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The TY3 Gag3 Spacer Controls Intracellular Condensation and Uncoating

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Cells expressing the yeast retrotransposon Ty3 form concentrated foci of Ty3 proteins and RNA within which virus-like particle (VLP) assembly occurs. Gag3, the major structural protein of the Ty3 retrotransposon, is composed of capsid (CA), spacer (SP), and nucleocapsid (NC) domains analogous to retroviral domains. Unlike the known SP domains of retroviruses, Ty3 SP is highly acidic. The current studies investigated the role of this domain. Although deletion of Ty3 SP dramatically reduced retrotransposition, significant Gag3 processing and cDNA synthesis occurred. Mutations that interfered with cleavage at the SP-NC junction disrupted CA-SP processing, cDNA synthesis, and electron-dense core formation. Mutations that interfered with cleavage of CA-SP allowed cleavage of the SP-NC junction, production of electron-dense cores, and cDNA synthesis but blocked retrotransposition. A mutant in which acidic residues of SP were replaced with alanine failed to form both Gag3 foci and VLPs. We propose a speculative “spring” model for Gag3 during assembly. In the first phase during concentration of Gag3 into foci, intramolecular interactions between negatively charged SP and positively charged NC domains of Gag3 limit multimerization. In the second phase, the NC domain binds RNA, and the bound form is stabilized by intermolecular interactions with the SP domain. These interactions promote CA domain multimerization. In the third phase, a negatively charged SP domain destabilizes the remaining CA-SP shell for cDNA release.

Most retroviruses and some retrotransposons (collectively referred to as retroelements) have functionally analogous capsid (CA) and nucleic acid-binding domains contained within the major structural Gag precursor polyprotein (9, 40). In addition to these domains, some retroelement structural polyproteins contain short spacers (SP) that have been implicated in particle morphogenesis but which do not code for mature protein derivatives with known functions (Fig. 1A to C). For example, the Gag structural protein of human immunodeficiency virus type 1 (HIV-1) contains SP domains between CA and NC (SP1 or p2). Rous sarcoma virus (RSV) and bovine immunodeficiency virus (BIV) Gag polyproteins have SP domains between CA and NC. HIV-1 mutants in which SP1 is deleted fail to condense properly (23). In the case of the alpharetrovirus RSV, short insertion mutations in SP result in budding tubules rather than production of spherical viruses (19), and RSV Gag mutants lacking SP are noninfectious (7). Thus, the presence of SP contributes to particle assembly. SP domains have also been proposed to influence Gag interaction with RNA (14, 18, 38), multimerization and membrane association (12, 13), particle size (24), and Gag processing rate (31, 32).

Protease (PR)-mediated production of SP from Gag is an essential step in morphogenesis for retrovirus Gag proteins containing discrete SP domains. HIV-1 PR processing of Gag polyproteins is ordered, with SP1-NC cleavage first, followed by matrix (MA)-CA cleavage, and finally CA-SP1 cleavage. Mutations that block processing decrease production of infectious virus (34; reviewed in reference 9). The intermediate MA-CA cleavage produces the amino terminus of CA to form a salt bridge required for in vitro assembly of mature cones (44). However, in vitro systems have also shown that Gag derivatives having amino-terminal extensions of CA require a free CA carboxyl terminus to form cones (10, 33). Furthermore, disruption of HIV-1 CA-SP1 cleavage in vivo by mutation (1, 23) or chemical inhibition (28) blocks the transition from immature to condensed conical particles.

Comparisons of retrovirus SP domains between CA and NC domains reveal little conservation of either length or sequence (Fig. 1C). Furthermore, Gag sequences spanning the CA-NC junction are not well conserved, so it is possible that where SP does not exist, its functions have been acquired by other domains. For example, Moloney murine leukemia virus (MoMLV) lacks an SP domain at the CA-NC junction but contains a charged helix domain in the carboxyl-terminal domain of CA that is important for virus-like particle (VLP) assembly and size determination (6). Similarly, Mason-Pfizer monkey virus lacks a canonical SP domain but does have an...
The budding yeast long terminal repeat (LTR) retrotransposon Ty3 (Fig. 1A) contains GAG3 and POL3 overlapping open reading frames (ORFs) that encode CA, SP, and NC major structural proteins (GAG3) and PR, reverse transcriptase (RT), and integrase (IN) catalytic proteins (POL3) (39). Like most retrotransposons, Ty3 undergoes an intracellular replication cycle and lacks matrix and envelope (Env) domains necessary for extracellular steps in the retroviral life cycle. Expression of Ty3 results in production of RNA, Gag3, and Gag3-Pol3 polyproteins. Gag3 multimerizes with Gag3-Pol3 and associates with genomic RNA to form VLPs. These particles are distributed in size, with most between 32 and 52 nm in diameter (15, 25). After VLP formation, Ty3 PR processes itself from Gag3-Pol3, cleaves Gag3 precursor proteins into CA, SP, and NC (Fig. 1B), and further processes Gag3-Pol3 into RT and IN. Processing and maturation of the VLP occur at cleavage sites similar to those targeted by retroviral PR (21) and result in condensation of the RNA without the massive CA reorganization that occurs within the retrovirus envelope (25–27). Protein sequence analysis of mature Ty3 CA and NC proteins allowed inference of a 26-residue SP domain separating CA and NC (21, 25). Ty3 SP has seven Glu and Asp residues, making it highly negatively charged. In addition, it contains eight Thr, Ser, and Tyr residues which are potentially phosphorylated. Comparison of Ty3 to a Saccharomyces paradoxus Ty3 (Ty3Sp), shows conservation of the acidic residues in SP although overall Gag and even SP sequences have diverged (Fig. 1C) (8).

