Mutating Conserved Residues in the Ribonuclease H Domain of Ty3 Reverse Transcriptase Affects Specialized Cleavage Events*

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ABSTRACT The reverse transcriptase-associated ribonuclease H (RT/RNase H) domains from the gypsy group of retrotransposons, of which Ty3 is a member, share considerable sequence homology with their retroviral counterparts. However, the gypsy elements have a conserved tyrosine (position 459 in Ty3 RT) instead of the conserved histidine in the catalytic center of retroviral RTs such as at position 539 of HIV-1. In addition, the gypsy group shows conservation of histidine adjacent to the third of the metal chelating carboxylate residues, which is Asp 426 of Ty3 RT. The role of these and additional catalytic residues was assessed with purified recombinant enzymes and through the ability of Ty3 mutants to support transposition in Saccaromyces cerevisiae. While all mutations had minimal impact on DNA polymerase function, amidation of D358, E401 and D426 eliminated Mg\(^{++}\) and Mn\(^{++}\)-dependent RNase H function. Substitution of H427, Y459 with Ala and D469 with Asn resulted in reduced RNase H activity in presence of Mg\(^{++}\), while in presence of Mn\(^{++}\) these mutants displayed lack of turnover. Despite this, mutations at all positions were lethal for transposition. To reconcile these apparently contradictory findings, the efficiency of specialized RNase H-mediated events was examined for each enzyme. Mutants retaining RNase H activity on a heteropolymeric RNA/DNA hybrid failed to support DNA strand transfer and release of the (+) strand polypurine tract primer from (+) RNA, suggesting that interrupting one or both of these events might account for the transposition defect.
Abbreviations used: CA, capsid protein; IN, integrase; IPTG, isopropyl-1-β-D-thiogalactopyranoside; LTR, long terminal repeat; NC, nucleocapsid protein; nt, nucleotides; PPT, polypurine tract; RT, reverse transcriptase; RNase H, ribonuclease H.
INTRODUCTION Although many steps in the life cycle of *Saccharomyces cerevisiae* LTR-containing retrotransposons are readily amenable to genetic analysis, there have been limited biochemical studies on enzymes supporting these events. With respect to reverse transcriptase (RT)-mediated conversion of the (+) strand RNA genome into integration-competent, double-stranded proviral DNA, several notable differences from their retroviral counterparts have been documented. Examples include (a), the use of a non-contiguous primer binding site (PBS) to initiate (-) strand DNA synthesis in Ty3 (1) (b), long-range interactions between D-loop nucleotides of the cognate tRNA primer (tRNA\(^{\text{Met}}\)) and the RNA genome controlling initiation of (-) strand DNA synthesis (2) (c), initiation of (-) strand DNA synthesis from an internal region of the tRNA primer in Ty5 (3) (d), an alternate model of tRNA primer inheritance in Ty1 (4,5) and (e), divergence in both the length and sequence of their (+) strand, polypurine tract (PPT) primers (6,7). In an initial step towards dissecting these complex events at the molecular level, we reported the purification of recombinant p55 Ty3 RT and preliminary characterization of its DNA polymerase and ribonuclease H (RNase H) activities (8). More recently, an active form of Ty1 RT has also been described by Wilhelm and co-workers (9,10). Although the Ty3 enzyme would recapitulate precise selection, extension and excision of its (+) strand PPT primer, we were unsuccessful in replacing Mg\(^{2+}\) in the Ty3 RNase H domain with Fe\(^{2+}\) to support hydroxyl radical-mediated cleavage of duplex DNA, a feature common to the RTs of human and feline immunodeficiency viruses (8,11). Such a result suggested that the mode of metal ion co-ordination in the Ty3 RNase H domain might differ from the extensively characterized retroviral enzymes.

Sequence alignment shows that a conserved histidine and a cluster of four carboxylate residues constitute the -D-E-D-H-D- motif common to the catalytic site of both retroviral and prokaryotic RNases H (12-14). In the proposed general acid-base model of catalysis (15,16) D134 of *E.coli* RNase H positions the attacking water molecule to donate a proton to H124, E48
anchors the water molecule acting as a general acid, while D10 and D70 co-ordinate divalent metal in the active site. Biochemical studies with HIV-1 and E.coli RNases H have indicated that only the first three carboxylate residues are critical for catalysis. Substituting HIV-1 H539 and E.coli H124 with N, D or A, only reduces RNase H activity (17,18) and a similar effect accompanies replacement of HIV-1 D549 and E.coli D134 with Asn (18-20). These findings suggest that the -H-D- component of the -D-E-D-H-D- motif can assist catalysis but are dispensable. In view of the sequence similarities between retroviral and retrotransposon RNases H, a surprising observation was the presence of tyrosine (Y459) in the Ty3 RNase H domain at a position generally occupied by histidine. A contribution of H124 of E.coli RNase H to catalysis has been proposed (15,21,22), but it was not immediately clear how this function might be fulfilled by tyrosine in the retrotransposon enzyme without invoking its activation by a nearby acidic residue. However, a compilation of RNase H sequences from the Gypsy group of retrotransposons (8) suggested a conserved histidine immediately adjacent to one of the catalytic carboxylate residues (D426) might be implicated in catalysis, leading to our proposal of a -D-E-DH-Y-D- motif (Fig. 1[A]). Interestingly however, a recent phylogenetic compilation of RNase H sequences from LTR- and non-LTR-containing elements suggests retrotransposon enzymes may lack the flexible “His loop” of retroviral and bacterial enzymes (13) (Fig. 1[B]), which may result in decreased RNase H activity. The same communication also indicates that while the DNA polymerase domains Ty3/Gypsy elements and retroviruses are closely related, their RNase H domains display much greater divergence. Such findings open the possibility that the retrotransposon RNase H domain could function in the absence of a “His loop”, and that Y459 of Ty3 RT need not be directly involved in catalysis. Thus, a more detailed biochemical analysis of the evolutionarily conserved residues of the Ty3 RNase H domain is clearly warranted.
In this communication, we investigated the role of several residues in the catalytic site of Ty3 RT–RNase H using purified, recombinant enzymes, in addition to monitoring transposition of the Ty3 element containing these mutations. Of the conserved carboxylate residues, RNase H activity is eliminated by replacement of D358, E401 and D426 with their amidated counterpart, while the equivalent replacement of D469 only partially reduced activity. Reduced activity was also noted for mutants H427A and Y459A. In contrast to our biochemical observations, all mutations prevented Ty3 transposition. Further evaluation of mutants H427, Y459 and D469 indicated they failed to support two specific events in Ty3 transposition, namely DNA strand transfer and processing of the (+) strand PPT primer. Taken together, our data suggests that while the D358/E401/D426 triad constitutes the biologically relevant metal binding site and is indispensable for RNase H function, H427, Y459 and D469 may be more important in positioning the substrate for specific cleavage events. Differences in RNase H activity between Ty3 and HIV-1 RT were also observed when Mn$^{2+}$ was substituted for Mg$^{2+}$ as the divalent cation, suggesting differences in the mode of metal ion co-ordination between retrotransposon and retroviral enzymes.
EXPERIMENTAL PROCEDURES

