

A Nucleocapsid Functionality Contained within the Amino Terminus of the Ty1 Protease That Is Distinct and Separable from Proteolytic Activity

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Ty1 is the most successful of the five endogenous yeast retrotransposons. The life cycle of Ty1 dictates that a number of nucleocapsid (NC)-facilitated events occur although the protein(s) responsible for these events has not been identified. The positioning of the NC peptide is conserved at the carboxy terminus of the Gag protein among most long terminal repeat (LTR)-containing retroelements. An analogous region of Ty1 that simultaneously encodes part of Gag, protease (PR), and the C-terminal p4 peptide was mutagenized. Some of these mutations result in smaller-than-normal virus-like particles (VLPs). The mutants were also found to impair an NC-like functionality contained within the amino terminus of the protease that is distinct and separable from its proteolytic activity. Remarkably, these mutants have distinct defects in reverse transcription.

Ty1, the most abundant retrotransposon in the yeast *Saccharomyces cerevisiae*, is present at 20 to 35 copies per haploid genome (4, 6, 9). These genomic elements are actively transcribed such that Ty1 mRNA represents approximately 1% of total poly(A)⁺ RNA within the cell (11). Transcription of genomic Ty1 elements requires transcription factor Spt3p (37). Since the *GAL1* promoter is active in *spt3* null cells, Ty1 elements driven by the *GAL1* promoter can be analyzed in a background free of endogenous element transcription (5).

Ty1 mRNA is translated into two proteins, Gag and the Gag-Pol polyprotein. The Gag protein is the major structural component of Ty1 virus-like particles (VLPs), replication intermediates in which reverse transcription occurs (13, 16, 25, 31). The Gag-Pol polyprotein contains the enzymatic components required for replication, including protease (PR), integrase (IN), and reverse transcriptase (RT). These enzymatic species are liberated from the Gag-Pol polyprotein by proteolytic processing. The element-encoded PR is responsible for proteolytic processing and is absolutely required for retrotransposition (1, 30, 41).

The stoichiometric ratio of Gag to Gag-Pol is determined by the efficiency of a 7-nucleotide (nt) frameshift signal contained within Ty1 mRNA. The frameshift is typically 5 to 10% efficient, which fixes the ratio of Gag to Gag-Pol at 10 to 20 to 1 (3). “Erasing” the frameshift signal by a single-nucleotide deletion blocks retrotransposition. Similarly, (i) altering expression levels of a single-copy tRNA gene that inhibits frameshifting or (ii) expression, in *trans*, of a protein known to inhibit +1 ribosomal frameshifting blocks retrotransposition (12, 18, 35, 38). Mutations that erase the endogenous frameshift signal can

be suppressed in *cis* by introducing a new frameshift signal at an ectopic site (21).

All of the functions typically associated with a nucleocapsid (NC) functionality such as RNA packaging, positioning of primer tRNA, and efficient strand transfer and reverse transcription occur during the Ty1 retrotransposition process. However, these functions have yet to be ascribed to a specific protein(s). Reverse transcription of Ty1 is primed by the initiator methionine tRNA (tRNA_{Met}ⁱ) (7). A basic region of Ty1 CA has recently been shown to facilitate this process in vitro, suggesting that the C-terminal basic region provides an NC-like function (8). Whether or not deletion of this region is compatible with Ty1 transposition in vivo remains to be determined. Unlike its retrovirus counterparts, Ty1 does not encode an NC peptide with a canonical zinc finger motif (15, 27). Indeed, it remains possible that some of the NC duties normally subserved by a single-element-encoded NC peptide in retroviruses are shared among host-encoded and Ty1 element-encoded protein(s).

NC protein coding regions can be readily identified in the *gag* genes of most retroviruses and retrotransposons, which contain one or two CX₂CX₄HX₄C “Zn knuckle” motifs. The position of the NC coding sequence in the genome of long terminal repeat (LTR)-containing retroelements is evolutionarily conserved. The NC peptide is located at or near the carboxy terminus of Gag downstream of CA and upstream of the Pol coding region. The p4 coding region occupies the analogous position in the Ty1 genome, and we hypothesized that it might specify an NC functionality. However, p4 is downstream of the basic region near the C terminus of CA identified by Cristofari et al. (8).

Certain mutations in the p4 region of Gag and the amino terminus of PR block transposition (27). However, interpretation of the mutant phenotype is complicated by the fact that these mutations simultaneously alter Gag, PR, and the p4 peptide sequences. The transposition defect observed in these

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TABLE 1. Primers used to construct PR mutants

Mutant	Sequence of primer ^a :	
	1	2
s9	GCGCGGATCCTTCAGCAAGTTTCTGGCCTAAGATGAA	GCGCGGATCCGCGGCCCATACTAATCATTCTGATGAT
s10	GCGCGGATCCGCGCGGCATGATTAGTATGATTTACTGTAGA	GCGCGGATCCGCGCGGCACACTCCTTCTCGATTCA
s11	GCGCGGATCCGCGCAGGGAGTTCATCATCAGAATG	GCGCGGATCCGCGCGGATTGAGGAGCATCACGAACC
s12	GCGCGGATCCGGAATCGTTGTCCGTGCTGGG	GCGCGGATCCCATCTTAGGCCATGAACCGATTCAATTGAACAAT

^a Primer 1 is used in conjunction with JB1993. Primer 2 is used with primer JB1994. *Bam*HI sites contained within the primers are underlined.

mutants could be secondary to loss or gain of function in any or all of these proteins. We demonstrated, through the application of a novel technique called frameshift transplantation, that these mutations specifically affect the Ty1 PR (21). While frameshift transplant mutants implicated the PR protein as being specifically affected, they did not offer any insight into the nature of the defect.

In this report we demonstrate that the amino terminus of the Ty1 PR serves an essential role in Ty1 replication that is separable and distinct from its proteolytic role. Mutations in this region give rise to a unique phenotype: a general failure in reverse transcription. Additionally, elements bearing these mutations produce smaller-than-normal VLPs (27). These findings support the assertion that the amino terminus of the Ty1 PR is required for at least one NC function: facilitating reverse transcription.