To better understand the role of the SP domain in morphogenesis of a retrotransposon, Ty3 was mutagenized to interrupt processing and to delete SP or neutralize its charge. The results of these studies showed that SP is not essential for assembly of particles, and production of NC in the presence or absence of SP is sufficient for a significant amount of cDNA synthesis.

FIG. 1. Ty3 genome and spacer constructs. (A) Ty3 genome organization. (B) Ty3 Gag3 with CA, SP, and NC domains. Intermediate processing product CA-SP (p27) is indicated and spans amino acids 1 to 233. SP is comprised of residues 208 to 233. Boxed residues are the SP domain and C-terminal CA and N-terminal NC regions flanking SP. Processing sites between CA-SP and SP-NC are demarcated by slashes. Alanine scanning mutations were introduced at bolded residues that are potential phosphorylation targets. Mutations to block processing at CA-SP or SP-NC sites (G207D, G207I, and H233V) or to neutralize charged residues (D/E → A) are indicated by arrows. Double mutations designed to block CA-SP processing, to concomitantly block processing at the CA-SP and SP-NC sites (G207D, G207I, and H233V) or to neutralize charged residues (D/E → A) are indicated by arrows. Double mutations designed to block CA-SP processing, to concomitantly block processing at the CA-SP and SP-NC sites, or to delete the SP domain (ΔSP) are indicated by brackets. (C) The amino acid sequences of retrovirus and retrotransposon SP domains at the CA-NC domain junction of Gag polyprotein. Amino acid sequences of the retroviral SP domains were obtained from the following references: HIV-1, Krausslich et al. (23); RSV, Craven et al. (7); BIV, Guo et al. (11); Ty3, Kuznetsov et al. (25); and Ty3Sp, Fingerman et al. (8). (D) Secondary structure of Ty3 Gag3 predicted by the PSIPRED server using default parameters. Predicted helices are shown as cylinders, and the predicted strands are shown as an arrow. The prediction confidence at each position is represented by a vertical bar, where the bar height corresponds to the prediction confidence. The SP domain is boxed. Note that the SP domain is predicted to contain no regular secondary structure. AA, amino acids.
However, failure to process CA-SP or the absence of SP results in a retrotransposition defect at some step subsequent to cDNA synthesis. An SP mutant in which acidic residues were replaced with Ala failed to form intracellular foci and VLPS. Based on these observations, we speculate that SP functions as a molecular “spring” in which the negatively charged SP and positively charged NC domains of Gag3 first interact intramolecularly to limit multimerization and then interact intermolecularly to promote correct multimerization of Gag3 on genomic RNA. After proteolytic processing of NC and formation of the electron-dense ribonucleoprotein core, a negatively charged CA-SP intermediate could promote processing and uncoating.

MATERIALS AND METHODS

Strains and culture conditions for Saccharomyces cerevisiae and Escherichia coli. The S. cerevisiae strain yTM443 (MATa ura3-52 trp1-H3 his3-D200 ade2-101 lys2-1 can1-100 bar1-12 COL1) was used as a template for QuikChange mutagenesis, previously described (27, 29). Cultures of E. coli strain DH5α [λ- φ80lacZ2M15 ΔlacZΔargF-U169 recA1 endA1 hsdR17 (R mB- mC-)] pSaA supE44 thi-1 gyrA96 relA1 (Invitrogen, Carlsbad, CA) was used for cloning and plasmid preparation.

Plasmid construction and mutagenesis. Mutagenesis of Ty3 Gag3 was performed as previously described (27). Vectors and base plasmids for some constructs and mutagenesis are described in Table 1. Plasmid pLZL2426 containing a region within GAG3 was used as a template for QuikChange mutagenesis, according to the manufacturer’s protocol (Stratagene, Cedar Creek, TX). Complementary primers for mutagenesis of the Ty3 SP-coding region (see data at https://webbli.ucdavis.edu/bsb/amc/lab) were obtained from Integrated DNA Technologies (Coralville, IA). The sequence of the subcloned, mutated region was determined (Genewiz Sequencing, South Plainfield, NJ), and the KpnI/BamHI (New England BioLabs, Ipswich, MA)-digested fragment was swapped into the wt Ty3 backbone as described above.

Transposition assay. Mutant Ty3 elements were assayed for the ability to transpose into a transfer DNA (TDNA)-containing target plasmid using a semi-quantitative patch assay as described previously (20). The Ty3 expression plasmid was a high-copy-number plasmid based on pDLC201 and marked with URA3. The target plasmid was a low-copy-number plasmid, pPK689, marked with HIS3. The wt or mutant Ty3 expression plasmid was cotransformed with the target plasmid into yeast strain yTM443. Transformants were selected, patched to SD medium lacking Ura and His (SD Ura “His”), and grown for one additional day. Patches representing four independent transformants were replated to SG Ura “His” medium and maintained at 24°C to allow Ty3 expression. Ty3 transposition into the target plasmid results in activation of transcription of a suppressor RNA gene and growth on SD Ade “Lys” medium. Transposition events were scored as the number of papillations relative to wt Ty3. For the quantitative transposition assay, the low-copy-number plasmid pNB2361, containing a galactose-inducible, HIS3-marked Ty3 element, was used. Mutations were introduced as described above, and transposition was monitored as described previously (17).

