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The Retrotransposon Tf1 Assembles Virus-Like Particles That Contain Excess Gag Relative to Integrase because of a Regulated Degradation Process

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The retrotransposon Tf1, isolated from *Schizosaccharomyces pombe*, contains a single open reading frame with sequences encoding Gag, protease, reverse transcriptase, and integrase (IN). Tf1 has previously been shown to possess significant transposition activity. Although Tf1 proteins do assemble into virus-like particles, the assembly does not require readthrough of a translational reading frame shift or stop codon, common mechanisms used by retroelements to express Gag in molar excess of the polymerase proteins. This study was designed to determine if Tf1 particles contain equal amounts of Gag and polymerase proteins or whether they contain the typical molar excess of Gag. After using two separate methods to calibrate the strength of our antibodies, we found that both *S. pombe* extracts and partially purified Tf1 particles contained a 26-fold molar excess of Gag relative to IN. Knowing that Gag and IN are derived from the same Tf1 primary translation product, we concluded that the excess Gag most likely resulted from specific degradation of IN. We obtained evidence of regulated IN degradation in comparisons of Tf1 protein extracted from log-phase cells and that extracted from stationary-phase cells. The log-phase cells contained equal molar amounts of Gag and IN, whereas cells approaching stationary phase rapidly degraded IN, leaving an excess of Gag. Analysis of the reverse transcripts indicated that the bulk of reverse transcription occurred within the particles that possess a molar excess of Gag.

The propagation of retroviruses and long terminal repeat (LTR)-containing retrotransposons requires the assembly of virus or virus-like particles (VLPs), which act as sites of reverse transcription and, in the case of retroviruses, serve as vectors of infection. The formation of particles depends on high levels of Gag expression to provide large amounts of capsid and nuclear capsid proteins relative to other particle components. Retroviruses, retrotransposons, and double-stranded RNA viruses express 10- to 50-fold more Gag than polymerase (Pol) protein as the result of inefficient translational reading frame shifts that occur between the Gag and Pol coding sequences (2, 6, 10, 14, 15). Other mechanisms for expressing a molar excess of Gag include the use of an inefficiently suppressed stop codon between Gag and Pol, as is the case for murine leukemia virus (12). These levels of Gag protein expression are consistent with the higher levels of Gag then of reverse transcriptase (RT) protein found in viral particles (31, 34). In the case of human immunodeficiency virus type 1, Ty1, and L-A, the relative excess of Gag protein expression has been shown to be required for normal particle assembly (7, 18, 19, 21, 37). Because the great majority of functional LTR-containing retroelements possess a mechanism to express high ratios of Gag to Pol, it is thought that all these elements require excess levels of Gag to allow for particle formation. In this regard, the LTR-containing retrotransposon Tf1 isolated from *Schizosaccharomyces pombe* poses an interesting problem.

Quantitative measurements of Tf1 activity indicate that as many as 20% of the cells in a culture suffer transposition events (26, 34a). Despite its high level of transposition activity, Tf1 ostensibly lacks a mechanism for overexpression of Gag. All of the coding sequences for Tf1 proteins are contained within a single open reading frame (ORF) and thus lack an internal reading frame shift and a suppressible stop codon (27). Using a version of the Tf1 ORF with an epitope tag fused at the N terminus of Gag, we showed that all Tf1 proteins are synthesized within a single primary translation product (28). This finding suggests that no transcriptional or translational mechanism provides independent expression of Tf1 Gag as a primary translation product. Even though Gag and Pol proteins appear to be synthesized in equal amounts, Tf1 VLPs have been detected in sucrose gradients and are typical of particles produced by other retrotransposons with respect to their rapid sedimentation velocity (28). One possible resolution of this paradox would be if Tf1 VLPs contain equal amounts of Gag and Pol proteins. Presumably, this would lead to a particle structure very different from that adopted by other LTR-containing retroelements which synthesize an excess of Gag. This novel type of particle might require high levels of RT and integrase (IN) because of unusually low specific activities for these enzymes, as is the case for avian protease (PR) (23). Alternatively, Tf1 particles may contain typical ratios of Gag to Pol protein but instead utilize a posttranslational mechanism to degrade the excess Pol products. In addition, Tf1 proteins might assemble into particles containing excess Gag despite the presence of equivalent amounts of Gag and Pol proteins.