MATERIALS AND METHODS

Yeast strains and plasmids. Yeast strain YH51 (*MATa ura3-52 his4-539 lys2-801 spt3-202*) was used for all experiments unless otherwise specified. Strain YH10 (*MATa ura3-52 his4-539 lys2-801*) was used in complementation studies. It is congeneric to YH51, differing only at the *SPT3* locus. Cells were grown on synthetic complete medium lacking Ura (SC-Ura). Transposition was induced by growth on medium containing 2% galactose.

Mutants s9 through s12 with linker insertion mutations in the p4 region were constructed via a three-piece ligation strategy with two PCR products and pJef1105 digested with *Hpa*I and *Bst*EII. The first PCR product was amplified using primer JB1993 (ATATGTCAGACCACCAAT) and primer 1 (Table 1). It was digested with *Hpa*I and *Bam*HI. The second product was amplified with primer 2 (Table 1) and primer JB1994 (TGTCTGGAAGTGAAATTGTA). It was digested with *Bst*EII and *Bam*HI. *d1*, *s7*, and *s8* mutations have already been described (27).

VLP preparation. VLPs were prepared via equilibrium density centrifugation on 20 to 70% linear sucrose gradients as described previously (13).

Immunoblotting. Cells were grown overnight in 10 ml of SC-Ura-2% raffinose overnight. Transposition was induced by diluting the culture to an A_{600} of 0.6, adding galactose to a final concentration of 2%, and incubating the culture at 26°C for 24 h or as indicated. Yeast lysates were prepared from approximately 2 A_{600} units of cells in a mini-bead beater (Biospec Products) in 40 μ l of 100% trichloroacetic acid (TCA) and 100 μ l of acid-washed glass beads. The beads were pelleted and washed once with 1 ml of ice-cold 5% TCA and once with 1 ml of ice-cold water. The lysates were removed from the beads by incubation in 100 μ l of 6% sodium dodecyl sulfate (SDS)-0.5 M Tris base at 55°C for 15 min. Five microliters of supernatant was used for immunoblotting.

After electrophoresis, proteins were transferred to Immobilon polyvinylidene difluoride membranes at 300 mA for 2 h. Membranes were blocked in 3% milk-0.1% Tween 20 in phosphate-buffered saline (PBS). Primary antibody R2-F (anti-VLP) was applied in blocking solution for 1 h at 1:10,000 dilution. Primary antibody 8B11 ascites fluid (anti-IN) was applied at 1:1,000 dilution. The blots were washed three times for 10 min in PBS-0.1% Tween 20 (PBS-T). The secondary antibody (horseradish peroxidase [HRP]-conjugated anti-rabbit antibody [used with R2-F] or HRP-conjugated anti-mouse antibody [used with 8B11]) (Amersham) was applied at a 1:7,500 dilution in 3% milk in PBS-T for 40 min. The membranes were washed as described above and developed with ECL (Amersham) in accordance with the manufacturer's instructions.

cDNA synthesis assays. Yeast cells harboring plasmids were grown for 24 h at 24°C in 10 ml of SC-Ura-2% raffinose. The cells were then diluted to an A_{600} of 0.6 and induced as described above. The cDNA synthesis is dependent on galactose induction (data not shown). Cells (0.5 ml) at an A_{600} of 2.0 were then pelleted and washed once in water. Nucleic acids were extracted from the cell pellet by agitation with glass beads in a solution consisting of 400 μ l of DNA extraction buffer (0.5 M NaCl, 20 mM Tris-HCl [pH 7.5], 1 mM EDTA, 1% SDS), 200 μ l of buffer-saturated phenol, and 200 μ l of chloroform using a mini-bead beater (Biospec Products). The lysate was centrifuged for 10 min in an Eppendorf model 5415 microcentrifuge at 14,000 rpm. The supernatant was extracted with an equal volume of chloroform before the nucleic acids were precipitated with ethanol. Ten micrograms of total nucleic acid was digested with 20 U of *Eco*RI and 2 μ g of RNase A in a 15- μ l reaction mixture. Southern blotting was performed as described previously (2).

RT assays. Endogenous and exogenous RT assays were performed essentially as described previously (16, 41).

RNA analysis. VLPs were prepared as described previously (27). The three sucrose fractions with peak RT activity were pooled and centrifuged at 250,000 $\times g$ at 4°C for 1 h to prepare a concentrated VLP pellet. This pellet was resuspended in 300 μ l of diethyl pyrocarbonate-treated water. The protein concentrations were determined, and 250 μ g of VLPs was used as an RNA source. Anti-Gag immunoblots confirmed that the amounts of Gag protein in the particles were comparable. RNA was extracted by incubating 100 μ l of VLPs with 50 μ l of buffer-saturated phenol-50 μ l of chloroform for 5 min at 65°C. The nucleic acids were ethanol precipitated with 2.5 M ammonium acetate, resuspended in load buffer, and resolved on a 1% agarose-formaldehyde gel.

For dot blots, the nucleic acids extracted as described above were treated with either DNase or RNase A and applied to a GeneScreen (NEN) membrane through a Schleicher and Schuell 96-well dot blot apparatus in 100 μ l of water. Northern and dot hybridizations were performed as described previously (2).

Analysis of reverse transcription intermediates. DNA was prepared from equivalent amounts of VLPs as described above. The resuspended nucleic acids were treated with 2 μ g of RNase A for 1 h at 37°C. The reaction mixture was reextracted with phenol and chloroform and precipitated. The DNA was resuspended in 20 μ l of water.

tRNA Northern blots. Total nucleic acids from equivalent amounts of wild-type and mutant VLPs were prepared as described above. The nucleic acids were separated in a 10% polyacrylamide-urea gel at 650 V. The nucleic acids were then transferred to a GeneScreen (NEN) membrane in 1 \times MOPS (morpholinepropanesulfonic acid) buffer at 20 V for 4 h at 4°C. The nucleic acids were cross-linked to the membrane for 12 min at 125 W. The membrane was then baked in vacuo at 80°C for 2 h. The membrane was prehybridized at 37°C in 10 ml of 6 \times SSC (1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-1% SDS containing 100 μ g of sheared herring sperm DNA/ml. ³²P-end-labeled oligonucleotide JB384 (10⁸ cpm; TTCCACTGCGCCACGGCGCT) was added and allowed to hybridize for 5 h. The blot was washed three times for 10 min in 6 \times SSC-1% SDS at 37°C, wrapped, and exposed to film.