Western blot analysis of Ty3 protein expression. Ty3 protein expression was monitored by Western blot analysis of cells transformed with pDLC201-based Ty3 expression plasmids. Western blotting was conducted as described previously (27). Fractionated proteins were transferred to Immobilon-P membrane (Millipore Corp., Billerica, MA) for 60 min using a semidry transfer apparatus with a discontinuous buffer system (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked in 5% nonfat milk in 1× phosphate-buffered saline (PBS)-0.1% Tween and incubated with primary polyclonal antibodies against yeast phosphoglycerate kinase 1 (Pgkl) (Molecular Probes-Invitrogen, Carlsbad, CA) for 1:500, followed by horseradish peroxidase-conjugated secondary antibody using an ECL Western blotting reagent kit according to the manufacturer’s protocol (GE Healthcare, UK, Ltd). An LAS-4000 Fuji Imaging system (Fujifilm Life Sciences) was used to detect protein bands, and Multigauge software was used for quantification. All measured bands were within the linear range of detection for the imaging system.

Southern blot analysis of Ty3 cDNA. Ty3 cDNA production was monitored by Southern blot analysis. Transformants were grown and induced as described above for Western blot analysis, except that cells were grown for 18 h in 5G medium to allow optimal detection of cDNA. DNA was extracted and analyzed as described previously (27), except that 10 µg of DNA was restricted with BamHI prior to agarose gel electrophoresis. The membrane-bound DNA was hybridized according to the manufacturer’s protocol (Millipore Corp., Billerica, MA) with a 32P-labeled DNA probe specific to a 2.9-kb region of the Ty3 genome. Hybridization was quantified using a Personal FX Molecular Imager and Quantity One software (Bio-Rad Inc., Richmond, CA). The cDNA level was normalized to the level of the Ty3 expression plasmid Ty3.

Indirect immunofluorescence analysis of Gag3 focus formation. Cultures of yeast transformed with pDLC201-based Ty3 expression plasmids were grown as described above and induced by growth in 5G medium for 6 h. Cells were processed as described previously (2). The primary antibody, anti-CA, was diluted 1:1,000. The secondary antibody was Alexa Fluor-488 (anti-rabbit fluorescein isothiocyanate [FITC]-conjugated fluorochrome) (Molecular Probes-Invitrogen, Carlsbad, CA). DAPI (4', 6-diamidino-2-phenylindole) was used at a concentration of 1 µg/ml in PBS to visualize nuclei. Microscopy was carried out essentially as described previously (2, 26).

RNA nucleic acid protection assay. Cells transformed with wt and mutant pDLC201-based Ty3 expression plasmids were induced for Ty3 expression for 6 h. A total of 20 OD units of cells was harvested and subjected to extraction of RNA and protein under native conditions. Nucleic acid protection assays were performed with TurboNuclease (Accelogel, San Diego, CA) but otherwise as previously described (27, 29).

TEM. BY4741 transformants carrying wt or pDLC201-based mutant Ty3 expression plasmids were grown at 24°C in SD Ura “His” medium to early log phase, induced by addition of galactose to a concentration of 2%, and maintained for
atomic force microscopy (AFM) analysis (25). Preparation of VLPs for AFM. Yeast cells transformed with wt and mutant pDLC201-based expression plasmids were grown in SR medium at 24°C and used to inoculate 400 ml of 5G medium. Cultures were grown for 18 h and 60 OD600 units of cells were harvested. VLPs were isolated by velocity sedimentation on sucrose step gradients using a modification of the previously described protocol (available at https://webfiles.uci.edu/sbsandme/lab/) and prepared for atomic force microscopy (AFM) analysis (25).

RESULTS

The lack of overall conservation between Ty3 and Ty3sp SP (Fig. 1C) suggested that the role of SP might be to control separation of NC and CA species rather than to provide a specific structure. We first mutated residues at the SP junctions with the intent of disrupting production of specific Gag3 processing intermediates. We then examined the requirement for the distinctive negative charge of this domain.

Immunoblot analysis of Ty3 mutants disrupted in CA-SP and SP-NC processing. The Ty3 SP domain was defined by Edman degradation and mass spectrometry of flanking regions (25). Previous analysis of Ty3 PR processing sites showed that, similar to retrovirus PR cleavage sites, they are embedded in uncharged amino acids spanning P3 to P2 residues (21). In the case of Ty3 SP, these contexts are IIG207/AT and YVH233/TV (where the slash indicates the cleavage position) (Fig. 1B). Analysis of Ty3 and retrovirus PR processing sites also suggested that beta-branched residues are excluded from the P1 position (21, 35) although this is not universal among retrovirus Gag processing sites (41).

6 h. Western blot analysis was performed as described above to confirm Ty3 expression (data not shown), and cells were processed for transmission electron microscopy (TEM) as described previously (27, 43). A total of 100 cells of each transformant type were imaged with a transmission electron microscope (Hitachi H7600; Tokyo, Japan).

FIG. 2. Effect of SP mutations on Ty3 Gag3 processing. Yeast cells were induced for expression of wt or mutant Ty3 elements by growth in SG Ura medium for 12 h. WCE were prepared, and 15 µg of total protein was fractionated on an SDS–4 to 20% polyacrylamide gradient gel. (A) Total protein from WCE probed with anti-CA antibody. (B) Total protein from WCE probed with anti-NC antibody. Eight Ala substitution mutants showed processing patterns similar to the pattern of wt Ty3 (data not shown). Pgk1 was monitored as a control for loading in blots of both panels. α, anti.

