). To compare directly the ability of the mutants to perform hairpin formation and transposition, we normalized the data shown in Figures 2D and 4C, setting wild-type activity at 100%



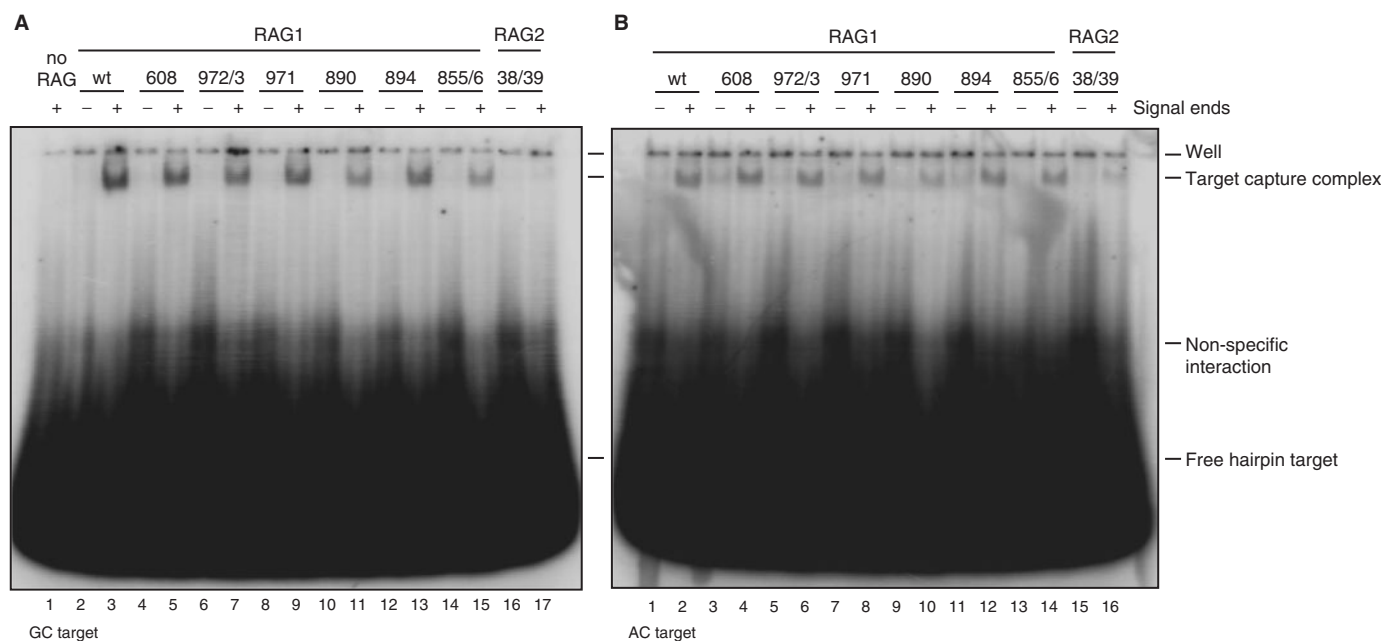
**Figure 4.** Several Rag1 nick-only mutants are able to transpose into different hairpin targets. (A) Schematic representation of *in vitro* transposition assay. Radiolabeled signal ends (\*) are incubated in  $\text{Ca}^{2+}$  with Rag proteins and HMG. Annealed hairpin target is then added. Following incubation in  $\text{Mg}^{2+}$ , transposition products are detected as signal ends covalently attached to the tips of hairpin targets. (B) *In vitro* transposition products were separated on a 15% sequencing gel, and visualized by autoradiography. (C) Quantitation of *in vitro* transposition assay shown in (B). Efficiency of transposition was determined as percentage of signal ends transposed by each of the mutants into AC and TG hairpin targets. Error bars represent SEM of five experiments. (D and E) Quantitation of activity of Rag1 mutants R972A/K973A, F971A, K890A, R894A, R855A/K856A as a percentage of wild-type activity in transposition into an AC or TG hairpin target (D) and hairpin formation on an uncleaved substrate with an AC or TG coding flank (E). Free SE, free (not transposed) signal ends; TnP, transposition products.