Transposition assays. Yeasts, grown as patches, were replica plated from SC-Ura-2% glucose to SC-Ura-2% galactose and grown at 26°C for 4 days. The patches were then replica plated to SC-Ura-2% glucose and grown overnight at 30°C. The patches were replica plated to yeast extract-peptone-dextrose (YPD) and, after overnight growth at 30°C, were replica plated to SC-0.1% 5-fluoroorotic acid. A portion of the patch was then diluted and plated on both YPD and YPD-75 μ g of G418/ml. The transposition frequency of the wild-type element is defined as 100%.

RESULTS

Mutations in the p4 region inhibit transposition. To define the essential role of the amino terminus of Ty1 PR, a series of

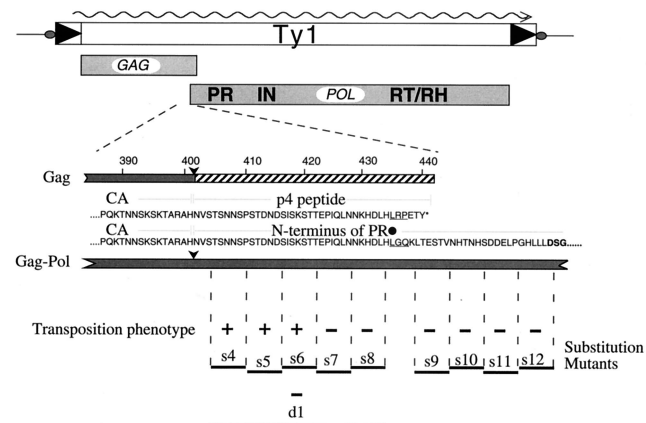


FIG. 1. Schematic of the Ty1 Gag-Pol overlap region. The Gag and Gag-Pol polyproteins as well as the relevant amino acid sequences are shown. Black dot, site of +1 translational frameshifting; *, Gag sequence position corresponding to the stop codon. The amino acids encoded fully or in part by the frameshift are underlined. PR active-site residues are in boldface. Arrowheads, PR cleavage sites in Gag and Gag-Pol. The extent of the p4 peptide is indicated. Block substitution mutants are shown under the amino acid residues they replace with the sequence AAGSAA. The transpositional competence of elements bearing these mutations is indicated. The s7, s8, and d1 mutants that block transposition are collectively referred to as the PR^{dsd} mutants.

block substitution mutants were constructed in this region (Fig. 1). The mutated sequences span the amino terminus of the PR up to, but not including, the active site. Some of the mutants (d1, s7, and s8) have been shown to cause a morphological change in Ty1 VLPs. Specifically, the VLPs produced by these mutant elements are smaller and migrate at a reduced rate through sucrose density gradients (27).

Ty1 PR activity is essential for transposition, and transposition frequency is reduced over 100-fold in Ty1 elements bearing a mutation in or near the PR active site (Table 2) (41). Transposition frequency is reduced over 20-fold in block substitution and deletion mutants s7 through s12 and d1 (Table 2) (27).

TABLE 2. Mutant phenotypes

Ty1 element	Proteolytic processing	Transposition frequency ^a for:	
		YH51	YH10
WT ^b	+	100	100
PR ⁻	-	<1	<1
d1	+	<1	7.6
s7	+	<1	9.4
s8	+	<1	9.1
s9	+/- ^c	<1	2.5
s10	+/-	<1	2.3
s11	+/-	<1	2.1
s12	-	<1	2.5

^a Transposition assays were performed and frequencies were measured as described in Materials and Methods. Strain YH10 is SPT3⁺ and expresses endogenous Ty1 elements. Strain YH51 is congenic to YH10 and is *spt3*. It does not express endogenous Ty1 elements.

^b WT, wild type.

^c +/-, partial processing phenotype.

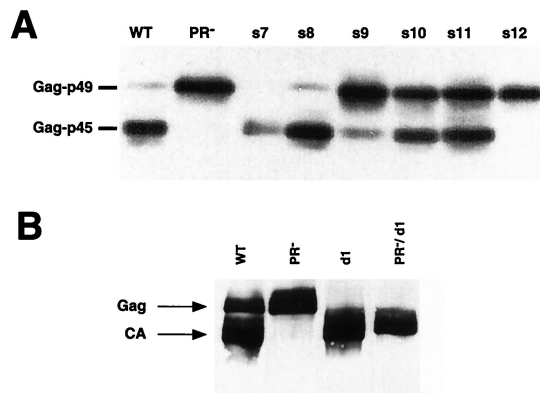


FIG. 2. Some block substitution mutants process Ty1 Gag. Lysates were prepared from equal numbers of YH51 cells harboring the indicated plasmids after 24 h of galactose induction. An SDS-10% polyacrylamide gel was used to separate the proteins. The immunoblot was probed with polyclonal antibody R2-F, which recognizes Ty1 Gag. (A) Variable processing in a series of block substitutions in the amino terminus of PR. Note that the extent of processing in mutants s7 and s8 is similar to that for the wild type (WT). (B) Processing of Gag is observed in d1 mutants.

Analysis of proteolytic processing. To explore the possibility that transposition is compromised in these mutants as a simple consequence of PR inactivation, we analyzed yeast lysates prepared from cells that were induced for transposition. The *in vivo* activity of the Ty1 PR can be conveniently assessed by determining the ratio of the Gag-p49 precursor (which has an electrophoretic mobility corresponding to a 58-kDa protein) to the mature form, Gag-p45 (CA), which migrates as a 54-kDa species.