Additional single and double mutations were introduced into Gag3 to more completely disrupt CA-SP processing. These included G207D, D203V G207V, and G207V H211V. G207D decreased amounts of both Gag3 and CA and resulted in elevated levels of a species with the expected mobility of CA-SP (Fig. 2A, lane 2). Disruption of PR processing by introduction of D59I in the PR active site (PR') (21) abolished this intermediate, indicating that it was a product of PR processing (Fig. 2A, lane 3). This species had the same mobility as a CA-SP species produced from an NC deletion mutant (ΔNC), supporting the interpretation that it was the CA-SP p27 precursor (Fig. 2A, lanes 2 and 7).

In order to test the effect of disrupting processing at the SP-NC junction, the His residue at the SP-NC P1 position was replaced with a beta-branched amino acid, creating mutant G207I. Extracts of cells expressing this mutant for 12 h were examined by Western blot analysis using anti-CA and anti-NC antibodies (Fig. 2A and B, lanes 4). Extracts showed increased unprocessed Gag3. However, rather than increased amounts of a p27 species, there were small amounts of CA. In addition, there was a species that reacted with the NC antibody but was larger than NC, potentially representing SP-NC. This suggested that the G207I mutation did not block CA-SP processing but, rather, enhanced production of an anomalous intermediate which could not be further processed.

FIG. 2A and B, lane 5 and 6). Retrotransposition of the G207D, G207V H211V, and G207V H211V mutants was not detectable using a qualitative genetic assay (Table 2).

In order to test the effect of disrupting processing at the SP-NC junction, the His residue at the SP-NC P1 position was replaced with the beta-branched amino acid Val (H233V) (Fig. 1B). This mutation eliminated production of NC and permitted production of only small amounts of CA (Fig. 2A and B, lanes 9). Introduction of the PR active site mutation into the H233V mutant did not...
significantly change the processing pattern (Fig. 2A and B, lanes 10). Thus, either H233V disrupts the Gag3 structure to an extent not compatible with recognition by PR, or SP maturation is ordered so that SP-NC processing creates the substrate for CA-SP processing. A mutant in which both processing sites were disrupted (G207D H233V) was also constructed. Western blot analysis of cells expressing this mutant with anti-CA and anti-NC antibodies confirmed the anticipated absence of Gag3 processing species (Fig. 2A and B, lanes 11). Retrotransposition of the wt Ty3 Gag3. Because this mutant appeared to correctly process Gag3 protein, retrotransposition was measured using both the qualitative genetic assay (Table 2) and a quantitative version of this assay (Table 3). The quantitative assay showed that retrotransposition was reduced by about 16-fold. Thus, deletion of the SP domain did not abrogate assembly but dramatically reduced retrotransposition.

Modeling based on cryo-electron tomography of the immature HIV-1 lattice predicted a bundle of SP alpha-helices spanning Gag3 is 34 kDa and SP is 3 kDa. Processed CA and NC species of the expected mobility were also detected, indicating that cleavage occurred at the chimeric site. To test the possibility that the unexpected species was an anomalous processing product, the PR catalytic site mutation was introduced into ΔSP, and the resulting mutant was analyzed (Fig. 2A and B, lanes 14). An elevated level of ΔSP PR− Gag3 with similar mobility to ΔSP Gag3 was observed. Although it is possible that this catalytically inactive chimeric protein could be cleaved by cellular proteases, Western blot analysis of cells induced for shorter time periods also displayed a Gag3 species of apparent mobility of 27 kDa (data not shown). These results argue that the detected species represents Gag3 and that, similar to what has been reported for the mobility of other proteins upon neutralization of negative charges, deletion of the negatively charged SP enhanced binding of SDS and increased relative mobility in SDS-PAGE by an amount disproportional to the decrease in molecular weight (MW) (46). Ty3 Gag3 processing is likely triggered, as with retroviral Gag maturation, during or subsequent to assembly (21, 27). Thus, correct processing of ΔSP Gag3 argues compellingly that this mutant assembles into a processing-competent species at a level comparable to that of wt Ty3 Gag3. Because this mutant appeared to correctly process Gag3 protein, retrotransposition was measured using both the qualitative genetic assay (Table 2) and a quantitative version of this assay (Table 3). The quantitative assay showed that retrotransposition was reduced by about 16-fold. Thus, deletion of the SP domain did not abrogate assembly but dramatically reduced retrotransposition.

### Table 2. SP mutant phenotypes

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<th>Gag3 focus formation</th>
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The presence (+) or absence (−) of a feature is indicated.

Integrase is present or absent with elevated minor cleavage products.

+++ indicates the wild-type pattern.