(Figure 4D and E). In this analysis, all Rag1 mutants except for R855A/K856A were much better at transposition than hairpin formation. R855A/K856A, however, was consistently defective for transposition (Figure 4B and C), and single mutant R855A also showed severely defective transposition (data not shown). This defect cannot be explained by its ability to capture target DNA, which is only mildly affected (Figure 5). We conclude that the hairpin formation defects of R972A/K973A,

F971A, K890A and R894A do not reflect fundamental problems with the chemistry of transesterification but that R855A/K856A (and perhaps specifically R855A) exhibits a defect in this step.

#### Effects of hairpin targets on transposition by the Rag1 mutants

Four Rag1 mutants (R972A/K973A, F971A, K890A and R894A) were quite proficient for transposition into



**Figure 5.** Signal end complexes formed by Rag1 mutants, but not Rag2 K38A/R39A, are able to capture target. (A) Rag-signal end complexes were formed *in vitro* and then incubated with radiolabeled GC hairpin target. After 2 h, complexes were separated on a native 5% polyacrylamide gel, which was visualized by autoradiography. (B) Rag-signal end complexes were formed and then incubated with radiolabeled AC hairpin target. After 2 h, complexes were separated on a native 5% polyacrylamide gel, which was visualized by autoradiography. TCC, target capture complex.

oligonucleotide targets. Given their severe defects in hairpin formation, we wondered whether any of these mutations might specifically affect recognition of hairpin targets, which strongly stimulate transposition by the wild-type Rag complex. To determine this, we used two previously described plasmid targets: pUC8 and pUC8 containing a 106-base pair inverted repeat (F14C) that forms a cruciform structure bearing hairpin tips [Figure 6A; (16,42,43)]. Three mutants (K608A, F971A and K890A), while somewhat impaired for transposition, were consistently and strongly stimulated by F14C (Figure 6B and C). These mutants thus retain the ability to recognize hairpin structures. One mutant (R894A) was modestly stimulated in some experiments. The hairpin target did not stimulate transposition by R972A/K973A, however (Figure 6B, lanes 7 and 8). R855A/K856A was inactive for transposition into both targets (Figure 6B and C, lanes 15 and 16), consistent with a defect in transesterification.

#### A Rag2 mutation affects both nicking and target capture

To better understand the involvement of Rag2 in hairpin formation and transposition, we analyzed the only Rag2 mutation suspected to affect catalysis, K38A/R39A (44). We found, surprisingly, that this mutation causes coding flank sensitivity: it forms hairpins near wild-type levels at an AC flank but is almost inactive with a TG flank (Figure 7A). This is a reversal of the coding flank preferences of Rag1 mutants, which form hairpins efficiently at TG, but not AC, flanks. Even more surprisingly, this conditional defect in cleavage mainly affects the nicking reaction. Total levels of nicking activity on a TG flank (reflected in the amounts of nicks plus