As shown in Fig. 2A, proteolytic processing in the wild-type element results in the conversion of the majority of the Gag-p49 species to the CA species. Processing is completely impaired in the PR active-site mutant and in mutant s12, in which the linker substitution lies closest to the active-site sequence DSG. Processing is impaired to various degrees in mutants s9, s10, and s11. However, mutants s7, s8, and d1 show processing phenotypes that are indistinguishable from that observed in wild-type elements. Importantly, no Gag immunoreactivity is detected in a control strain containing an empty vector, which independently confirms that *spt3* mutants do not express endogenous Ty1 Gag proteins (data not shown).

The transposition defect observed in mutants s9 through s12 may be explained in part by impaired processing. However, the severe transposition defects of mutants s7 and s8 (mutants s7, s8, and d1 are referred to collectively as the PR^{dsd} mutants, for DNA synthesis defective) cannot be explained by a proteolysis defect because we have shown that they process Ty1 Gag and Gag-Pol normally (see below).

Because the d1 mutant lacks a majority of the p4 region that distinguishes Gag from CA, processing in it cannot be readily assessed by a conventional anti-Gag immunoblot assay. A doublet of bands, one of which comigrates with p45 and one of which migrates slower (by an amount equivalent to an ~0.5-kDa increase in size), is typically seen in d1 lysates probed with an anti-Gag antibody. The p45.5 species could represent the precursor form of Gag in this d1 mutant; partial processing of the carboxy-terminal five amino acids would yield a mixture of

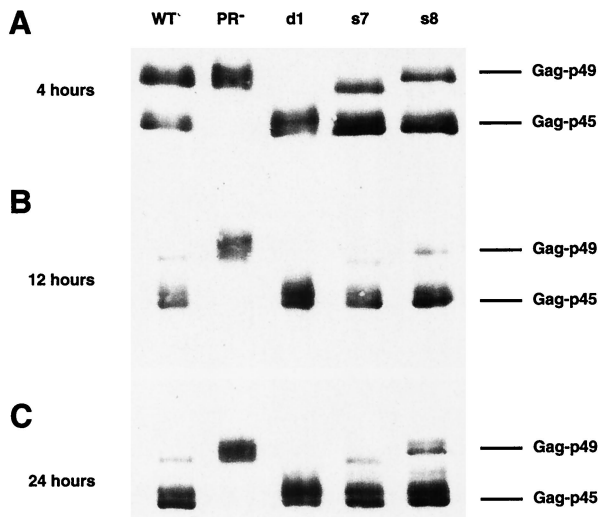


FIG. 3. PR^{dscd} mutants process Gag. Lysates were prepared from equal numbers of YH51 cells harboring the indicated Gal-Ty1 plasmids after 4 (A), 12 (B), or 24 (C) h of galactose induction. SDS-10% polyacrylamide gel electrophoresis was used to separate the proteins. The immunoblot was probed with polyclonal antibody R2-F, which recognizes Ty1 Gag. Fourfold more protein was loaded in the 4-h time point to facilitate detection.

CA and the uncleaved precursor species and account for the observed difference in electrophoretic mobilities. Ty1 Gag is also known to be phosphorylated (39). The doublet observed in *d1* lysates could in theory also be the consequence of Gag phosphorylation; however we were unable to resolve the doublet into a singlet via treatment with a variety of phosphatases (J. F. Lawler, Jr., and J. D. Boeke, unpublished results). To demonstrate that this doublet results from partial proteolytic processing, we created a double mutant in which a PR⁻ active-site mutation was created in the context of pJef1105-*d1*. This construct, pJL527 (pJef1105PR⁻/*d1*), produces only the p45.5 species. We therefore conclude that the doublet observed in *d1* lysates is the result of partial proteolytic processing (Fig. 2B).

Proteolytic processing of the Gag-Pol polyprotein proceeds through an ordered (or semioordered) pathway with regard to cleavage site utilization (17, 26). The Gag-p4/Gag-PR site is cleaved first. This cleavage is required for subsequent processing of the PR-IN and/or IN-RT sites. The *d1* mutant produces an IN species that comigrates with authentic IN produced by the wild-type element (27). The appearance of this mature IN species is evidence of Gag-Pol processing in the *d1* mutant.

VLP assembly is a dynamic process and, as such, might be sensitive to minor variations in the time course of Gag and Gag-Pol processing (31). To determine whether the transposition defect observed in the PR^{dscd} mutants is the consequence of an altered time course of PR activity, we prepared lysates from cultures at various time points after the onset of galactose induction. Anti-Gag immunoblots of these cultures are shown in Fig. 3A to C. No difference between the wild type and PR^{dscd} mutants can be detected at any of the time points examined. If anything, in this experiment, the PR^{dscd} mutants apparently process Gag faster than the wild type; however, this modest difference is not reproducible (data not shown). Similarly, the time course of processing at the PR-IN and IN-RT junctions

did not vary between wild-type and *d1* mutant elements (Fig. 4).

We also determined that introduction of the *s7* or *s8* mutation does not lead to a change in site selection by the PR at the Gag-PR junction (21). Gag-PR is cleaved between the histidine (amino acid 401) and asparagine residues in the sequence TARAHNVSTS in the *s7* and *s8* mutants as well as the wild-type element (21). It therefore seems unlikely that a qualitative or quantitative difference in processing is responsible for the transposition defect observed in the PR^{dscd} mutants. We note, however, that introduction of the *s7* mutation causes a slight increase in the electrophoretic mobility of Gag-p49. This is also seen when an amino-terminal truncation of the *s7* mutant Gag protein is expressed in *Escherichia coli* (J. F. Lawler, Jr., and J. D. Boeke, unpublished observations).

Galactose-induced overexpression of Ty1 PR active-site mutants is not complemented in *trans* by endogenous elements (10). To test whether the PR^{dscd} mutants could be complemented in *trans*, we assayed transposition in congenic yeast strains differing only at the *SPT3* locus. *SPT3* encodes a transcription factor that is required for the expression of endogenous Ty1 elements but not Gal-Ty1 elements (5, 37).