**Ty3 ΔSP assembles and undergoes proteolytic maturation.** To test whether the Ty3 SP domain is essential for assembly of VLPs or for retrotransposition, the SP domain was deleted, creating a chimeric processing site (H207V/T235V) at the synthetic CA-NC junction (Fig. 1B). This mutant was designated ΔSP. Western blot analysis of extracts from cells expressing ΔSP revealed a smaller Gag3 species that reacted with antibodies against CA and NC (Fig. 2A and B, lanes 13). However, the mutant Gag3 had an apparent mobility similar to that of the processed CA-NC junction (Fig. 1B). This mutant was designated ΔSP. Western blot analysis of extracts from cells expressing ΔSP revealed a smaller Gag3 species that reacted with antibodies against CA and NC (Fig. 2A and B, lanes 13). However, the mutant Gag3 had an apparent mobility similar to that of the expected mobility were also detected, indicating that cleavage occurred at the chimeric site. To test the possibility that the unexpected species was an anomalous processing product, the PR catalytic site mutation was introduced into ΔSP, and the resulting mutant was analyzed (Fig. 2A and B, lanes 14). An elevated level of ΔSP PR− Gag3 with similar mobility to ΔSP Gag3 was observed. Although it is possible that this catalytically inactive chimeric protein could be cleaved by cellular proteases, Western blot analysis of cells induced for shorter time periods also displayed a Gag3 species of apparent mobility of 27 kDa (data not shown). These results argue that the detected species represents Gag3 and that, similar to what has been reported for the mobility of other proteins upon neutralization of negative charges, deletion of the negatively charged SP enhanced binding of SDS and increased relative mobility in SDS-PAGE by an amount disproportional to the decrease in molecular weight (MW) (46). Ty3 Gag3 processing is likely triggered, as with retroviral Gag maturation, during or subsequent to assembly (21, 27). Thus, correct processing of ΔSP Gag3 argues compellingly that this mutant assembles into a processing-competent species at a level comparable to that of wt Ty3 Gag3. Because this mutant appeared to correctly process Gag3 protein, retrotransposition was measured using both the qualitative genetic assay (Table 2) and a quantitative version of this assay (Table 3). The quantitative assay showed that retrotransposition was reduced by about 16-fold. Thus, deletion of the SP domain did not abrogate assembly but dramatically reduced retrotransposition.

Modeling based on cryo-electron tomography of the immature HIV-1 lattice predicted a bundle of SP alpha-helices spanning...
ning the CA-SP boundary of HIV-1 Gag, which was proposed to contribute to HIV-1 Gag multimerization and particle assembly (47). However, PSIPRED modeling (5) does not predict an alpha-helical structure for Ty3 Gag3 SP (Fig. 1D). HIV-1 SP1 is proposed to act as a relatively independent structural element. Thus, if Ty3 SP and HIV-1 SP1 perform similar functions, it was possible that transposition of the Ty3 ΔSP mutant would be rescued by introduction of the HIV-1 SP1 domain. wt and PR− Ty3 elements in which SP1 replaced SP were constructed. The novel Gag species contained chimeric processing sites at the SP1 junctions. This construct produced and processed chimeric Gag3 protein and produced an amount of cDNA equivalent to 36% of the amount produced by wt Ty3. A quantitative assay showed that despite production of cDNA by the Ty3 HIV-1 SP1 mutant, it displayed a 50-fold-lower transposition frequency than wt Ty3. Therefore, the substitution of HIV-1 SP1 did not disrupt the HIV-1 SP1 domain. wt and PR− Ty3 elements in which SP1 replaced SP were constructed. The novel Gag species contained chimeric processing sites at the SP1 junctions. This construct produced and processed chimeric Gag3 protein and produced an amount of cDNA equivalent to 36% of the amount produced by wt Ty3. A quantitative assay showed that despite production of cDNA by the Ty3 HIV-1 SP1 mutant, it displayed a 50-fold-lower transposition frequency than wt Ty3. Therefore, the substitution of HIV-1 SP1 did not rescue the Ty3 ΔSP mutant (data not shown).

Pol3 processing is not dependent upon Gag3 processing. Identification of mutations that blocked processing at one or both of the SP junctions allowed us to test whether processing of Gag3-Pol3 within the Pol3 domain is sensitive to the state of Gag3 maturation. This was assessed by Western blot analysis using anti-IN antibodies (Fig. 3A). Neither of the single mutations that disrupted SP production, G207D or H233V, disrupted production of mature IN (Fig. 3A, lanes 2 and 6). However, mutations with more severe effects, including G207I (Fig. 3A, lane 3) and G207V H211V (Fig. 3A, lane 5), which failed to produce NC but produced CA, and H233V (Fig. 3A, lane 6) and G207D H233V (Fig. 3A, lane 7), which failed to produce NC or CA, each produced a doublet of the approximate mobility (~100 kDa) of an RT-IN fusion in addition to IN. This species has been observed for wt Ty3 (22) although it was present in greater amounts in extracts of cells expressing these Gag3 processing mutants. Only in extracts of cells expressing the D203V G207V mutant was IN production undetectable (Fig. 3A, lane 4). wt levels of IN were also observed for the ΔSP mutant, indicating that the SP domain itself is not critical for production of IN from Gag3-Pol3 (Fig. 3A, lane 15). Because at least some mutations that affected production of CA and NC did not affect processing of IN from Gag3-Pol3, these results indicate that Gag3 processing products are unlikely to be required for processing of Gag3-Pol3. The D203V G207V mutations could have interfered with Gag3-Pol3 maturation and affected Gag3 folding or assembly and, therefore, only indirectly affected processing.

Charged residues, but not single, potentially phosphorylated residues, are essential for full-length Gag3 particle production. The ability of the ΔSP mutant to process precursor polyproteins (Fig. 2A and B, lanes 21, and 3A, lane 15), combined with the decrease in retrotransposition (Tables 2 and 3), suggested that SP is required downstream of proteolytic maturation. As noted above, this domain has a significant number of potentially phosphorylated and negatively charged residues, and the latter are conserved between Ty3 and Ty3Sp. To test for an essential role of any single residue, each of the eight potential phosphorylation targets within the SP domain (Fig. 1B) was individually replaced with Ala. In addition, residues S218 and S222 were mutated to Glu to mimic phosphorylation. The resulting mutants were tested for expression and processing of Ty3 Gag3 by Western blot analysis. In all cases, amounts of CA and IN were similar to those produced by wt Ty3 (data not shown). Retrotransposition frequency of these mutants was also similar to that of the wt (Table 2). Correct protein processing and wt levels of retrotransposition indicated that particle assembly and cDNA synthesis occurred normally. Although these results do not exclude the possibility that phosphorylation contributes to SP function, they indicated that phosphorylation of any individual residue is not essential.