Strains and culture conditions - *E. coli* and *S. cerevisiae* strains were cultured and transformed by standard methods. *S. cerevisiae* yTM443 (23) (*MATa trp1-H3 ura3-52 his3 Δ200 ade2-101 lys2-1 leu1-12 can1-100 ΔTy3 bar::hisG Gal3*), a derivative of yVB110 containing no endogenous copies of Ty3 (24), was used for transposition assays, whole-cell extract DNA and protein analyses. *E. coli* CJ236 (New England Biolabs) (F' *cat* (=pCJ105; M13*scm*/*Cm*)/ *dut ung-1 thi-1 relA1 spoT1 mcrA*) was used for production of single-stranded DNA for site-directed mutagenesis (25).

Modeling the Ty3 RNase H Domain - The C-terminal Ty3 RNaseH domain (residues 341-476) was modeled using the RNaseH domain of p66 HIV-1 RT (Protein Data Bank ID 1HYS, residues 427-553) as the reference protein. The Ty3 structure was generated using the Modeller function within the Homology module of InsightII (Accelrys). The conserved carboxylates D358, E401, D426 and D469 were manually aligned to the equivalent RT residues (D443, E478, D498 and D549, respectively). Optimization was set to high while all other options remained at the defaults.

Site-directed mutagenesis - Point mutations in the RNase H domain of Ty3 RT expressed on plasmid p6HTy3RT (8) were introduced by one of two PCR strategies. For mutants E401Q, D426N and H427A, 3' and 5' primers homologous to the mutation site, each containing the desired mutated codon, were used in separate PCR reactions (26), paired with the appropriate primer for either an upstream or downstream restriction site (5' Ty3 RT *Sall* and 3' Ty3 RT *HindIII*, respectively). The PCR products generated overlap at the ends containing the mutation site; these products were then used as template to amplify the entire mutated construct with
outside primers. Mutants D358N, Y459A and D469N were generated using the appropriate 5' and 3' primers containing the desired mutated codon. The resulting fragments were cleaved and subcloned into p6HTy3RT. The final constructs were completely sequenced in the region derived by PCR amplification. These same mutations were introduced into plasmid pEGTy3-1 (24). 3' primers homologous to the mutation site were used for site-directed mutagenesis as described (25).

**Expression and Purification of Ty3 RT mutants** - Ty3 RT variants were purified from logarithmically grown and IPTG-induced *E.coli* cultures by a combination of metal chelate (Ni\textsuperscript{2+}-NTA Sepharose, Qiagen) and size exclusion chromatography (Superdex 200, Pharmacia). Purified enzymes were free of contaminating nucleases and stored at -20°C in a 50% glycerol-containing buffer (50mM NaH\textsubscript{2}PO\textsubscript{4}/Na\textsubscript{2}HPO\textsubscript{4} (pH 7.8), 0.7 M NaCl). Under these conditions we observed minimal loss of activity over several months.

**DNA Polymerase Activity** - RNA-dependent DNA polymerase activity was evaluated on a 138-nt RNA template (prepared by *in vitro* transcription), corresponding to nucleotides 4851-4977 of Ty3 genome plus 12 additional nucleotides, hybridized to a 5' \[^{32}\text{P}\] end-labeled 20 nt DNA primer (Integrated DNA Technologies). Template-primer was annealed by incubation at 95°C in 10 mM Tris/HCl (pH 7.8), 2.5 mM MgCl\textsubscript{2} and slow cooling to room temperature. A reaction mixture containing 50 nM template-primer and 250 \(\mu\)M dNTPs was prepared in a buffer comprising 10 mM Tris/HCl (pH 7.8), 9 mM MgCl\textsubscript{2}, 80 mM NaCl and 5 mM dithiothreitol. DNA synthesis was initiated at 30°C by addition of wild type or mutant RT to a final concentration of 50 nM in a final reaction volume of 10 \(\mu\)l. Aliquots were removed after 5 min and mixed with an equal volume of 89 mM Tris borate, pH 8.3, 2 mM EDTA, and 7M urea containing 0.1%
bromophenol blue and xylene cyanol. Polymerization products were resolved by high voltage denaturing polyacrylamide gel electrophoresis and evaluated by autoradiography.

**RNase H Activity** - RNase H activity was initially evaluated concomitant with polymerization, using the substrate indicated above, but relocating radiolabel to the 5' terminus of the RNA template. A reaction mixture containing 50 nM template-primer and 250 µM dNTPs was prepared in a buffer of 10 mM Tris/HCl (pH 7.8), 9 mM MgCl₂, 80 mM NaCl, 5 mM dithiothreitol. Hydrolysis (and polymerization) was initiated by adding wt or mutant RT to a final concentration of 250 nM in a 10 µl reaction and allowed to continue at 30°C for 20 min. Hydrolysis was terminated as above. Products were resolved by high voltage denaturing electrophoresis and evaluated by autoradiography. In absence of polymerization, RNase H activity was evaluated on a 5' end-labeled 40 nt RNA template (Dharmacon Research) annealed to a 30 nt DNA primer (Integrated DNA Technologies). A reaction mixture containing 50 nM template-primer was prepared in a buffer of 10 mM Tris/HCl (pH 7.8), 80 mM NaCl, 5 mM dithiothreitol. Nine mM MgCl₂ or 1 mM MnCl₂ was used for Mg⁺⁺- or Mn⁺⁺-dependent hydrolysis, respectively. Hydrolysis was initiated by addition of enzyme to a final concentration of 50 nM in a final volume of 60 µl. Ten µl aliquots were removed at times indicated and processed as above.