In the experiments described here, we first identify a new mutation in Tf1 PR that resulted in the accumulation of the full-length primary translation product as the sole Tf1 protein. This finding supports our earlier conclusion that Tf1 synthesizes all of its proteins in a single primary product. We then used pulse-chase labeling and immunoprecipitation of Tf1 proteins to characterize the order of the PR cleavage events that result in the mature Tf1 proteins. To address the question of how these proteins assemble into VLPs, quantitative immunobloting was used to measure the levels of Tf1 Gag and IN

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TABLE 1. Yeast strains used

Strain	Genotype	Source or reference	Plasmid description
YHL912	*h⁻ ura4-294 leu1-32*	J. Boeke, 21X5A	
YHL1032	YHL912/pFL20	28	Vector
YHL1051	YHL912/pHL411-62	28	*nmt1*-Tf1
YHL1836	YHL912/pHL490-80	This report	*nmt1*-Tf1-*neoAI* with PR frameshift
YHL1282	YHL912/pHL449-1	25	Wild-type *nmt1*-Tf1-*neoAI*
YHL3128	YHL912/pHL857-1	This report	*nmt1*-Tf1-*neoAI* with point mutation in PR

proteins in *S. pombe* extracts as well as in Tf1 VLPs. We found that Tf1 particles did contain a large excess of Gag protein and that a posttranslational mechanism was responsible for degradation of the majority of the IN protein. In addition, we observed that the bulk of Tf1 reverse transcripts were produced after the IN was degraded.

MATERIALS AND METHODS

The *S. pombe* minimal liquid and plate media were composed of EMM (30). Selective plates contained EMM and 2 g of dropout mix, which contained all amino acids, adenine, and uracil except for the nutrient that was absent for selection, per liter. A final concentration of 10 μM vitamin B_1 (thiamine) was added to EMM plates when indicated.

Strains and plasmids. The yeast strains and plasmids used are described in Table 1. Strain YHL3128 contains pHL857-1, which is the same as pHL449-1 (*nmt1*-Tf1-*neoAI*) except it contained a point mutation in PR. pHL587, the original plasmid with the PR mutation, was isolated as the result of a large screen of hydroxylamine-mutagenized pHL449-1 plasmids for those that showed transposition defects (34a). The *Avr*II-*Bsr*GI restriction fragment that was sequenced and found to contain the PR point mutation was replaced with the large *Avr*II-*Bsr*GI fragment of pHL449-1 to produce pHL857-1. YHL1836 contained pHL490-80, a plasmid identical to pHL449-1 except for a frameshift mutation at the *Sac*I site located in PR. pHL490-80 was made in a three-piece ligation that contained the *Xho*I-*Bam*HI vector fragment of pHL414-2 (28), the *neoAI*-containing *Apa*I-*Bam*HI fragment of pHL449-1, and the *Xho*I-*Apa*I fragment of pHL415-2 that included the PR frameshift (28).

Immunoprecipitations. The protocol used was adopted from a previously published version (22). Five-milliliter cultures of YHL1032 and YHL1051 were grown to mid-log phase, and 2.5 mCi of Trans label ([^{35}S]methionine and [^{35}S]cysteine; ICN) was added to each. After 20 min, 1.0 ml of cells for the first time point was harvested, and unlabeled methionine and cysteine were added to a final concentration of 2 mM. At each time point, 1.0 ml of cells were removed from the cultures and incubated for 20 min on ice with 100 μl of 50% trichloroacetic acid. The cells were then pelleted, washed with cold acetone, and dried. These cells were resuspended in 50 μl of boiling buffer (50 mM Tris-Cl [pH 7.5], 1 mM EDTA, 1% sodium dodecyl sulfate [SDS]), and glass beads were added to the meniscus. The mixtures were vortexed for 1 min and boiled for 4 min. Then 800 μl of IP (immunoprecipitation) buffer (50 mM Tris-Cl [pH 7.5], 150 mM NaCl, 0.5% Tween 20, 0.1 mM EDTA) was added to the boiled cells, and this mixture was spun for 10 min in a microcentrifuge. Supernatant containing 10^7 cpm was mixed with 1 μl of anti-Gag antiserum (anti-CA660-2) and with 1 μl of anti-IN antiserum (anti-IN655-3). After incubations for 1 h, the solutions were spun for 10 min and the supernatants were agitated for 1 h on ice with 70 μl of protein A-Sepharose beads (CL-4B; Pharmacia). The beads were then washed twice with IP buffer, twice with IP urea buffer (100 mM Tris-Cl [pH 7.5], 200 mM NaCl, 0.5% Tween 20, 2 M urea), once with 1% β-mercaptoethanol, and once with 0.1% SDS. The beads were ultimately resuspended in 40 μl of sample buffer and boiled for 3 min in preparation for loading onto an SDS-polyacrylamide gel. After the gels were run, they were treated with Amplify (Amersham) and dried before being exposed to film.