In complementation assays, mutants *s9* through *s12* behave more like the PR active-site mutant (PR⁻) in that their transposition frequencies are markedly reduced (approximately 40- to 47-fold) in both *SPT3* and *spt3* backgrounds (Table 2). In contrast, mutants *s7*, *s8*, and *d1* show more-significant residual levels of transposition in the *SPT3* background (11- to 13-fold reduced from the wild-type level). This suggests that *s7*, *s8*, and *d1* are complemented to a greater extent by endogenous elements than the PR⁻ and *s9* through *s12* mutants and that the functionality they lack can be supplied in *trans*. In this type of system, it should be noted that the mutant elements are overexpressed and likely constitute the bulk of Ty1 proteins in the cell.

This provides a second piece of evidence that the PR^{dscd}

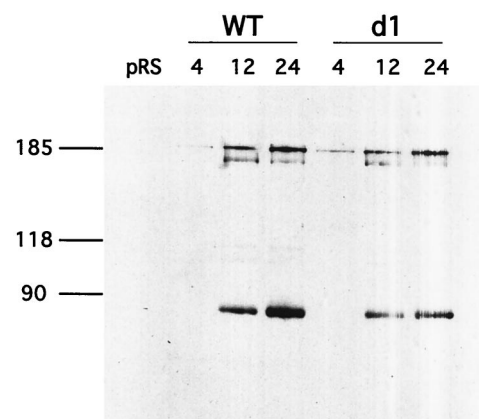


FIG. 4. *d1* mutants competently process Gag-Pol. Lysates were prepared from equal numbers of YH51 cells harboring the indicated Gal-Ty1 plasmids after 4, 12, or 24 h of galactose induction. SDS-8% polyacrylamide gel electrophoresis was used to separate the proteins. The immunoblot was probed with monoclonal antibody 8B11, which recognizes an epitope between amino acids 310 and 435 in Ty1 IN (J. F. Lawler, Jr., and J. D. Boeke, unpublished observations; 13). WT, wild type.

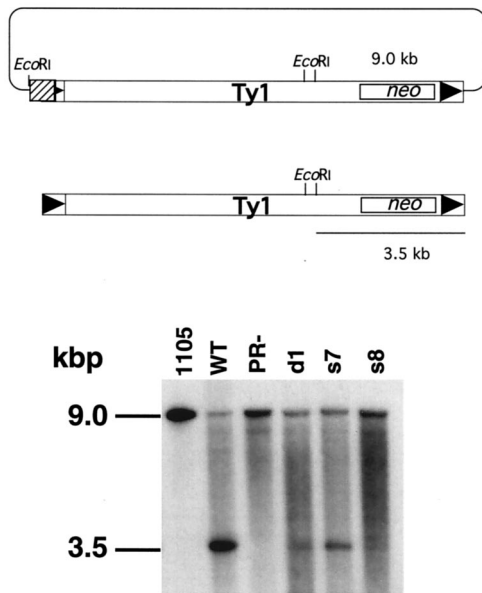


FIG. 5. PR^{dsd} and PR^- mutants have a cDNA synthesis defect. (Top) Sizes of *EcoRI* fragments containing the *neo* gene in the donor plasmid (upper portion) and in Ty1-*neo* cDNA (lower portion). (Bottom) Total yeast nucleic acids from strain YH51 were digested with *EcoRI*, treated with RNase A, and separated on a 1% agarose gel. The nucleic acids were transferred, and the membrane was probed with ^{32}P -labeled DNA generated from an *XhoI-HindIII* fragment of the Tn903 *neo* gene (boxed) via random hexamer labeling. The upper band (9.0 kbp) represents the Gal-Ty1-*neo* plasmid product. The lower band represents Ty1-*neo* cDNA. Equal amounts of DNA were loaded in all lanes. WT, wild type.

mutants cannot be grouped into the same phenotypic class as mutants with impaired processing and suggests a function separable from catalytic activity that can be complemented *trans*. In contrast, other mutations in the Gag-PR cleavage site that modestly reduce processing efficiency have only very minor effects on transposition frequency (27). Therefore, the PR^{dsd} mutants are a functionally distinct class of PR alleles that define a separate nonproteolytic activity of the Ty1 PR.

We have previously demonstrated that the amino terminus of the PR and the carboxy terminus of Gag are generated by a single autoproteolytic event (21, 27). The sequences affected by *s7* and *s8* mutations lie in a region where *GAG* and the portion of *POL* encoding PR overlap. We have also shown that *s7* and *s8* mutations exert their deleterious effect on transposition through some effect on the PR, as opposed to an effect on Gag. This was done by creating viable frameshift transplant mutants in which the sequences affected by *s7* and *s8* mutations were only translated as part of *POL* (21).

Effects on reverse transcription. Experiments in which the frameshift was transplanted indicated that PR sequences (and not Gag-p49 or the C-terminal Gag-p4 peptide sequences) are the required p4 region sequences defined by mutants *s7*, *s8*, and *d1*. However, those studies did not shed light on the nature of the transposition defect observed in the PR^{dsd} mutants. The Ty1 life cycle serves as a useful framework to begin to address this question. One requirement for successful retrotransposition is the synthesis of Ty1 cDNA from an mRNA template. We analyzed this stage of the Ty1 replication cycle in the PR^{dsd}

mutants by determining whether they are capable of cDNA synthesis.

A cDNA synthesis assay developed for a distantly related transposon of fission yeast *Schizosaccharomyces pombe* was adapted for this purpose (23) (26). In this assay, marked Ty1-*neo* cDNA can be distinguished from the plasmid-borne copy by digesting total cellular DNA with *EcoRI*. The Ty1-*neo* donor plasmid (pJef1105) and Ty1-*neo* cDNA migrate at 9.0 and 3.5 kbp, respectively, in this assay. A *neo* probe was used to detect both species. The presence of the donor plasmid signal serves as an internal control for events such as plasmid loss or integration. The transcription factor encoded by the *SPT3* gene is required for the synthesis of full-length Ty1 mRNA (5, 37). Therefore an *spt3* mutant host strain was used to permit interpretation of the mutant phenotype in the absence of an endogenous wild-type Ty1 transcript.