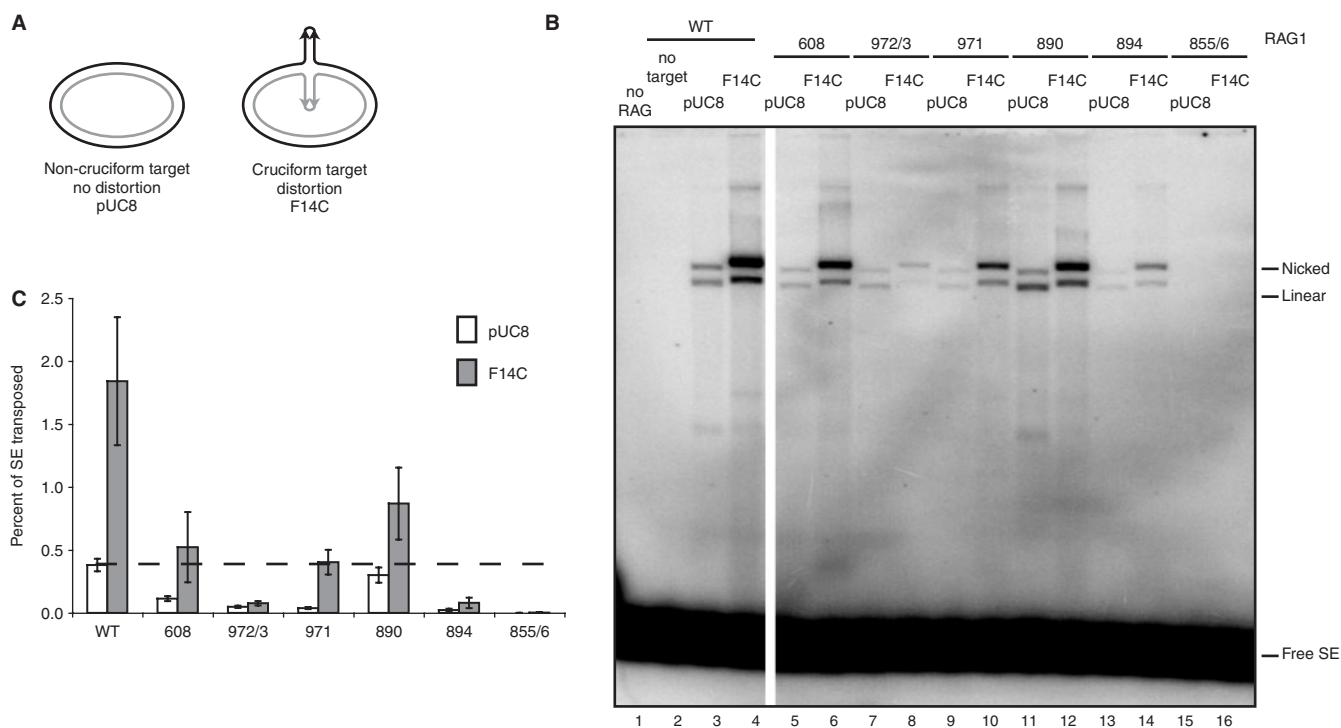
hairpins) were only 10% of levels on other substrates (Figure 7A), and a prenicked substrate fully rescued hairpin formation (Figure 7B). Consistent with results in Qiu *et al.* (44), mismatched base pairs at the coding flank actually inhibited nicking and hairpin formation by K38A/R39A, again in direct contradistinction to the behavior of the Rag1 mutants (Figure 7C); four different pairs of mismatched sequences produced similar results (data not shown). These data indicate that Rag2 plays a previously unsuspected role in cleavage.

Lastly, we investigated the effects of the K38A/R39A mutation on transposition. This mutant showed no detectable transposition of precleaved signal ends into a number of hairpin target sequences and was only weakly active using the most favored targets (Figure 7D). Transposition into plasmid targets was quite weak, although some activity was observed with the F14C cruciform target (Figure 7E). K38A/R39A was severely defective for capture of two hairpin target sequences (Figure 5).

## DISCUSSION

### Rag1 amino acids critical for transesterification

We previously identified residues in Rag1 involved in hairpin formation and provided evidence that Rag1 possesses a YKEFRK motif that may be functionally similar to the YREK motif found in IS4 family transposases (20). Now, data in the present work have identified amino acids in the Rag complex that are specifically required for transesterification (R855/K856). Transposition by IS4 family transposases, which involves hairpin formation, also requires basic residues: the structure of the



**Figure 6.** Rag1 K608A, F971A and K890A are capable of wild-type levels of transposition when provided a cruciform target. **(A)** Schematic representation of transposition targets. The pUC8 plasmid was used as a nondistorted target control. F14C contains a 106 bp inverted repeat sequence that adopts a cruciform structure. **(B)** Products of *in vitro* transposition assay using targets in **(A)** were separated on a 1% agarose gel and visualized by autoradiography. All lanes were run on the same gel, but a few lanes between WT and 608 were removed for demonstration purposes. **(C)** Quantitation of percentage of signal ends transposed into either nondistorted pUC8 target or distorted F14C target in **(B)**. Dashed line marks wild-type level of transposition into pUC8 target. Error bars represent SEM of five experiments. Nicked, products of a single signal end insertion into plasmid yield a nicked plasmid transposition product; linear, products of a double signal end insertion into plasmid target yield a linearized plasmid transposition product; free SE, free (un-transposed) signal ends.