Protein preparations. Crude protein extracts were made from 5-ml cultures grown without thiamine in EMM minus uracil dropout medium. The cells were washed first with water and then with extraction buffer consisting of 15 mM KCl, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-KOH (pH 7.8), and 5 mM EDTA. The cells were then resuspended in 0.4 ml of extraction buffer plus 5 mM dithiothreitol and 2 mM phenylmethylsulfonyl fluoride. Acid-washed glass beads (0.4 mm in diameter) were added to the meniscus, and the sample was vortexed for 5 min; 0.1 ml of extraction buffer was mixed into the extract, and the supernatant was removed. An equal volume of 2× sample buffer was added to the supernatant, and the mixture was boiled in preparation for SDS-gel electrophoresis.

The preparations of large-scale yeast extracts and the subsequent analysis on sucrose gradients were based on previously published protocols (8, 9, 13). Five hundred milliliters of EMM–Leu (250 μg/ml) medium (32) was inoculated at an optical density at 600 nm (OD_{600}) of 0.05 and grown at 32°C for 2 days to reach stationary phase. The cells were harvested, washed, and broken as previously described (8, 9, 13) except that higher (>90%) efficiency of *S. pombe* cell breakage required that the cell suspension be vortexed with the glass beads in a 25-mm glass test tube for about 20 min. Beads loaded to just above the meniscus greatly increased the efficiency of breakage and recovery of VLPs. Then 2.5 ml of harvested cells was resuspended in 5 ml of buffer B-EDTA (15 mM KCl, 10 mM HEPES-KOH [pH 7.8], 5 mM EDTA) containing 3 mM dithiothreitol and 2 mM phenylmethylsulfonyl fluoride. Five milliliters of supernatant recovered from a 3,000-rpm spin (5 min) of the cell extract was loaded onto a 20 to 70% linear gradient of sucrose in buffer B-EDTA. The gradients were spun for 24 h at 25,000 rpm in a Beckman SW28 rotor. Fractions of 1.2 ml were collected, and 100 μl from each was precipitated in 10% trichloroacetic acid. The pellets were washed in cold acetone, resuspended in sample buffer, and loaded onto SDS-polyacrylamide gels for immunoblot analysis.

The protein preparations used to produce the material for the immunoblots were loaded onto an SDS–10% polyacrylamide gel with equal amounts of total Coomassie blue-staining material in each lane. Standard electrotransfer techniques were used (35), with Immobilon-P (Millipore) as the membrane. The detection method used was the ECL (enhanced chemiluminescence) system as described by the manufacturer (Amersham) except that the secondary antibody, horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin, was used at a dilution of 10,000-fold. The primary polyclonal antisera used for each filter were from bleeds 660-2 (anti-Gag) and 655-3 (anti-IN) (28).