As discussed above, Ty3 SP contains a disproportionate number of acidic residues that would confer a high negative charge density. To test the contribution of this property, these residues were replaced with Ala (D/E → A). In order to distinguish any defects created by PR-mediated destabilization from assembly defects, the PR catalytic site mutation was introduced into this mutant (D/E → A PR−). Cells expressing these mutants were examined for protein production and processing using antibodies against CA, NC, and IN. Extracts analyzed for CA and NC production showed that both mutants produced greater than wt levels of Gag3, but Gag3 processing

<table>
<thead>
<tr>
<th>Mutation(s)</th>
<th>Transposition frequencya</th>
<th>Fold decrease from wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>2.90 × 10⁻³ ± 0.39</td>
<td>1</td>
</tr>
<tr>
<td>ΔSP</td>
<td>0.18 × 10⁻³ ± 0.06</td>
<td>16</td>
</tr>
<tr>
<td>ΔSP PR−</td>
<td>0.01 × 10⁻³ ± 0.00</td>
<td>290</td>
</tr>
<tr>
<td>D/E → A</td>
<td>&lt;10⁻³</td>
<td>NA</td>
</tr>
</tbody>
</table>

a NA, not applicable.

b Frequency of cells that underwent transposition out of total cells plated.

FIG. 3. Effect of SP mutations on Gag3-Pol3 processing and cDNA synthesis. (A) Western blot analysis of IN protein processed from Gag3-Pol3. Yeast cells were induced for expression of wt Ty3 or mutant derivatives and monitored for protein expression as described for Fig. 2 except that WCE were probed with anti-IN antibody. (B) Southern blot analysis of Ty3 cDNA. Transformants expressing wt and mutant Ty3 were grown in SG Ura medium for 18 h. Extracted DNA was digested with BamHI to linearize the Ty3 plasmid. A [32P]-labeled probe to a 2.9-kb internal region of Ty3 was used to visualize Ty3 cDNA (5.4 kb) and the expression plasmid. The ratio of wt cDNA to plasmid signals was set to 1.0, and other samples were normalized to this value. Measurements are representative of two or more independent transformants tested.
However, the PR detected only low levels of Gag3-Pol3 (Fig. 3A, lane 13). Western analysis using the anti-IN did not detect IN relative to Gag3 is due to the reduction in negative charge.

The proteolytic maturation observed for SP mutants suggested that a significant subset was assembly competent. However, retrotransposition was undetectable. In order to determine whether relatively subtle defects in processing could have more profound consequences for reverse transcription, cDNA production was examined. Cells were induced for 18 h to express wt and mutant Ty3 elements. DNA was extracted, and Southern blot analysis was performed using a Ty3-specific probe. Comparison of the ratio of extra-chromosomal Ty3 cDNA to Ty3 donor plasmid in cells expressing wt and mutants (Fig. 3B) showed that the G207D mutant—the only processing mutant that generated wt levels of NC—produced a higher ratio of cDNA to plasmid than wt Ty3 (Fig. 3B, lane 12). It was striking that the reduction in the amount of cDNA generated for G207D and ∆SP mutants was much less severe than the decrease in retrotransposition. The D/E → A mutant produced no cDNA, consistent with its failure to process Gag3 (Fig. 3B, lane 10). Thus, overall, levels of cDNA generated by a mutant correlated with the ability to generate mature NC.

The role of the negatively charged SP domain in focus formation and RNA packaging. The Gag3 processing and cDNA levels observed for the ∆SP mutant indicated that SP is dispensable for assembly and cDNA synthesis but is critical for some later step. However, the absence of D/E → A Gag3 processing raised the possibility that the charged residues in SP play a critical early role if SP is present. Cells induced for Ty3 expression form foci containing Ty3 Gag3 and Gag3-Pol3 and Ty3 RNA (2). Focus formation is one of the earliest correlates of assembly (26, 27). In order to gain insight into how SP might contribute to assembly, we examined the abilities of the D/E → A and ∆SP mutants and their PR− derivatives to form foci (Fig. 4A). Indirect immunofluorescence studies using antibodies to detect CA showed that cells expressing wt Ty3 displayed the characteristic one or two large foci, and PR− derivatives were similar, but with less diffuse cytoplasmic fluorescence. In contrast, cells expressing the D/E → A mutant had considerable cytoplasmic fluorescence, consistent with the Western blot analysis showing wt levels of Gag3. However, this fluorescence was diffuse, with many small speckles, and was not readily distinguishable from the D/E → A PR− derivative. Cells expressing ∆SP showed foci indistinguishable from those of cells expressing wt Ty3. Together these results indicated that SP is not essential for formation of Ty3 assembly foci but that if SP is present, replacing the charged residues within it disrupts focus formation.