**DNA Strand transfer** - Strand transfer reactions were performed using a 40 nt donor RNA template annealed to a 5' [³²P] end-labeled 20 nt DNA primer (Integrated DNA Technology) and a 40 nt acceptor RNA (Dharmacon Research). Donor and acceptor templates were designed to share 20 nt of homology at their 5' and 3' termini, respectively. Polymerization and successful strand transfer produces a 60 nt cDNA product. A reaction mixture containing 50 nM donor RNA template/DNA primer, 250 nM acceptor RNA template and 250 µM dNTPs was prepared in a
buffer of 10 mM Tris/HCl (pH 7.8), 9 mM MgCl₂, 80 mM NaCl, 5 mM DTT. Polymerization was initiated by addition of Ty3 RT to a final concentration of 250 mM in a final volume of 60 µl. Ten µl aliquots were removed at times indicated in the text and processed as above. The same system was adapted to evaluate RNase H activity during DNA synthesis, using a 5' [³²P] end-labeled 40 nt donor RNA annealed to the 20 nt DNA primer.

**Polypurine Tract Selection** - To evaluate Ty3 PPT selection a 65-nt, (-) strand DNA template (corresponding to nucleotides 4848-4913 of the Ty3 genome) containing the PPT complement was hybridized to a variety of 5' end-labeled (+) strand RNA primers spanning the PPT by heating to 90°C and slow cooling in 10 mM Tris/HCl (pH 7.5), 2.5 mM MgCl₂. A reaction mixture containing 50 nM template-primer was prepared in a buffer of 10 mM Tris/HCl (pH 7.8), 9 mM MgCl₂, 80 mM NaCl, 5 mM dithiothreitol. Hydrolysis was initiated by addition of RT to a final concentration of 50 mM in 10 µl volume and allowed to continue at 30°C for 20 min. Reactions were stopped and hydrolysis products resolved as above.

**Transposition assays** - Qualitative plasmid-based suppressor target assays were performed as previously described (27). The assay is based on expression of Ty3 under control of the GAL1-10 promoter on a URA3-marked donor plasmid (pEGTy3-1) and subsequent integration of the replicated Ty3 into a HIS3-marked target plasmid (pCH2bo19V) (28). The target plasmid contains two divergent tRNA genes. One of these tRNA genes acts to recruit Ty3 to the target site. The other is a transcriptionally inactive ochre suppressor tRNA^{Tyr} gene (sup2-o), which is activated by Ty3 integration into the target site. Transposition is scored by suppression of the ochre nonsense mutations, ade2-101 lys2-1, in yeast strain yTM443. Suppression in cells that have undergone transposition results in papillations on synthetic complete medium containing...
glucose (SD) and lacking adenine and lysine. yTM443 cells were transformed with pEGTy3-1, carrying wild-type or mutant Ty3, and the target plasmid pCH2bo19V and plated onto SD medium lacking uracil and histidine. Three independent colonies from each transformation were patched onto SD -URA -His. Plates were incubated at 30°C for 24 h, and the cells were replica-plated to SD medium -ADE -Lys and to synthetic complete medium containing galactose (SG) lacking uracil and histidine to induce Ty3 transposition. After 48 h at 30°C on SG medium, the patches were replica-plated onto SD medium -ADE -Lys and incubated at 30°C for 6 days. Transposition was scored as papillations on SD medium -ADE -Lys.

**Whole-cell extraction** - Cultures (10 ml) of yTM443 cells transformed with pEGTy3-1, carrying wild-type or mutant Ty3, were grown in SG medium to an absorbance of ~1.0 at 600nm and the cells collected by centrifugation. Whole-cell extracts were prepared essentially as described previously (27). Briefly, pelleted cells were resuspended in 1.2 ml of whole-cell extract buffer (0.1 mM EDTA, 25 mM HEPES (pH 7.5), 50mM KCl, 5 mM MgCl₂, 10% glycerol) containing 1 µg/ml of leupeptin and pepstatin, and 1mM PMSF. Cells were lysed by vortexing in presence of glass beads; the lysate was clarified by centrifugation and protein concentration was determined using the micro BCA assay kit (Pierce).

**Virus-like particle (VLP) preparation** - One liter cultures of yTM443 cells transformed with pEGTy3 derivatives were grown to late log phase in SG medium to induce Ty3 expression. A mock VLP preparation was made with non transformed yTM443 cells. VLPs were partially purified from whole-cell extract as previously described (29). Briefly, the cells were harvested, washed in buffer, digested with zymolyase and lysed by vortexing with glass beads. Whole cell extract was fractionated over a 70%, 30% and 20% (5, 5 and 15 ml, respectively) sucrose step gradient by centrifugation in a Surespin 630/36 ml rotor (Sorvall) at 22,000 rpm for 3 h at 4°C.
Four ml of the 70%/30% interface, where VLPs sediment, was collected and divided in two portions. Each portion was concentrated by centrifugation in a Surespin 630/17 ml rotor (Sorvall) at 24,000 for 1 h at 4°C and the pellet was resuspended in 100 µl of buffer containing 9mM HEPES (pH 7.8), 13.5 mM KCl, 4.5 mM MgCl₂ and 10% glycerol. VLP proteins were used for IN immunoblot analysis.

**Immunological analysis** - Proteins from whole-cell extracts and VLPs were fractionated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes (Hybond ECL; Amersham), and probed with antibody to Ty3 NC (a generous gift of J.L. Darlix, ENS-INSERM U412, Lyon, France), CA or IN (23). Secondary antibodies to rabbit IgG were detected by chemiluminescence, using the ECL system as described by the manufacturer (Amersham).
RESULTS

Preliminary Characterization of Ty3 RNase H Mutants – The recombinant enzymes were purified and their purity was estimated by Coomassie Brilliant Blue staining (Fig. 1[C]). Prior to evaluating Ty3 RNase H function, it was important to confirm that altering conserved residues in this domain did not induce global changes in enzyme structure. Using the approach of Figure 2[A], RNA-dependent DNA polymerase and RNase H activity were monitored concomitantly by locating the $[^{32}\mathrm{P}]$ label to the 5' terminus of the 20 nt primer or 138 nt RNA template, respectively. The DNA polymerase profiles of Fig. 2[B], Panel [i], obtained after 5 min incubation, indicate minimal differences between the recombinant enzymes. The exception to this was mutant D358N, which consistently displayed lower activity with extended incubation. Equivalent results were obtained on several different substrates (data not shown). Thus, to a first approximation, mutating conserved residues of the Ty3 RNase H domain had minimal effects on the structure of the DNA polymerase catalytic center.