Gag and IN expression. Tf1 Gag and IN were expressed in *Escherichia coli* BL21 from pET-15b (Novagen) vectors that contained inserts of either Gag or IN coding sequence. The C terminus of Gag and the N terminus of IN in these expression clones were approximated on the basis of the molecular weights of the yeast-derived proteins and the published protease cleavage sites of a closely related Gypsy family retrotransposon, Ty3 (20). PCR fragments containing the Gag and IN sequences were ligated into pET-15b. The Gag expressed from the pET-15b vector had six histidines and the thrombin site added to the N terminus and had His-Leu-Lys attached to Tyr-235 at the C terminus. The sequence encoding the N terminus of Gag was ligated into the *Bam*HI site of pET-15b, using a *Sal*I site included in the 5′ PCR primer HL48 (5′TCATTAGTCGACA TGAAAAACTCATCACAGAAA3′). The sequence encoding the C terminus also contained a *Sal*I site that was incorporated in the 3′ PCR primer HL49 (5′CACTAGCGTCGACTTAAATTTTCAGATGGTATTCGA3′). The IN expressed from pET-15b had six histidines with the thrombin site attached to Thr-854 at the N terminus and ended with the native C terminus of the Tf1 ORF. The PCR fragment that contained the IN sequence was flanked with *Sal*I sites that were ligated into the *Bam*HI site of pET-15b. The 5′ PCR primer was HL50 (5′AATCTAGTCGACACAGATGATTTTAAAAACCAAG3′), and the 3′ PCR primer was HL51 (5′ATTCATGTCGACGTTCTCAGATATTTAGAT TATTG3′).

BL21 cells containing the pET-15b Gag or IN expression vector and the resulting extracts were treated as suggested by the supplier (Novagen) throughout the purification of the two proteins. Cultures of 100 ml were grown to an OD_{600} of 0.4 and induced with 1.0 mM isopropylthiogalactopyranoside (IPTG) for 3 h at 37°C. These cells were then harvested and resuspended in 40 ml of binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl [pH 7.9]). The cells were lysed, and the DNA was broken by sonication. The extracts were spun at 39,000 × *g* for 20 min, and the pellets were resuspended in 5 ml of binding buffer that contained 6 M urea. The six-histidine-containing Gag and IN were bound to 10 ml of prepared His-Bind resin in batch in a tube on a lab shaker at room temperature for 45 min. After the beads were pelleted, the bound protein was washed with 15 ml of wash buffer (20 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl [pH 7.9], 6 M urea) and eluted with 13 ml of 300 mM imidazole solution that contained 6 M urea.

cDNA preparations and blots. About 10^9 cells (100 OD_{600} units) from the culture used for the protein samples in Fig. 7B were harvested for each time point in Fig. 9A. The cells were resuspended in a glass tube (13 by 100 mm) with 200 μl of EB (0.5 M NaCl, 0.2 M Tris-Cl [pH 7.5], 10 mM EDTA, 1% SDS), and 200 μl of PICA (1:1 mixture of EB equilibrated phenol and chloroform that contained 1/24 isoamyl alcohol) was added to enough acid-washed glass beads to fill past the miniscus. The mixture was vortexed vigorously for 30 min in a multivortexer (Baxter Scientific Products), and an additional 400 μl each of EB and PICA was added before a final 30-s vortex. The sample was centrifuged, the supernatant was ethanol precipitated, and the pellet was resuspended in 50 μl of

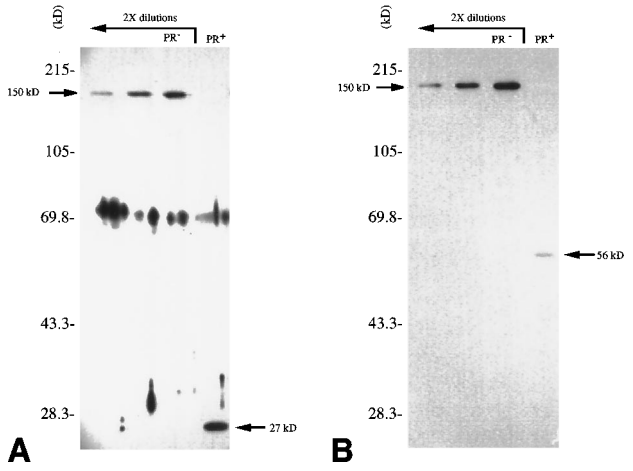

FIG. 1. Immunoblots of extracts made from *S. pombe* expressing wild-type Tf1 or Tf1 with a PR mutation. (A) The immunoblot contained material from cells that expressed Tf1 with a PR mutation (YHL3128). Lanes marked PR^- have twofold dilutions of this protein. The PR^+ lane has material from a strain that expressed wild-type Tf1 (YHL1282). The blot was probed with the anti-Gag antiserum. The 150-kDa band is the primary Tf1 translation product, and the 27-kDa band is mature Gag. The positions of molecular mass markers are indicated. The diffused signal at approximately 70 kDa was a background band that is seen with the anti-Gag antiserum and is seen in all lanes, including those containing cells not expressing Tf1. (B) Samples used for panel A were loaded, and the filter was probed with the anti-IN antiserum. The 150-kDa band was the primary Tf1 translation product, and the 56-kDa band was mature IN.