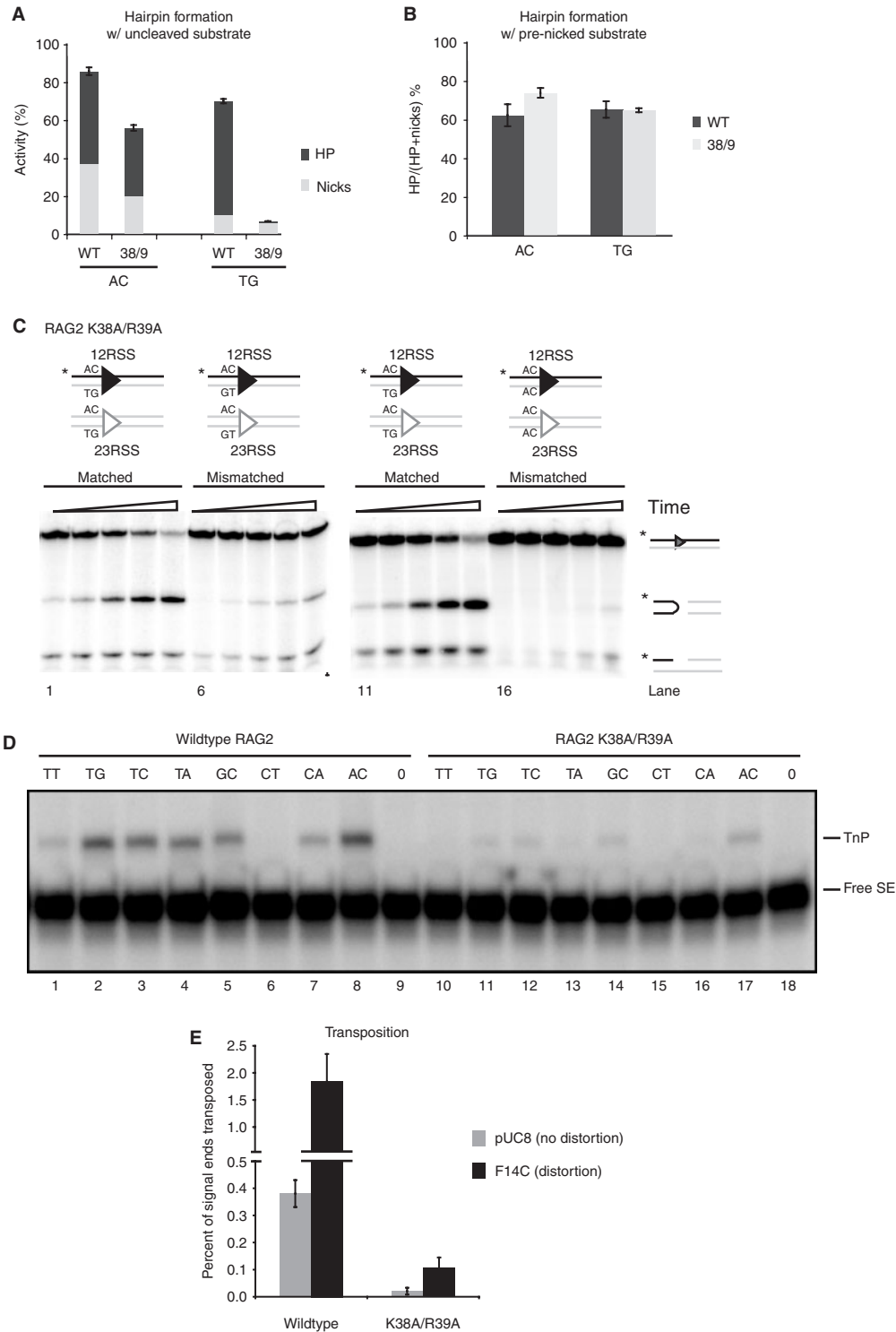
Tn5/DNA complex shows that the 5' phosphate group of thymine 2 of the transferred strand is held in place by specific contacts with R210 and R322 (35). R322 is part of the YREK motif (45), and mutation of this residue diminishes hairpin opening and strand transfer activity (46). We speculate that R855 in Rag1, which, similar to R210 in Tn5, is located N-terminal to and apart from the catalytic E residue, may likewise interact with the phosphate backbone of the DNA, and that the R855A/K856A mutant may fail to correctly position the DNA substrate in the catalytic pocket.

#### Novel roles for Rag2 in DNA cleavage and transposition

Although it has long been known that Rag2 is required for recombination and transposition, its mechanistic contributions to these reactions have remained unknown. Our analyses of the K38A/R39A Rag2 mutant revealed an unexpected sensitivity to coding flank sequence. [R39A was previously noted to cleave at wild-type levels, but this study was performed using favorable coding flank sequences (47); C.P.L. and D.B.R., unpublished data.] Our data provide strong evidence that Rag2 plays a critical role both in contacting DNA at the RSS-coding flank junction and in site-specific nicking.