In a second experiment, relocating radiolabel to the template 5' terminus allowed RNase H activity to be evaluated during RNA-dependent DNA synthesis. Under such conditions, the primary hydrolysis products generated by wild type Ty3 RT are 21 and 12 nt (region “a”), which would be the expected products from a template on which the primer had been fully extended. Despite prolonged incubation, these products are absent for mutants D358N, E401Q and D426N (Fig. 2[B], Panel [ii], Lanes 3-5, respectively). By analogy with a recent model for RNase H-mediated catalysis (16), D358 and D426 would directly coordinate the divalent metal, with E401 positioning the water molecule acting as a general acid. Altering any of these three residues might be expected to eliminate hydrolysis. In contrast, H427, Y459 and D469 appear dispensable for Ty3 RNase H activity (Fig. 2[C], Panel [ii], Lanes 6-8, respectively). In addition to the 21- and 12 nt hydrolysis products, cleavage at two additional regions of the template is
evident. The larger of these products (region “b”) is only slightly smaller than the intact template, suggesting longer residency of these mutants on the initial template/primer duplex and low-level template hydrolysis prior to polymerization. Further downstream, a second region of the RNA template is susceptible to hydrolysis. Since the DNA synthesis profiles of these mutants shows no major termination products in this region, this might reflect (i), transient pausing at a region of template secondary structure (ii), resolution of this structure via RNase H-mediated hydrolysis and (iii), continued polymerization. Such a mechanism has in fact been proposed for HIV-1 RT (30-32) and RNA folding programs indicate a stable stem-loop structure in this region \(\text{(data not shown)}\). Thus, while presenting a more complex hydrolysis profile, the cumulative data with mutants H427A, Y459A and D469N indicates they retain significant RNase H activity on a heteropolymeric RNA/DNA hybrid.

**Divalent Cation Requirement of Ty3 RT Mutants** – Previous work indicated that the HIV-1 RNase H mutant p66\[^{E478Q}\]/p51 (33) recovered polymerization-dependent RNase H activity in Mn\[^{++}\] on both a random heteropolymeric RNA/DNA hybrid and a second substrate mimicking release of the tRNA primer (33,34). We therefore determined whether such a phenotype could be reproduced with Ty3 RT, and if this was restricted to the equivalent catalytic residue, E401. For this analysis, a \[^{32}\text{P}\]-labeled 40 nt RNA/30 nt DNA hybrid (Fig. 3[A]) was employed. In keeping with our recent studies (8) and the data of Fig. 2, the primary Mg\[^{++}\]-dependent products with wild type Ty3 RT indicate cleavage at template nucleotides –21 and –13 (Fig. 3[B], Lane W and Fig. 3[C], Panel [I]), corresponding to the polymerase-dependent and -independent modes of hydrolysis, respectively, as described by Gopalakrisnan and collaborators (35,36). However, in contrast with what is typically seen with HIV RT (8,33), polymerase-independent cleavage appears to predominate over polymerase-dependent cleavage when multiple binding events are permitted. However, in experiments conducted in the presence of heparin (which restricts Ty3
RT to a single binding event), the more established pattern of hydrolysis emerges: polymerase-dependent (-21) and polymerase-independent (-13) cleavages represent 65 and 35% of the total product, respectively (data not shown). Though speculative, it is possible that when Ty3 RT cleaves at position –21, this is immediately followed by cleavage of the same substrate at position –13 when multiple binding events are permitted. This subsequent cleavage, however, is suppressed in the presence of heparin because re-binding of Ty3 RT cannot occur. Experiments to address this possibility are being considered.

As expected, substitution within the D358/E401/D426 triad eliminated Mg\(^{++}\)-dependent RNase H activity (Fig. 3 [B], Lanes 1-3), and this was reduced upon replacement of H427, Y459 and D469 (Fig. 3[C] Panels (ii) – (iv)). Surprisingly, introducing Mn\(^{++}\) as the divalent cation (Figs. 3[B] and [D]) had several consequences for the latter mutants. Firstly, in contrast to HIV-1 RT, Mn\(^{++}\) failed to stimulate any mutant of the D358/E401/D426 triad (Fig. 3[B], Lanes 4-6, respectively). Based on data from *E.coli* (37) and HIV-1 RNase H (38), Mn\(^{++}\) might be expected to occupy two sites in the Ty3 RNase H domain, *i.e.* at Site 1, coordinated via D358/E401/D426 and Site 2 via D358/D469. Since both potential sites share D358, amidation of E401 or D426 appears to affect D358 geometry such that the occupancy by either divalent cation is affected. Secondly, the Mn\(^{++}\)-dependent activity of mutants H427A, Y459A and D469N exceeded that observed in Mg\(^{++}\) (Fig. 3[D], Panels [ii] – [iv]). Retention of Mn\(^{++}\)-dependent activity again suggests these residues are less critical for catalysis. Thirdly, wild type Ty3 RT exhibited relaxed RNase H specificity in Mn\(^{++}\), hydrolyzing the template at almost every position between nucleotides –24 and –6 (Fig 3[C], Lane 2). Although indirect, lack of Mn\(^{++}\)-dependent activity with mutants of the D358/E401/D426 triad (Fig 3 [B]) ruled out the trivial possibility *E.coli* RNase H contamination in our enzyme preparations. Finally, the data of Fig. 3 [D] indicate that in the presence of Mn\(^{++}\), cleavage products accumulate rapidly during the first minute of the reaction. However, almost no additional cleavage is observed at subsequent time points, and hydrolysis
does not reach the level observed in presence of Mg\(^{++}\) (compare hydrolysis profile of WT Ty3 RT in Fig. 3[D] with that of Fig. 3[C]). This suggests slower turnover, i.e. the enzyme, having cleaved the substrate, fails to dissociate from the product and, as a result, additional cleavage is not observed. Enhanced Mn\(^{++}\)-dependent activity, for mutants H427A, Y459A, and D469N, was directed primarily on \(-13\) cleavage, yielding hydrolysis profiles qualitatively similar to that observed with wild type enzyme in Mg\(^{++}\). Stated differently, if the H427/Y459/D469 triad is implicated in Site 2 metal binding, abrogating this event results in catalysis mediated by metal bound exclusively at the biologically relevant site.

**RNase H Mutations are Lethal for Ty3 Transposition** – Although the data of Figs. 2 and 3 indicate retention of RNase H activity with Ty3 RT mutants H427A, Y459A and D469N, it was unclear whether these levels could support transposition, which requires a combination of non-specific and highly accurate processing of the replication intermediate. Consequently, all mutations were introduced into the RT domain of pEGTy3-1, a plasmid harboring a replication-competent Ty3 element (39), to be tested in a transposition assay. The assay is based on expression of Ty3 RNA upon induction with galactose and subsequent insertion of its double stranded DNA genome into the target plasmid pCH2bo19V (28), (Fig. 4[A]). Integration occurs between two divergent tRNA genes, sup2bo and tRNA\(_{\text{Val}}\) (AAC). The sup2bo gene is a transcriptionally inactive ochre suppressor tRNA\(_{\text{Tyr}}\), which is activated by Ty3 integration into the target site, and suppresses the ade2-101, lys2-1 ochre nonsense mutations in the yeast host yTM443. Therefore, transposition is scored as papillations on a synthetic complete medium (SD) lacking adenine and lysine.