Tris-EDTA. Five microliters of this mixture was restriction digested and subjected to agarose gel electrophoresis for DNA blot analysis. After transfer, the filters were hybridized with a 1-kb *neo* probe derived from a *Bam*HI digest of pGH54 (17).

RESULTS

Identification of the single Tf1 primary translation product. The expression of Tf1 protein as a single primary translation product is unusual and suggests that the particle structure or the assembly may be different from that of other retrotransposons and retroviruses. A fusion of the Tf1 ORF to the fructose bisphosphatase promoter and mRNA leader produced a single translation product that was observed on immunoblots when PR was inactivated by the deletion of three amino acids in the PR active site (28). Although this previous experiment supported the sequence-derived prediction that Tf1 contains only one ORF, (28), the importance of this question to particle structure led us to seek independent confirmation of this conclusion.

To determine if the PR deletion affected only PR activity and not also an unidentified translational terminator, we sought a different PR mutant. Here we have independently identified and sequenced a mutant of Tf1 that contains a single base change near the PR active site that resulted in the lack of Tf1 protein processing. The *Avr*II-*Bsr*GI restriction fragment of pHL857-1 that contained the mutation was sequenced, and the defect in protein processing was found to be the conversion in PR of glutamic acid 249 to a lysine. Strain YHL3128 contained plasmid pHL857-1 with the mutant Tf1 expressed from the *nmt1* promoter with the natural Tf1 mRNA leader. We expressed this PR^- mutant and analyzed the resulting yeast extract on immunoblots that were probed with either an anti-Gag (Fig. 1A) or an anti-IN (Fig. 1B) antiserum. The single 150-kDa band in both panels indicated that in the context of the natural Tf1 transcript and a single base change, Tf1 protein was expressed from within a single primary translation product. The translation of wild-type Tf1 transcript resulted in the accumulation of the 27-kDa Gag and the 56-kDa IN.

Processing events resulting in mature Tf1 protein. To characterize the processing events that result in the cleavage of the primary translation product into the mature Tf1 proteins, we used antisera raised against Gag and IN to immunoprecipitate Tf1 proteins from cells that were pulse-labeled with [^{35}S]methionine and [^{35}S]cysteine. Extracts were made from cells that contained a plasmid version of Tf1 expressed from the *nmt1* promoter (YHL1051) and from cells that contained the vector without Tf1 (YHL1032). Figure 2A is an autoradiogram of an SDS-polyacrylamide gel of denatured Tf1 protein immunoprecipitated with the anti-IN antiserum from extracts of expressing and nonexpressing log-phase cells. Twenty minutes after labeled methionine and cysteine were added to the cultures, unlabeled methionine and cysteine were added and, simulta-