Wild-type Ty1 elements synthesize approximately 4- to 65-fold more cDNA than the PR^- and PR^{dsd} mutants, which synthesize very little (Fig. 5). Note that the donor plasmid signal does not vary appreciably across the lanes. Mutants *s7* and *d1* reproducibly synthesize slightly more cDNA than *s8* and PR^- mutants, although they are all significantly impaired with respect to the wild type. This observation implies that mutants *s7* and *d1* are "leaky" with respect to cDNA synthesis compared to the other mutants. The marked reduction in the amount of cDNA synthesized by these mutants points to a defect in the Ty1 life cycle before completion of cDNA synthesis.

It is tempting to speculate that the smaller size and reduced migration of the PR^{dsd} mutant particles are a direct consequence of their inability to synthesize cDNA. This is not the case however. Elements bearing inactivating point mutations in RT, which are incapable of any cDNA synthesis, form VLPs that comigrate with wild-type VLPs on sucrose density gradi-

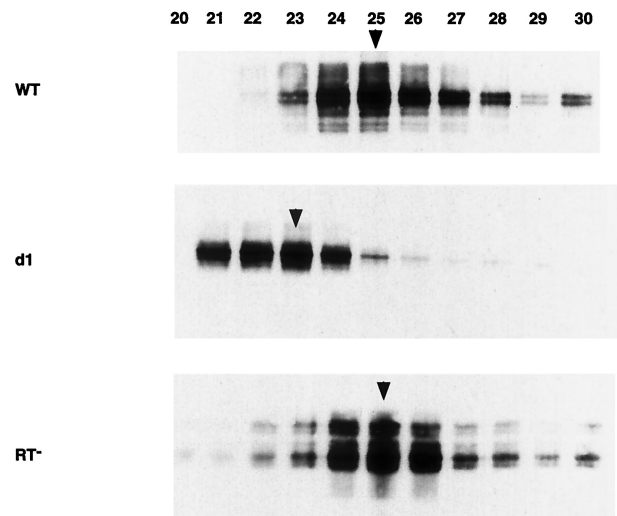


FIG. 6. VLP migration. Samples (2 μ l) of each 1.2-ml sucrose gradient fraction were mixed with sample buffer and loaded in each lane. SDS-10% polyacrylamide gel electrophoresis was used to separate the proteins. The immunoblot was probed with polyclonal antibody R2-F, which recognizes Ty1 Gag. Arrowheads, fractions containing peak RT activity. The numbering of the gradient proceeds increasingly from the top (lightest) to the bottom (heaviest). WT, wild type.

TABLE 3. RT activities

Sample	RT activity	
	Normalized ^a	% of WT ^b
WT	2,577	100
PR ⁻	2,518	97
d1	2,242	87
s7	2,554	99
s8	2,155	83

^a Anti-Gag immunoblotting was performed on serial twofold dilutions of peak VLP fractions. A dilution factor was calculated based on the amount of Gag immunoreactivity in that fraction. Raw RT activity was normalized by multiplying by the dilution factor.

^b WT, wild type.

ents (Fig. 6). Functionally inactivating mutations in the RNase H domain of RT also produce VLPs that migrate normally (J. F. Lawler, Jr., and J. D. Boeke, unpublished results).

RT activity on an exogenously supplied substrate is used to monitor VLP purification from sucrose gradients. We performed an immunoblot with an anti-Gag antibody on fractions with peak RT activity to compare RT activities of wild-type and mutant VLPs. This normalization step is necessary because VLP yield can vary with certain experimental factors such as cell lysis efficiency. As shown in Table 3, the wild-type and PR^{dscd} mutant VLPs all possessed nearly equivalent amounts of RT activity when normalized to the amount of Gag immunoreactivity present in the particles. In contrast, the amount of cDNA produced was reduced 4- to 65-fold (Table 4).

The retrovirus NC functions, which include RNA packaging and facilitation of strand transfer, are clearly required for Ty1 replication (15, 19, 22, 42). Retrovirus NC peptides are typically encoded in the region between Gag and PR, which may suggest an evolutionary conservation of NC placement in this region. We next began to look for a defect in functions normally ascribed to NC proteins such as Ty1 mRNA packaging.

VLPs from wild-type and mutant elements were prepared, and their nucleic acids were extracted. Northern blots of the contents of these VLPs show a discrete, *neo*-hybridizing band corresponding to the marked Ty1-*neo* mRNA at approximately 6.5 kb and a second band at approximately 1.7 kb (Fig. 7A). The latter band likely corresponds to transcripts that initiate from within the *neo* gene that marks the Ty1 element. Consistent with previous reports, less Ty1 mRNA is observed in PR⁻ particles (14, 41). In contrast, the PR^{dscd} mutants encapsidated normal amounts of Ty1-*neo* mRNA. This observation can be

TABLE 4. Fold decrease of primer RNA and reverse transcription products

Sample	Fold decrease of:		
	Primer tRNA ^a	Strong-stop DNA ^b	cDNA ^c
WT ^d	1.00	1.00	1.00
PR ⁻	4.69	11.4	65.4
d1	2.64	4.53	3.68
s7	2.55	1.98	4.40
s8	1.81	1.75	8.17

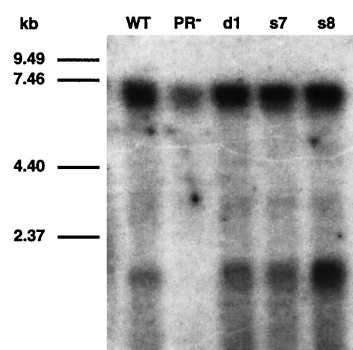
^a Data from Fig. 9.

^b Data from Fig. 8A.

^c Data from Fig. 5.

^d WT, wild type.