The results of this assay are presented in Figure 4[B]. The upper panel ("non transposed") shows that, prior to induction, none of the constructs used permitted growth on selective medium. In the lower panel ("transposed") transcription of Ty3 RNA was induced by
galactose and, in the case of the WT Ty3 element, papillations were observed. As expected from the analysis of recombinant Ty3 RT, RNase H-inactivating mutations D358N, E401Q and D426N were lethal for transposition. Surprisingly, the same was true for mutants H427A, Y459A and D469N. In order to determine whether the barrier to transposition reflected imprecise maturation of either the Gag3 (CA, NC) or Pol3 polyproteins (PR, RT, IN and RT/IN), an immunological evaluation of whole cell (Figure 5[A]) or VLP proteins (Fig. 5[B]) was undertaken. Due to the absence of specific antibodies, RT could only be evaluated in the context of the RT/IN polyprotein. In general, the relative CA and NC levels in cell extracts (Fig. 5[A]) and VLPs (data not shown) were not significantly influenced, while reduced amounts of IN and RT/IN were noted for mutants Y459A and D469N in VLPs. However, in no case accumulation of an aberrant maturation intermediate was evident. Identification of appropriately-sized IN and RT/IN also excluded the possibility that loss of transposition activity did not reflect frameshifting errors inadvertently introduced into the Ty3 clones with the RNase H point mutations. The inability of mutants retaining RNase H activity to transpose suggests they fail to provide a “threshold” hydrolysis level required in vivo, and that the Mn$$^{2+}$$-dependent activity observed in vitro is not biologically relevant. Alternatively, while Ty3 RT mutants H427A, Y459A and D469N could process random, heteropolymeric substrates, the same might not hold true for precise RNase H-mediated events required during replication. This notion was investigated in the next sections.

**RNase H-Proficient RT mutants Fail to Support DNA Strand Transfer** – DNA strand transfer, i.e. relocation of nascent DNA to an acceptor template (35), is a specialized event in Ty3 replication requiring RNase H activity. Although this has been studied in retroviruses (35,40,41), model Ty3 systems to investigate the mechanism and its dependence on RNase H function have not been reported. The features of our DNA strand-transfer system (Fig. 6 [A]) are similar to those described by Peliska and Benkovic (35), where extension of a 20-nt DNA primer
to the 5' terminus of the donor RNA template yields a 40-nt strand transfer intermediate, and transfer to the acceptor template, followed by resumption of DNA synthesis, yields a 60-nt strand transfer product. In this model system, efficient strand transfer requires that polymerization-independent RNase H activity reduces the donor RNA template to a size permitting its dissociation and relocation of nascent DNA onto the acceptor (33,35,42,43).

Figure 6 [B] summarizes the strand transfer activities of wild type Ty3 RT and mutants retaining RNase H function. In each case we observed efficient DNA synthesis on the donor template to produce the 40 nt strand transfer intermediate. Although a slight degree of pausing was evident in the immediate vicinity of the primer 3' OH, the activity of these enzymes was comparable, confirming that their DNA polymerase domains were not structurally compromised. However, while strand transfer product accumulates with wild type Ty3 RT, it is barely detectable with mutants H427A, Y459A and D469N. Figure 6[C] follows the same reaction using a 5' end-labeled donor RNA template rather than radiolabeled primer, which allowed us to monitor RNase H function prior to and concomitant with DNA strand transfer. The accumulation of a 30 nt hydrolysis product early in the time course (Fig. 6 [B], panel [I]) correlates with transient pausing shortly after DNA synthesis is initiated. Thereafter the primary hydrolysis products are 18 nt and shorter, each of which results from an enzyme which has completed DNA synthesis to the 5' terminus of the donor template. A comparison of the hydrolysis and polymerization products gives insight into the size to which the donor template must be reduced to allow strand transfer. For example, although we observe rapid accumulation of an 18 nt hydrolysis product, strand transfer and continued synthesis is not evident. With time, the 18 nt RNA diminishes and is replaced with fragments of 11 and 10 nt. As these accumulate, there is a parallel rise in strand transfer activity, suggesting the donor RNA template must be reduced to ~10 nt to permit its displacement and relocation of the growing point onto the acceptor template.
Despite retaining both DNA polymerase and RNase H activity, the three mutant enzymes barely support strand transfer (Fig. 6 [C], Panels [ii] – [iv]). This is particularly significant for mutant Y459A (Fig. 6 [C], Panel [iii]), since this enzyme yields appreciable amounts of 18 nt hydrolysis product. Data with this enzyme support our contention that cleaving the donor template 18 nt from its 5’ terminus leaves an RNA fragment stably bound to nascent DNA, denying access to the acceptor template and preventing strand transfer. Low level transfer activity with this mutant correlates well with the rate at which the 11/10 nt hydrolysis product appears. Trace amounts of strand transfer product visible with mutants H427A and D469N (Fig. 6 [C], Panels [ii] and [iii]), despite undetectable 11/10 nt RNase H hydrolysis product might be explained by low level dissociation of the residual 18 nt donor template from nascent DNA over the course of the 1-hour reaction.