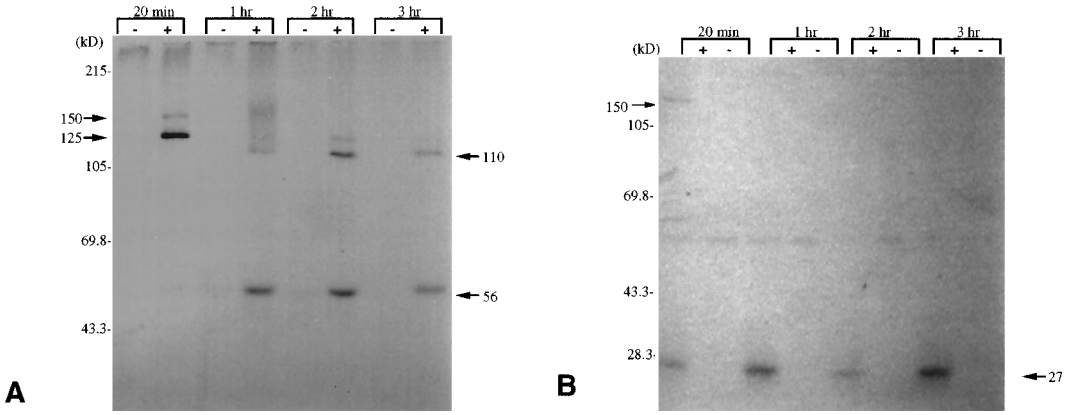

FIG. 2. Immunoprecipitations of Tf1 proteins from pulse-chased cultures. (A) SDS-polyacrylamide gel containing radioactive material from immunoprecipitations. Log-phase *S. pombe* cultures were labeled with [^{35}S]methionine and [^{35}S]cysteine. After 20 min, unlabeled methionine and cysteine were added, and cells were harvested at various times afterwards. The cells were lysed, and the anti-IN antiserum was used to precipitate any Tf1 protein products containing IN. The times above the lanes refer to the lengths of time after label was added to the cultures that the cells were harvested. +, lanes containing extract from Tf1-expressing cells (YHL1051); −, lanes with material from cells not expressing Tf1 (YHL1032). The arrows indicate the molecular masses of precipitated Tf1 protein, and the positions of molecular mass markers are shown. (B) SDS-polyacrylamide gel of immunoprecipitates from the samples used for panel A except that the anti-Gag antiserum was used. The arrow marks the position and size of the mature Gag protein. The varying intensity of the Gag band was the result of sample loss. We therefore drew no conclusions from the strength of these signals.

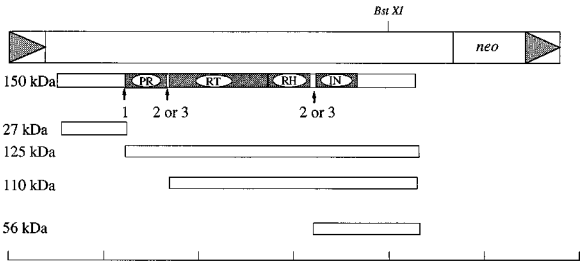

FIG. 3. Processing model for the order of cleavages that results in mature Tf1 protein. The largest rectangle represents the Tf1 element, and significant restriction sites are shown. Triangles indicate the LTRs. The narrower rectangles indicate the sizes of Tf1 proteins, and their approximate molecular masses are shown on the left. The shaded rectangles depict the positions of the PR, RT, RNase H (RH), and IN domains. The arrows labeled 1, 2, and 3 indicate the order of the cleavages that resulted in the mature Tf1 proteins. The line at the bottom is a scale that delineates 1-kb intervals.

neously, cells were removed for the first time point. Successive samples were removed at various times. The anti-IN antiserum precipitated a 150-kDa primary product and a 125-kDa species. The latter most likely represented the primary product minus the Gag domain, since the 125-kDa product was not detected by the anti-Gag antiserum (Fig. 2B). From the 20-min sample, the anti-Gag antiserum precipitated the 27-kDa Gag protein and a barely detectable amount of the 150-kDa primary translation product, indicating that the Gag is removed from the primary product within the first 20 min (Fig. 2B). As cells were allowed more time to process Tf1 protein in the presence of unlabeled methionine and cysteine, the 150-kDa primary product and 125-kDa protein precipitated by the anti-IN serum were chased first into a 110-kDa protein and then into the mature 56-kDa IN protein. The size of the 110-kDa species and its reactivity with only the anti-IN antiserum suggested that it represented a processing intermediate composed of the RT and IN domains. When the same extracts were precipitated with anti-Gag antiserum, the minor band of primary translation product chased directly into the Gag species without the production of any visible intermediates (Fig. 2B). The 150-, 125-, 56-, and 27-kDa bands seen in the YHL1051 lanes were not produced by the nonexpressing control strain YHL1032, indicating that they are Tf1-specific proteins. The data in Fig. 2 indicated that the processing of the Tf1 polypeptide is ordered as depicted in Fig. 3, with the first cleavage occurring between Gag and PR. The 110-kDa RT-IN protein is a prominent IN-containing species in the 2-h lane (Fig. 2A), and it indicates that a large fraction of the 125-kDa protein suffered a second cleavage between PR and RT. Because our antisera could not detect a PR-RT protein, we could not rule out the possibility that as much as half of the 125-kDa protein received a second cleavage between RT and IN. Therefore, the model indicated in Fig. 3 shows that cleavages between PR and RT and between RT and IN could occur in either order.