A



B

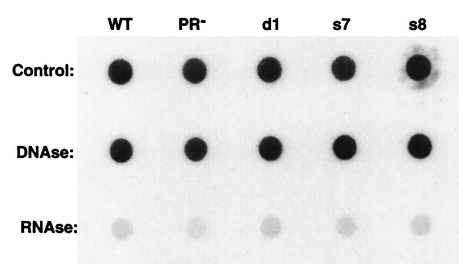


FIG. 7. (A) RNA blot of VLP-extracted nucleic acids. The amount of VLP sample used was normalized via anti-Gag immunoblots. The nucleic acids were separated on a 1% agarose-formaldehyde gel, transferred to a GeneScreen membrane, and probed as for Fig. 4. (B) Dot blot of VLP-extracted nucleic acids. Nucleic acids from the same extraction were divided into three pools and treated with DNase or RNase A or were not treated and were probed as described above. WT, wild type.

taken as further evidence that the PR^{dscd} mutants, although affecting the PR molecule, do not behave as simple PR active-site mutants.

Various cellular messages have been detected in Ty1 VLPs (J. F. Lawler, Jr., and J. D. Boeke, unpublished data; 40). The unexpected absence of the 1.7-kb *neo* band in the PR⁻ lane might suggest some reduction in this mutant's ability to non-specifically package certain non-Ty1 mRNAs (14, 40, 41). These *neo* transcripts are sensitive to RNase A treatment. Dot blots of nucleic acids extracted from VLPs also show that most of the *neo*-hybridizing material is sensitive to RNase A treatment but not DNase treatment (Fig. 7b).

A specific defect in reverse transcription. The observation that the PR^{dscd} mutant VLPs package normal amounts of Ty1 mRNA implies a defect during the reverse transcription process. The first step in the reverse transcription process is minus-strand strong-stop cDNA synthesis. In this step, tRNA_{Met}ⁱ is bound to the primer binding site within Ty1 mRNA and serves to prime reverse transcription. It has recently been demonstrated, using T7 transcripts, that the primer tRNA-Ty1 RNA annealing process can be facilitated in vitro by a basic domain in the carboxy terminus of CA (8). The general reverse transcription defect that we observed in our mutants could, in theory, be due to a specific defect in minus-strand strong-stop cDNA synthesis or in its transfer. We designed an experiment to distinguish between these two possibilities.

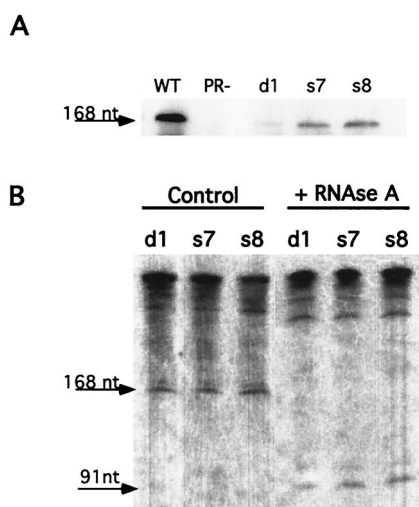


FIG. 8. (A) DNA blot of VLP-extracted nucleic acids. This blot was transferred to a GeneScreen membrane and subsequently probed with a random-primed ^{32}P -labeled probe corresponding to minus-strand strong-stop cDNA. These samples were normalized to the Gag content of the VLPs. WT, wild type. (B) Blot of VLP-extracted nucleic acids that were either treated with RNase A or not treated. The corresponding shift in electrophoretic mobility is consistent with the presence of a covalently bound $\text{tRNA}_{\text{Met}}^{\text{i}}$. These samples were normalized to A_{260} .

Ty1 RT synthesizes a cDNA strand complementary to the first 91 nt of the Ty1 mRNA (7). This DNA/RNA hybrid is digested by a Ty1-encoded RNase H activity, which permits the minus-strand strong-stop cDNA to be transferred to the 3' LTR of a Ty1 message and continue minus-strand synthesis (28, 29). Various reverse transcription intermediates have been detected in Ty1 VLPs (20, 32).

VLPs from wild-type, PR^- , and PR^{dsd} mutant cells were purified and assayed for minus-strand strong-stop cDNA. As shown in Fig. 8A, when equal amounts of VLPs were analyzed, there was significantly more minus-strand strong-stop cDNA in wild-type particles than in the PR^{dsd} mutant particles. These results were quantified (Table 4).

As expected from the transposition frequency, the *d1* mutant had the "tightest" phenotype in terms of amount of strong-stop DNA. Interestingly, PR^- VLPs contained no detectable minus-strand strong-stop cDNA, consistent with their inability to incorporate label in endogenous reverse transcription reactions (41). This observation extends the recent finding that PR^- VLPs do not possess dimeric RNA whereas wild-type VLPs do and suggests that the dimers might be required for efficient primer tRNA packaging and strong-stop DNA synthesis (14). The initial minus-strand strong-stop cDNA product contains a covalently bound $\text{tRNA}_{\text{Met}}^{\text{i}}$ primer that is removed at a later step in the reverse transcription process. Relatively little of this product exists within PR^{dsd} mutant VLPs. The product detected in both wild-type and mutant particles is 168 nt long and can be shifted to 91 nt long following treatment with RNase A. Thus no qualitative difference between the strong-stop DNAs produced in wild-type and mutant VLPs was detected (Fig. 8B).

Thus there is a clear defect in reverse transcript abundance in PR^{dsd} mutants. However, a formal possibility is that the PR^{dsd} mutant particles do not package sufficient primer tRNA.

To explore this possibility, we performed RNA blotting to quantitate the amount of $\text{tRNA}_{\text{Met}}^{\text{i}}$ present in the wild-type and mutant particles. Wild-type particles contain about twofold more $\text{tRNA}_{\text{Met}}^{\text{i}}$ than PR^{dsd} mutant particles (Fig. 9; Table 4). The PR^- mutant has even less $\text{tRNA}_{\text{Met}}^{\text{i}}$ than the PR^{dsd} mutants. However, it also packages less Ty1 mRNA. Indeed, a comparison of the signal intensities shows that there is twofold more $\text{tRNA}_{\text{Met}}^{\text{i}}$ signal in the wild-type particles than in the PR^{dsd} mutants. Unlike what is observed in minus-strand strong-stop synthesis, where the *d1* mutant phenotype is the most severe and the *s8* mutant is the least severe, there is no apparent difference in $\text{tRNA}_{\text{Met}}^{\text{i}}$ levels between the various PR^{dsd} mutants.