Ty3 NC zinc finger mutants do not form cytoplasmic foci and are defective in packaging Ty3 genomic RNA. NC zinc finger mutant Gag3 concentrates in the nuclei (26), presumably because it fails to recognize genomic Ty3 RNA. The retention in
the cytoplasm of the D/E → A mutant Gag3 suggested that the Gag3 NC domain engages RNA. Because retrotransposons are intracellular, RNA packaging must be assessed using a nuclease protection assay to remove contaminating unpackaged Ty3 RNA. Nuclease protection assays were performed to test RNA protection of the D/E → A and ∆SP mutants. In extracts of cells that expressed wt Ty3 for 8 h, 23.4 ± 10.3% of Ty3 RNA was protected from nuclease digestion, and protection was greater for the PR mutant (Fig. 4B). However, extracts of cells expressing the D/E → A or the PR derivative did not show significant protection of Ty3 RNA. In contrast, the ∆SP mutant extracts protected a higher fraction of RNA than the wt, and this was further enhanced in the PR derivative. These results suggest that the D/E → A mutant failed prior to or at the stage of protein condensing around the genomic RNA. The ∆SP mutant not only assembled but also formed particles which were more resistant to nuclease than wt particles. Because the level of protection afforded by the ∆SP PR mutant was higher than the level of protection by the PR mutant, this difference is not likely to be attributable to differences in RNase H activity.

SP processing mutants have aberrant particle morphologies. The ability of G207D and ∆SP Ty3 mutants to undergo proteolytic processing, produce NC, and generate cDNA contrasted with the defective retrotransposition of these mutants. This suggested that SP-NC cleavage is required for cDNA synthesis but that both SP and CA-SP cleavage have roles subsequent to cDNA synthesis. One possibility would be that they function to facilitate uncoating, thus making cDNA accessible for integration. TEM and AFM were utilized to examine Ty3 wt and mutant particles for differences in particle structure.

When examined by TEM, cells expressing Ty3 show large clusters of VLPs (Fig. 5), corresponding to the assembly foci visualized by indirect immunofluorescence (Fig. 4A). The PR− mutant was examined to exemplify VLPs in the immature state. In cells expressing the PR− mutant, particles occurred in chains and appeared to be overlapping like flattened VLP doublets with outer, electron-dense regions. In contrast, cells expressing wt Ty3 had more irregularly sized and distributed VLPs with electron-dense centers, representing the condensed ribonucleoprotein cores. Cells expressing the G207D mutant contained clusters of tightly packed VLPs in disordered arrays. Consistent with the ability of this mutant to produce mature NC, many particles had electron-dense centers. The H233V mutant particles were most similar to those of the PR− mutant in that VLPs lacked electron-dense centers and were in a highly ordered matrix. This phenotype was consistent with undetectable Gag3 processing. However, these particles lacked the compressed overlapping appearance of the PR− mutant, suggesting a difference possibly related to Gag3-Pol3 processing. Inspection of cells expressing the ∆SP mutant showed that this mutant formed particles but that these particles were elongated and appeared connected in chains rather than large orderly matrices. Electron-dense regions were more unevenly distributed for wt and G207D. Overall, these results showed that maturation of NC, rather than processing of CA-SP or any function of SP, is the key step to allow electron-dense core formation. In these mutants, this property correlated with production of cDNA. Out of over 100 cells expressing the D/E →

FIG. 5. Transmission electron microscopy of Ty3 VLPs. Cells transformed with wt Ty3 and mutant derivatives were grown in SG medium for 6 h to allow Ty3 expression. Cells were harvested and processed for imaging as described in Materials and Methods. Cells were imaged at 100 kV. Magnification (e.g., 10Kx, magnification of ×10,000) and scale bars are indicated in the individual panels. Insets are at a magnification of ×2.5 of the original images.
A mutant, relatively few showed evidence of particle formation. However, in a fraction of the samples, low-density collections of partially condensed particles were observed (Fig. 5). These structures had a bubbly, uneven appearance and lacked the integrity of wt particles.

**AFM visualization of particle surfaces.** TEM showed that the G207D mutation, which disrupted CA-SP but not SP-NC processing, was associated with VLPs having dense centers and tightly packed VLP clusters. This observation, coupled with the ability of this mutant to make cDNA but an inability to retrotranspose, raised the possibility that processing of SP affects particle uncoating. AFM was previously used to examine the external structure of Ty3 particles (25). This analysis showed that wt VLPs are heterogeneous but that PR\(^{-}\) mutants are relatively uniform, with capsomer features characterized by both pentagonal and hexagonal symmetry. If CA-SP processing is required to achieve the heterogeneous state of wt particles, then the individual G207D and H233V mutants and the double mutant would have regular surface structures, similar to the structure of the PR\(^{-}\) mutant. AFM analysis showed that the single mutants had regular structures that did not change significantly with inactivation of PR (Fig. 6). G207D H233V mutant particles appeared to be irregular, but mutant G207D H233V PR\(^{-}\) particles were more regular. Although this result did not conform to the anticipated appearance of unprocessed particles, it could also be that the G207D H233V mutations destabilized particles through another mechanism. This interpretation is supported by the observation that this mutant produced less IN than the other mutants and that PR inactivation increased the amount of Gag3-Pol3 (Fig. 3A, lanes 7 and 8). Despite the apparent abundance of ΔSP mutant protein, Ty3 protein in the VLP fraction was significantly reduced compared to similar preparations from cells expressing wt Ty3 (data not shown). Ordered particles were observed in the absence or presence of the PR\(^{-}\) mutation.

**DISCUSSION**

Some retrovirus Gag proteins contain an SP domain between CA and NC domains. Where it has been studied, this domain is important for assembly and core condensation. Retrotransposons undergo intracellular assembly and replication. The existence of a SP domain in the Ty3 retrotransposon allowed exploration of the role of this domain in retrotransposition. We found that the negative charge of Ty3 SP is essential for assembly of the normal amount of particles. However, Ty3 particles can be assembled in the absence of this domain. Our results showed that these particles produce about half the amount of cDNA produced by wt particles but are 16-fold less active for transposition. Thus, Ty3 SP must also play a critical postreplication role in retrotransposition.