**PPT Selection by Ty3 Mutants** – During Ty3 replication, the PPT primer must be (i), excised from (+) RNA (ii), extended into (+) DNA and (iii), excised from (+) DNA to provide a correct 5’ LTR terminus for integration. PPT processing thus represents a second specialized RNase H-mediated event. The experimental protocol for examining PPT utilization is shown in Fig. 7 [A], and follows processing of RNA primers hybridized to different positions of the same (-) DNA template. The non-PPT primer (Fig. 7 [A], panel [i]) served as a control, while the PPT/5’ and PPT/3’ primers (Figs. 7 [A], panels [ii] and [iii]) flank the 13 nt PPT at its 5’ or 3’ terminus with 11 ribonucleotides, respectively. PPT/5’-3’ primer flanks the PPT with 5 and 6 nt at the 5’ and 3’ termini, respectively. On the non-PPT template, wild type Ty3 RT produces a major 21 nt fragment, suggesting the DNA polymerase catalytic center is positioned at the template 3’ OH. A second series of hydrolysis products corresponding to cleavage between –11 and –14 is also evident (Fig. 7 [B], Panel [i]). Although the latter products are absent with mutants H427A, Y459A and D469N, each supports hydrolysis at position –21. However, only Y459A RT yields
equivalent levels of -21 product as the wild type enzyme. A different picture emerges when these mutants process the PPT from adjacent 5' (+) RNA. Fig. 7 [B], Panel [ii] indicates removal of this RNA would yield an 11 nt radiolabeled fragment. While this is achieved by wild type RT, mutant enzymes are virtually inactive. Precise processing of the PPT flanked by 11 ribonucleotides at its 3' terminus was predicted to yield a 13 nt radiolabeled fragment (Fig. 7 [A], Panel [iii]). 13- and 20-nt fragments are produced by wild type Ty3 RT (Fig. 7 [B], Panel [iii]), the latter most likely arising from positioning of its polymerase domain over the 5' terminus of the PPT-containing primer (44-46). Interestingly, although the three mutants hydrolyze the non-PPT portion of the primer, cleavage at the PPT/U3 (+) RNA junction is impaired. Finally, release of the PPT 3' OH on substrate PPT/5'-3' is predicted to generate an 18 nt radiolabeled fragment. This is the primary product observed with wild type RT (Fig. 7 [B], Panel [iv]). Additional fragments of 21/22 nt most likely reflect positioning of the polymerase on 5' terminus of the PPT-containing primer and cleavage in a polymerization-dependent fashion. Internal cleavage of the PPT is indicated by the 12/11-nt fragments (Fig. 7 [B], Panel [iv]), a feature which was also observed with both the HIV-1 and murine leukemia virus enzymes (47,48). Again, Ty3 mutants fail to select the PPT 3' OH of this substrate. Thus, in keeping with data of Fig. 6, the stringency imposed by the conformation of PPT-containing substrates has a significant impact on selection by RNase H mutants.
DISCUSSION

The availability of recombinant RT from the *S. cerevisiae* retrotransposons Ty1 (9,49,50) and Ty3 (1,8) has spurred efforts to define a unifying mechanism of RNase H-mediated hydrolysis for bacterial, retroviral and retrotransposon enzymes, as well as understand how specific RNase H-dependent events are achieved in the context of non-specific hydrolysis of the RNA/DNA replication intermediate. In this communication, we describe how altering conserved residues of the Ty3 RNase H domain affects both metal ion dependency and hydrolysis of a variety of biologically relevant substrates. However, before comparing or contrasting the Ty3 RNase H domain with more extensively studied systems, a recent phylogenetic study conducted by Malik and Eickbush (13) is particularly relevant. These authors have documented that while the DNA polymerase domain of retroviral RTs is a sister group to the Ty3/gypsy elements and caulimoviruses, their C-terminal RNase H domains are only distantly related. More importantly, the flexible “His-loop” of bacterial and retroviral RNases H, the role of which is still unresolved, is absent from LTR-containing retrotransposon enzymes when the conserved carboxylates are aligned (Fig. 1 [B]). Thus, the catalytic mechanism for Ty3 RNase H need not be strictly reconciled with other more extensively studied bacterial and retroviral enzymes, perhaps exemplified by recent hydroxyl radical footprinting efforts (8) and in this report by the activity of wild type and mutant enzymes as a function of divalent cation requirement.

One unequivocal feature of our data is complete loss of Mg$^{++}$- and Mn$^{++}$-dependent RNase H activity when any residue of the D358/E401/D426 triad is altered, while substitutions in the H427/Y459/D469 triad are only partially inhibitory. Thus, in keeping with bacterial and retroviral RNases H, we propose that D358/E401/D426 constitute the primary metal binding site of Ty3 RNase H, which presumably is occupied with Mg$^{++}$ in vivo. Relaxed RNase H specificity and reduced enzyme turnover with wild type Ty3 RT in the presence of Mn$^{++}$ was surprising, but...
not entirely without precedent. This profile is reminiscent of the activity of EcoRV (51) and TaqI endonucleases (52) when Mn\textsuperscript{++} substitutes Mg\textsuperscript{++} in the active site. In the presence of Mn\textsuperscript{++} both endonucleases show increased phosphodiester bond hydrolysis and lower $K_m$ for the substrate, i.e. tighter binding that slow down product release, with the consequence of decreasing enzyme turnover and inducing relaxed specificity. Indeed, for EcoRV, Mn\textsuperscript{++} was shown to accelerate the chemical reaction and stabilize the enzyme-substrate complex. Under these conditions, extended residency at a non-cognate site might be predicted to enhance hydrolysis, thus accounting for relaxed specificity (51). Invoking this argument, data of Fig. 3 [D] suggests the relaxed specificity of wild type Ty3 RT in Mn\textsuperscript{++} reflects enhanced affinity for the substrate, prolonged residency and accelerated hydrolysis. Moreover, in the presence of Mn\textsuperscript{++}, two divalent metals can occupy the RNase H domain, while a third will be coordinated by the carboxylate triad of the DNA polymerase catalytic center. Mn\textsuperscript{++} occupancy at both catalytic centers may increase the affinity for nucleic acid at both domains. For mutants H427A, Y459A and D469N, we predict that the biologically relevant metal binding sites of the RNase H (Site 1) and DNA polymerase catalytic centers will be occupied by Mn\textsuperscript{++}. Single-metal occupancy at the RNase H domain would favor correct positioning on the substrate, while coordination of Mn\textsuperscript{++} at the polymerase catalytic center increases the $K_m$ to reduce the rate of product release. This scenario would account for a Mn\textsuperscript{++}-dependent hydrolysis profile of mutants His427/Y459/D469, which is qualitatively similar to wild type enzyme in Mg\textsuperscript{++}. Finally, retention of RNase H activity on several substrates, despite the absence of a flexible RNase H “His-loop” in Ty3 RT, suggests Y459, H427 and possibly D469 may participate in catalysis by positioning the substrate such that the RNA strand of an RNA/DNA hybrid finds the appropriate trajectory into the RNase H catalytic center for hydrolysis.