Quantification of Gag-to-IN ratios. The results presented above indicated that all Tf1 proteins are derived from a single primary translation product that is subsequently processed into the components of Tf1 particles. This represents a significant departure from the expression strategies of other LTR-containing retrotransposons and retroviruses. The lack of a reading frame shift or stop codon separating the Tf1 Gag from the Pol coding sequences resulted in the synthesis of equal molar amounts of Gag and Pol, whereas other retroelements known to form particles produce 10- to 50-fold more Gag than Pol. We decided to measure the ratio of Gag to IN in Tf1 particles because of the seeming paradox that Tf1 synthesizes equal amounts of Gag and Pol proteins but nevertheless produces VLPs.

We measured the relative amounts of Gag and IN by using quantitative immunoblots calibrated with known amounts of antigen. We have calibrated the antibody reactivities by using *S. pombe* extracts and protein purified from bacteria as the source of Gag and IN antigens. Since the Tf1 primary translation product contained equal amounts of Gag and IN by definition, our first source of antigen was an *S. pombe* extract from the PR-defective Tf1 strain YHL3128, for which we compared the signal intensities on immunoblots produced by anti-Gag and anti-IN antisera. Figure 1 shows two identical immunoblot filters with twofold serial dilutions of the same extract probed with either the anti-Gag antiserum (Fig. 1A) or the anti-IN antiserum (Fig. 1B). Densitometric analysis of this figure revealed that the signal intensities on the autoradiogram increased linearly with the amount of extract and that the anti-IN antiserum produced 1.6 times more signal per polypeptide than did the anti-Gag antiserum.

One potential problem with this antiserum calibration was that the Gag and IN epitopes in the primary translation product might have differed structurally from the antibody binding sites in the mature Gag and IN proteins that we ultimately wished to compare. To address this issue, we decided to independently calibrate the antisera with known amounts of purified, mature-sized Tf1 Gag and IN. We expressed the Tf1 proteins in bacteria by using the six-histidine vector pET15b and purified Gag and IN with the nickel column technique. The exact positions of the Tf1 protease cleavage sites at the C terminus of Gag and the N terminus of IN are unknown. However, the positions of the cleavage sites in the closely related element Ty3 are known and could be aligned with the Tf1 sequence to predict with reasonable accuracy the cut sites in the Tf1 proteins (20). Figure 4A is a Coomassie blue-stained gel of purified Tf1 Gag and IN shown in separate lanes and also in a series of lanes containing equal amounts of Gag with 1.3-fold dilutions of IN. As seen in the Gag lane, there was only a single purified protein species in the Gag preparation, while the IN lane showed a few impurities. Because Coomassie blue staining is proportional to the total number of arginine and lysine amino acids, and there are only 8% fewer of these amino acids in Gag compared with IN, we reasoned that the band intensities of these proteins would provide a good estimate of their quantities in the gel (5). Lane 2 represents the dilution that most closely approximates equal amounts of Gag and IN Coomassie blue staining. Figure 4B is an immunoblot of the same samples probed simultaneously with the anti-Gag and anti-IN antisera. Lane 6 represented the sample that came closest to producing equal levels of immunoreactive signals for Gag and IN. By comparing the results in Fig. 4A and B, we estimate that the anti-IN antiserum was 2.9-fold more active than the Gag antiserum with these purified proteins. The difference between the calibrations using purified protein and yeast extract was less than a factor of 2; for the purposes of the remaining calculations, we will use the 1.6-fold figure for the relative sensitivity of the IN antiserum compared with the Gag antiserum in order to produce the most conservative estimates of excess Gag levels. Despite the differences in the estimate of the greater sensitivity of the IN antiserum, the PR^+ lanes in Fig. 1 and another wild-type *S. pombe* extract shown in Fig. 4B both showed that Gag protein accumulated to much higher molar amounts than IN.