The phenotype of Rag2 K38A/R39A differs from that of the coding flank sensitive Rag1 mutants in two

interesting respects. K38A/R39A shows diametrically opposite coding flank sequence preferences, and it displays its sequence sensitivity at the nicking step of the reaction rather than the hairpin formation step. The nicking defect could reflect less stable binding of the mutant complexes to particular coding flank sequences. Another (nonexclusive) possibility is that nicking, like hairpin formation, may be promoted by formation of a distorted DNA structure, whose stability is influenced by the coding flank sequence. A precedent for DNA distortion prior to nicking is provided by another hairpin-forming transposase, Tn5: mutation of W323 affects both distortion prior to nicking and the nicking step itself (48). Our observation that unpaired coding flank nucleotides inhibit nicking by K38A/R39A suggests that these unpaired substrates prevent this mutant from creating the proper distortion for nicking. These findings, in conjunction with the previous observation that Rag2 contacts DNA at the coding flank (28,49,50), strongly suggest that the N-terminus of Rag2 contacts the cleavage site and that these contacts are critical for catalysis of nicking. Our data also provide insight into the role of Rag2 in transposition. The severe target binding defect of K38A/R39A indicates Rag2 involvement at an early stage in the recognizing or binding of DNA, and, in particular, participation of the N-terminal region of Rag2 in making critical contacts with target DNA.



**Figure 7.** Rag2 K38A/R39A forms hairpins but is deficient for transposition. **(A)** Quantification of *in vitro* cleavage reaction to measure hairpin and nick formation was performed using oligonucleotide substrates containing either an AC or TG flank (as in Figure 2A). Reaction products were separated on 12% sequencing gel, visualized by autoradiography and quantified using ImageQuant software. Top part of bar represents the percentage of hairpin products converted from nicks, and bottom part represents the percentage of nicked products. Height of each bar (top + bottom) represents total nicking activity. **(B)** *In vitro* cleavage reaction using oligonucleotide substrates containing a nick (as in Figure 2C), with either AC or TG flanks. The reaction was visualized and quantified as in (A). The quantification shows the percentage of nicks converted to hairpins by wild-type and Rag2 K38A/R39A. **(C)** Cleavage reaction by mutant K38A/R39A, using oligonucleotide substrates containing a matched (M) or mismatched (MM) coding flank. Two different pairs of MM sequences were used as shown schematically above gels. **(D)** Reaction products from *in vitro* transposition into multiple targets by Rag2 K38A/R39A. Reaction products were separated on a 15% sequencing gel and visualized as in (A). **(E)** Quantification of *in vitro* transposition into nondistorted (pUC8) or distorted (F14C) plasmid targets comparing wild-type and Rag2 K38A/R39A. Reaction products were separated on a 1% native agarose gel, visualized by autoradiography and quantified using ImageQuant software. Graph represents percentage of signal ends transposed into target; error bars show SEM over three experiments. TnP, transposition products; free SE, free (untransposed) signal ends.



### Differences between hairpin formation and transposition

We have found that a number of Rag1 mutants with severe defects in hairpin formation remain remarkably competent for transposition. The K890A mutant is particularly striking in that it combines a profound defect in hairpin formation with near-wild-type transposition activity. This suggests that the mutations affect generation of distorted DNA intermediates, while retaining the ability to bind to hairpin targets. In addition, mismatches in the coding flanks facilitated hairpin formation by R894A and R972A/K973A; yet, these mutants failed to show a corresponding increase in transposition activity when presented with hairpin targets, which are normally quite conducive to this reaction. Together, these data suggest that the Rag complex may utilize different combinations of residues to engage unpaired or distorted DNA in the two reactions. It is instructive in this regard to consider studies of phosphorothioate stereoselectivity in Tn10, which suggest that the transesterification steps of hairpin formation and transposition occur with significant conformational differences at the active site (51).

Further analyses of postcleavage and target capture complexes should help define the molecular basis for our observations. Moreover, our identification of mutants that alter the balance between the two alternative transesterification reactions, hairpin formation and transposition, raises the interesting possibility that some Rag mutants could promote increased transposition, perhaps at the expense of efficient V(D)J recombination.

### SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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*Conflict of interest statement.* None declared.

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