**Cleavage of SP is context dependent and ordered.** wt Gag3 is processed into major CA-SP, CA, and NC species. The current study showed that processing within the Pol3 domain of Gag3-Pol3 requires assembly but is independent of Gag3 processing. Mutations that disrupted Gag3 processing had nonreciprocal effects. The G207D mutation, which disrupted processing at CA-SP, did not interfere with production of NC. In contrast, the H233V mutation at the SP-NC junction blocked processing at that cleavage site and also interfered with CA-SP processing. Although this could result from effects of the H233V mutation on the overall folding of Gag3, the fact that the H233V mutant was competent for particle formation, and even Gag3-Pol3 processing of IN, argued against this mutation having a long-range, deleterious effect on Gag3 folding. A simpler explanation, consistent with the presence of the CA-SP intermediate in extracts of cells expressing wt Ty3, is that processing is ordered, with the SP-NC cleavage not only preceding CA-SP cleavage but also potentially required for that cleavage. A requirement for cleavage of Ty3 SP-NC prior to CA-SP would make it similar to the processing of HIV-1 Gag at the SP1-NC junction prior to processing at the CA-SP1.
foci formed in cells expressing foci of Ty3 protein and RNA and P body components (2). Induction of Ty3 expression is the appearance of cytoplasmic into the functions of Ty3 SP. One of the earliest events after charge bias led to distinct phenotypes that provided insights of mutant and visualized by TEM and AFM. Initially, the ability is in contrast to the foci and particles formed by the /H9004

assembly. The particles that formed differed from those formed by wt Ty3 and were heterogeneous in size and shape. Although this could result from gross misfolding of Gag3, some other mutations that interfered with assembly destabilized Gag3 (26, 27), but the D/E → A mutation did not significantly reduce amounts of Gag3.

We propose that domains of Ty3 Gag3 change conformation during assembly and that mutations affecting SP and NC charge or binding to RNA disrupt this progression. Taylor et al. (42) used nuclear magnetic resonance (NMR) to analyze a fragment comprised of the RSV CA C-terminal domain (CTD)-SP-NC in the presence and absence of oligonucleotides. On the basis of these experiments, they proposed that the CA CTD and neighboring SP interact to antagonize multimer formation in the absence of RNA but that binding of RNA by NC disrupts this conformation, allowing initiation of assembly. A related sequence of events could occur during Ty3 assembly. An intramolecular interaction between the acidic SP domain and the basic NC domain could limit intermolecular CA domain interactions prior to the NC domain binding RNA (Fig. 7). Upon binding of RNA, the basic NC domain might be more available for intermolecular interactions with SP, thereby allowing intermolecular interactions of CA and even promoting multimerization of Gag3 on the RNA. An intermolecular interaction involving NC would be analogous to the model proposed for human T-cell lymphotropic virus type 1. That NC has an acidic domain, which NMR studies have shown stabilizes NC binding to RNA. Stabilization is proposed to be mediated by intermolecular interactions between the acidic and basic domains of NC, which favor multimerization on the RNA (36). The Ty3 model would differ from that model in that the acidic domain of SP, rather than NC, would provide intermolecular interactions that promote assembly.

The defective assembly of the Gag3 mutant with neutral SP is in contrast to the foci and particles formed by the ΔSP mutant and visualized by TEM and AFM. Initially, the ability of ΔSP to form particles might seem at odds with the idea that intermolecular SP-NC interactions promote assembly. However, it is possible that in addition to promoting multimerization, the negative charge on SP is important for folding of a spacer domain into Gag3 multimers. If SP is lacking, NC domain binding to RNA might be sufficient to promote enough CA intermolecular interaction to support Gag3 multimerization.

Ty3 SP is apparently not essential for gross assembly of particles or even cDNA synthesis. The ΔSP Gag3 protected RNA from nuclease digestion to a greater extent than wt Ty3 Gag3, suggesting that it forms compact particles, and TEM showed that G207D and H233V Ty3 produced relatively uniform VLPs compared to wt. However, ΔSP Ty3 Gag3 protected cDNA but failed to retrotranspose. Together, the ΔSP and G207D phenotypes argue that SP must be both present and
cleaved from CA to allow some step in morphogenesis during or subsequent to reverse transcription. One candidate for such a step would be rearrangement of CA to allow uncoating. For example, a negatively charged SP domain could destabilize a CA-SP/CA-SP network to promote uncoating. For example, a negatively charged SP domain could destabilize a CA-SP/CA-SP network to promote uncoating.

**Summary.** We speculate that Ty3 SP acts as a molecular spring, promoting first Gag3 assembly and then VLP uncoating. In the first stage, the acidic SP domain of Gag3 has intramolecular interactions with the basic NC domain. In the second stage, the NC domain binds RNA nonspecifically, and intermolecular interactions occur between it and the SP domain. Such interactions could support multimerization of Gag3 on the RNA, thereby promoting CA-CA interactions. In a third phase, which could overlap with reverse transcription, the negatively charged SP domain destabilizes CA-SP interactions, allowing disassembly to a stage that releases an integration-competent form of cDNA. A role for SP in assembly through RNA binding and multimerization and then in dissociation may have evolved in a retrotransposon in order to allow differential regulation of events required for concentration of structural subunits, assembly of a stable particle, and reverse transcription and events involved in uncoating.

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**REFERENCES**