This study also provides the first in vitro demonstration of strand transfer activity for a retrotransposon RT and the requirement for RNase H activity. A lag between completion of DNA
synthesis and appearance of transfer product with wild type Ty3 RT suggests RNase H-mediated removal of the donor template and relocation of nascent DNA on the acceptor is rate-limiting step. At this stage, coordination between polymerization-dependent and -independent template hydrolysis is important, and most likely involves distinct binding modes. While the 18 nt product reflects an enzyme whose polymerase and RNase H catalytic centers remain in close contact with the RNA/DNA duplex, hydrolysis at positions -11/-10 suggests relocation of the enzyme beyond the duplex terminus such that polymerase domain contacts are largely lost and the interaction with the RNase H domain becomes more critical. Although Y459A RT catalyzes polymerization-dependent cleavage, critical nucleic acid contacts within the RNase H domain may have been altered or lost, destabilizing the nucleoprotein complex during translocation to the duplex terminus. Dissociation and loss of polymerization-independent hydrolysis would leave the fragmented template stably bound to the growing point, thereby interrupting strand transfer. Although indirect, data with this mutant also indicates that Ty3 RT lacks any helicase activity to induce removal of residual template. The ability of mutants H427A and D469N to cleave the template/primer duplex immediately following the onset of DNA synthesis contrasts with reduced activity when the polymerizing enzymes reach the template 5' terminus. Since extended contact with the single-stranded portion of the template are possible during initiation, the activity of these mutants suggests that, once synthesis to the template 5' terminus is complete, the resulting nucleoprotein complex is unstable and cannot translocate to catalyze polymerization-independent hydrolysis. These results could explain the in vivo defects we observed for all RNase H mutants. Based on published observations with MLV (53-55) HIV-1 (56, 57) and Ty1 (49), we predict that transposition of Ty3 mutants whose RNase H domain is completely (D358N, E401Q and D426N) or partially inactivated (H427A, Y459A and D469N) would be interrupted at (-) strand DNA transfer.
Finally, the inability of otherwise RNase H-proficient Ty3 RT mutants to hydrolyze RNA/DNA hybrids with a more unique structure is best exemplified in PPT selection. Here, while cleavage at the junction with U3 RNA is severely impaired, non-PPT portions of the same template are efficiently hydrolyzed. Although the Ty3 PPT sequence (5′-GAGAGAGAGGAAGA-3′) differs significantly from its retroviral counterparts (e.g. 5′-AAAAGAAAAAGGGGG-3′ for HIV-1), selective processing by wild type enzyme infers an unusual structure which (a), renders it RNase H-resistant yet (b), allows precise cleavage at its 3′ terminus to liberate U3 DNA sequences. We recently used chemical footprinting to demonstrate that, in the absence of protein, the HIV-1 PPT RNA/(-) DNA hybrid (58) contains two structural distortions, namely the A:T base pair adjacent to the 3′ terminus and within a distal r(A)₄:d(T)₄ duplex. Although the precise mechanism remains to be elucidated, the interdependence between these unique structures clearly contributes to the selectivity of PPT processing. Preliminary studies suggest similar structural anomalies may also be a feature of the Ty3 PPT (M. Kvaratskhelia and S.F.J. Le Grice, unpublished observations), although the extent of distortion may be less severe. We therefore suggest that, similar to the RNase H primer grip of HIV-1 RT (59) H427, Y459 and D469 constitute a subset of residues whose interaction with an RNA/DNA hybrid induces the appropriate trajectory of the RNA template into the RNase H catalytic center for hydrolysis. Although speculative, Y459 of Ty3 RT, a residue conserved in many LTR-containing retrotransposons, may be the counterpart of Y501 of the HIV-1 RNase H primer grip, alteration of which to alanine yields an enzyme exhibiting an abnormal PPT processing phenotype (60). Experiments to better understand the role of this Ty3 residue are currently underway.

In summary, our data shows that D358, E401 and D426 constitute the primary Mg²⁺ binding site of the Ty3 RNase H domain and are required for catalysis. A second triad of conserved residues, H427, Y459 and D469, may interact with substrate to ensure that the RNA template is appropriately positioned for catalysis. Based on phylogenetic (13), biochemical and...
modeling data (this work), a catalytic role for Y459 akin to that proposed for H539 of HIV-1 RT (15) seems highly unlikely. Thus, whether these distantly-related RNase H domains follow different catalytic mechanisms, remains an open question. Finally, although we propose a single metal binding site in the Ty3 RNase H domain, this does not necessarily mean that the catalytic mechanism is single-metal catalyzed, since a second metal could be introduced by the substrate.

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FIGURE LEGENDS

Fig. 1. [A] Comparison of HIV-1 catalytic residues with Ty3 RNase H conserved amino acids. Amino acid substitutions introduced into the Ty3 enzyme are indicated. [B], Spatial distribution of conserved residues constituting the active center the HIV-1 and Ty3 RT-RNase H. A partial superimposition of the peptide backbones is illustrated. HIV structural elements and amino acid numbering is in yellow, while those of Ty3 are in red. [C], Purity of Ty3 enzymes. One µg of total protein was fractionated by SDS-polyacrylamide gel electrophoresis and stained with Coomassie Brilliant Blue. Lane M, molecular weights markers (in kDa).

Fig. 2. RNA-dependent DNA polymerase and RNase H activities of Ty3 RT variants. [A], schematic representation of the RNA-dependent DNA polymerase (RDDP) and RNase H assay. The substrate is a 138 nt RNA template annealed to a 20 nt DNA primer. [i] In the RDDP assay, the 20 nt DNA primer is 5' radiolabeled to allow visualization of DNA synthesis. [ii] In the RNase H assay the 138 nt RNA is 5' radiolabeled and annealed to the 20 nt DNA primer. Ty3 RT RNase H activity, during DNA synthesis, hydrolyzes the radiolabeled RNA. Arrows indicate potential cleavage sites at the 3' and 5' end of the RNA. [B], [i], RNA-dependent DNA polymerase activity catalyzed by Ty3 RT WT and mutants. Full-length product (cDNA, 138 nt) and the ^32P-20mer DNA primer are indicated on the right. [ii] RNase H hydrolysis profiles. The full-length uncleaved RNA template is indicated on the left. The 21 nt and 12 nt products (a) represent cleavage by enzymes have polymerized to 5' end of the template. Additional hydrolysis products derived from enzymes stalled on the initial template/primer duplex (b) and during polymerization (c) have bee indicated Lane 1, substrate in absence of enzyme; Lane 2, WT Ty3 RT; Lane 3, Ty3 RT D358N; Lane 4, Ty3 RT E401Q; Lane 5, Ty3 RT D426N; Lane 6, Ty3 RT H427A; Lane 7, Ty3 RT Y459A; Lane 8, Ty3 RT D469N.
Fig. 3. Mg** and Mn**-dependent RNase H activities of Ty3 RT variants. [A], schematic representation of the substrate used for analysis. The major Ty3 RT derived cleavage sites on the 40-nt RNA template are indicated, designating the first base pair of the RNA-DNA hybrid in the DNA polymerase catalytic center -1. The shaded ellipse indicates the enzymatic footprinting of Ty3 using nucleases S1 and DNase I (8). [B], Hydrolysis profiles of Ty3 RT mutants D358N (Lanes 1 and 4), E401Q (Lanes 2 and 5) and D426N (Lanes 3 and 6) in Mg** and Mn**. The major Ty3 RT derived cleavage sites are indicated. [C], [D], Hydrolysis profiles of Ty3 RT mutants His427A, Y459A and D469N in Mg** or Mn** respectively. For each enzyme, a time course is presented. Lanes 1-7 represent samples analyzed after 0, 1, 2, 5, 10, 30 and 60 min, respectively. Lanes C, alkaline hydrolysate of RNA template. RNase H activity was evaluated in the absence of DNA synthesis.