DISCUSSION

Genome organization in retroviruses and retrotransposons is evolutionarily conserved and NC (identified by its Zn knuckle motif) is always found at the carboxy terminus of the Gag protein. We have previously shown that a function essential for successful Ty1 replication is contained within the region common to the C terminus of Gag, PR, and the p4 peptide. This region, which we refer to as the p4 region, was analyzed via a series of block substitution mutations to determine whether an essential Ty1 NC functionality resides there. Mutations in the carboxy terminal portion of this region block transposition, whereas mutations residing in the amino-terminal portion do not. We have previously demonstrated, through the use of a frameshift transplant technique, that these mutations cause a transposition defect via an effect they exert on the Ty1 PR (21).

The most trivial explanation for the transposition defect seen in these mutants is that they inactivate the Ty1 PR, which is required for transposition. However, we are unable to detect any alterations in proteolytic processing in the mutant elements. Samples harvested at various points after the induction of transposition revealed no differences in the kinetics of processing. Samples harvested after 24 h of induction, a time point by which Ty1 protein levels have likely reached equilibrium, indicate no defect in the extent of processing.

Ty1 elements containing mutations in or near the Gag-PR cleavage site have been described (27). One of these mutants, s3.4, contains an isoleucine substitution at position +1 relative

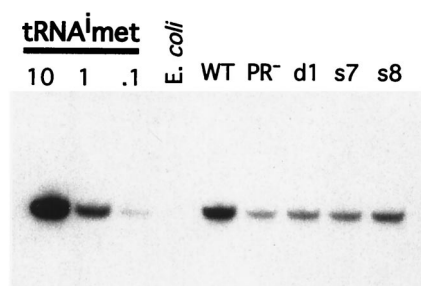


FIG. 9. Mutant elements package $\text{tRNA}_{\text{Met}}^{\text{i}}$. Total nucleic acids were prepared from equal amounts of Ty1 VLPs and separated on a 10% polyacrylamide-urea gel. The amount of VLP sample used was normalized via anti-Gag immunoblots. A standard curve of purified yeast $\text{tRNA}_{\text{Met}}^{\text{i}}$ was also resolved on this gel. The gel was exposed overnight with an intensifying screen.

to the His-Asn scissile bond and exhibits partially impaired processing of Gag but transposes with only a very modest reduction in efficiency. The existence of this class of mutant implies that, even if a kinetic defect in PR activity that is below our threshold of detection exists, it is unlikely to impair transposition.

The PR^{d_{sd}} mutants are partially complemented by endogenous elements whereas mutations in or near the PR active site are not. We therefore conclude that this essential "activity" of PR is distinct and separable from its proteolytic activity.

The reduced size of PR^{d_{sd}} mutant particles that was previously reported and confirmed in this study may suggest a defect in VLP maturation (27). It is clear from comparisons between wild-type, PR^{d_{sd}} mutant, and RT⁻ particles that the synthesis of full-length Ty1 cDNA is not required for normal particle morphology.

PR^{d_{sd}} mutant elements have a significant defect in synthesis of cDNA but package Ty1 mRNA in normal quantities, pointing to defects in reverse transcription. This is another aspect of the PR^{d_{sd}} mutants that distinguishes them from PR⁻ mutants, which package far less Ty1 mRNA than wild-type elements. An analysis of reverse transcription intermediates indicates that these mutants fail to synthesize normal amounts of cDNA products despite the presence of nearly normal amounts of primer tRNA^{i_{Met}}. The *d1* mutant appears to be defective in initiating reverse transcription, whereas *s7* and *s8* appear defective in transfer or extension of the strong-stop DNA (Table 4).

There are a number of potential explanations consistent with these data. First, it has recently been shown that Ty1 mRNA is dimeric in wild-type particles and monomeric in PR⁻ particles (14). It is possible that the Ty1 mRNA in PR^{d_{sd}} mutant particles, although normal in abundance, remains monomeric despite encoding a functional PR. Monomeric Ty1 mRNA may not be a suitable substrate for Ty1 RT or may not anneal properly with its primer tRNA^{i_{Met}}.

This explanation raises the larger question of why this might be so. One explanation is that normal particle morphology may be required for proper mRNA folding and primer tRNA^{i_{Met}} annealing in vivo. Experiments designed to test this hypothesis were attempted by supplying PR^{d_{sd}} mutant VLPs with an exogenous oligonucleotide to prime reverse transcription (28, 29). We were never able to detect synthesis of minus-strand strong-stop cDNA by PR^{d_{sd}} mutant elements in these "in viro" assays (data not shown).

It has recently been shown that a 103-amino-acid carboxy-terminal fragment of Ty1 CA from amino acids 299 to 401 possesses primer tRNA annealing activity and can initiate the Ty1 reverse transcription reaction in vitro (8). It is possible that the PR^{d_{sd}} mutations, by altering either the kinetics or spatial parameters of particle assembly, prevent this part of the protein from accessing its substrates. This hypothesis is consistent with the observation that PR^{d_{sd}} mutants are partially complemented in *trans*. Alternatively, normal particle morphology may be required for Ty1 RT to be appropriately positioned. In the absence of appropriate positioning, Ty1 RT may be unable to extend an existing primer template. Previous work (27) has shown, and this work confirms, that PR^{d_{sd}} mutant VLPs are smaller than wild-type VLPs.

The observation that an NC function is contained within a

viral PR is unprecedented, although not for retroelements (24, 33). Some plant viruses, including potyviruses, also encode NC functions within their PR gene open reading frame (34). In one case, it has been shown that this NC functionality contained within the PR is essential for systemic movement of viral RNA (36). The inclusion of an NC functionality in the context of an active PR is likely a genome-streamlining strategy that enables the virus and element to maintain a smaller genome. It is not clear whether the NC function assigned to the p4 region in this work is direct or indirect.

In conclusion, we have shown that the N terminus of the Ty1 PR plays an essential role in facilitating reverse transcription. This activity is distinct and separable from its proteolytic activity and can be partially complemented in *trans*.

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