Fig. 4. Ty3 RNase H mutants fail to support transposition. [A], Schematic representation of the transposition assay. [B], Transformants were patched onto selective (-His, -URA) SD to repress transposition, and grown for 2 days. Cells were then replica plated onto SD medium lacking lysine and adenine (upper panel) and onto selective SG medium to induce transposition. Cells, grown on selective SG medium, were then replica plated onto SD medium lacking lysine and adenine (lower panel). Papillations on SD medium lacking lysine and adenine indicates transposition.

Fig. 5. Immunological analysis of Ty3 proteins. [A], Analysis of Ty3 structural proteins from whole-cell extract isolated from yTM443 cells overexpressing WT or RT mutant Ty3. Proteins were detected using a polyclonal rabbit anti-CA IgG antibody and polyclonal rabbit anti-NC IgG antibody. Molecular weights markers (in kDa) are indicated on the left. The positions of the
Gag3 precursor protein (38 KDa), mature CA (26 KDa) and NC (7.9 KDa) are indicated on the right. [B]. Analysis of Ty3 IN from virus-like particles isolated from yTM443 cells overexpressing WT or RT mutant Ty3. The amount of VLP protein was normalized to mature capsid protein. Protein was detected using a polyclonal rabbit anti-IN IgG antibody. For both panels, Lanes 1-7 represent WT, D358N, E401Q, D426N, H427A, Y459A and D469N Ty3 RT, respectively. Molecular weights markers are indicated on the left. The positions of the RT-IN fusion protein (115 KDa) and mature IN (61 KDa) are indicated on the right.

Fig. 6. Strand transfer activity of mutant Ty3 reverse transcriptase [A]. Schematic representation of the strand transfer system, comprising 40 nt donor and acceptor RNAs templates sharing 20 nt of homology. Extension of the 20 nt DNA primer (solid arrow) to the 5' terminus of the donor template (14) generates a 40 nt strand transfer intermediate. Homology between the donor and acceptor templates allows transfer and continued synthesis, resulting in a 60 nt strand transfer product. [B], Strand transfer activity of Ty3 RT variants. Migration positions of the DNA primer (20 nt), strand transfer intermediate (40 nt) and full length, 60 nt strand transfer product are indicated. Lane 1-7, represent 0, 1, 2, 5, 10, 30 and 60 min time points, respectively. [C], RNase H cleavage of the 40 nt donor template during polymerization. The full length (uncleaved) 40 nt RNA and the major Ty3 RT derived cleavage sites are indicated on the left. Lanes 1-7 represent samples evaluated after 0, 1, 2, 5, 10, 30 and 60 min, respectively.

Fig. 7. Selection of the Ty3 polypurine tract. [A]. Schematic representation of the model PPT selection system, comprising a 65 nt DNA template to which one of three RNA primers is hybridized. The Non-PPT primer (Panel [i]) corresponds to the 24 nt RNA sequence immediately upstream the PPT. The arrows indicate the major Ty3 RT WT derived cleavage
sites. PPT/5’ (Panel [ii]) and PPT/3’ (Panel [iii]) represent the PPT primer extended at its 5’ and 3’ terminus, respectively, by 11 nt. In Panel [iv], the PPT is flanked at its 5’ and 3’ termini by 5 and 6 nt, respectively. Arrows indicate predicted cleavage sites. [B], Hydrolysis profiles from substrates [i] – [iv]. The major Ty3 RT derived cleavage sites are indicated on the left of each panels. Lanes 1, no enzyme; Lane 2, Ty3 RT WT; Lanes 3, Ty3 RT H427A; Lanes 4, Ty3 RT Y459A; Lanes 5, Ty3 RT D469N. The junction between the PPT 3’ terminus and U3 RNA sequences is indicated on each panel.
Lener et al., Fig. 2

[Diagram A] 138 nt RNA template
RNA-Dependent DNA Synthesis
RNase H

[Diagram B] cDNA (138 nt)
DNA primer (20 nt)
RNA Template (138 nt)
RNase H

[Diagram i] 21 nt
12 nt
Lener et al., Fig. 3
Lener et al., Fig. 4

[A] 

Replica plate to SD-Lys-ADE → NON TRANSPOSED → Replica plate to SG-URA-His → Replica plate to SD-Lys-ADE → TRANSPOSED

[B] 

wt D358N E401Q D426N wt H427A Y459A D469N

Lener et al., Fig. 4
Lener et al., Fig. 5

[A] Cell extracts

[B] Virus-like particles
Lener et al., Fig. 6

[A] 20 nt DNA primer

[B] DNA Polymerase

[C] RNase H

WT Y459AH427A D469N

Strand Transfer Intermediate

DNA primer (20 nt)

40 nt Donor RNA template

40 nt Acceptor RNA template

Mg^{++}/RT/
dNTPs

60 nt DNA Strand transfer product

40 nt DNA Strand transfer intermediate

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Lener et al., Fig. 7

[A] 

[i] Non-PPT

![Diagram of Non-PPT](image)

[ii] PPT/5'

![Diagram of PPT/5'](image)

[iii] PPT/3'

![Diagram of PPT/3'](image)

[iv] PPT/5'-3'

![Diagram of PPT/5'-3'](image)

[B] 

[i] Non-PPT

![Gel Image of Non-PPT](image)

[ii] PPT/5'

![Gel Image of PPT/5'](image)

[iii] PPT/3'

![Gel Image of PPT/3'](image)

[iv] PPT/5'-3'

![Gel Image of PPT/5'-3'](image)
Mutating conserved residues in the Ribonuclease H domain of Ty3 reverse transcriptase affects specialized cleavage events
Daniela Lener, Scott R. Budihas and Stuart F. J. Le Grice

J. Biol. Chem. published online May 6, 2002

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