To determine the ratio of Gag to IN in an extract of *S. pombe* YHL1282, we exposed an immunoblot that contained serial twofold dilutions of extract so that both IN and Gag bands could be seen on the same film with intensities in the

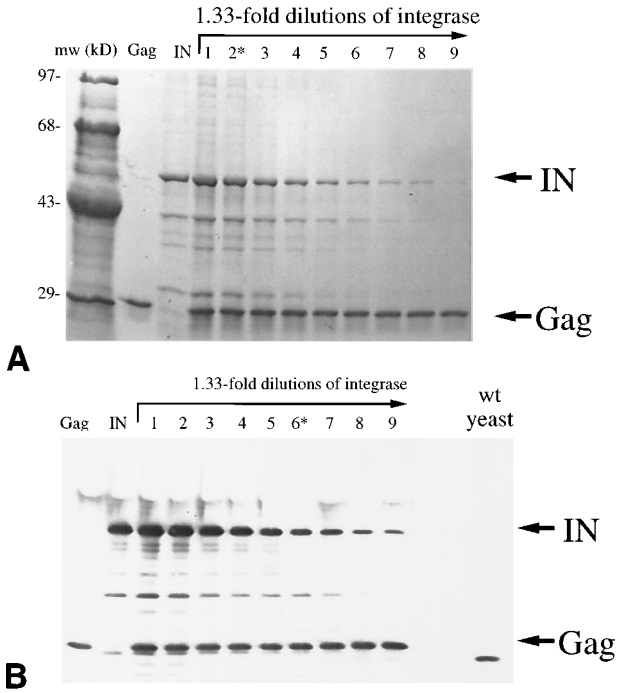

FIG. 4. Coomassie blue-stained gels of purified Gag and IN. (A) Coomassie blue-stained gel containing Tf1 Gag and IN purified from bacteria that were mixed together in various ratios. The lanes marked Gag and IN contain only the purified material indicated. The remaining lanes contain equal amounts of Gag mixed with 1.3-fold dilutions of IN. Lane 2 is indicated with an asterisk because it was the lane that came the closest to containing equal amounts of Gag and IN. The arrows marked IN and Gag show the positions of the IN and Gag proteins. The lane labeled mw contained the molecular mass markers, and their effective molecular masses are indicated. (B) The protein samples shown in panel A were loaded onto an immunoblot that was probed with the anti-Gag and anti-IN antisera. The lane marked wt yeast is an *S. pombe* extract made from a strain expressing Tf1 (YHL1282).

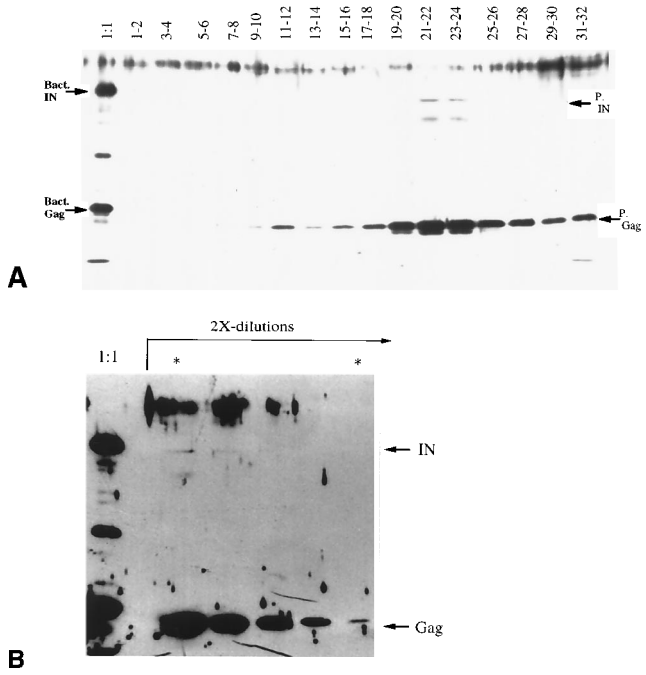

FIG. 6. (A) Immunoblot of a sucrose gradient that contained Tf1 VLPs. This blot contained material from a sucrose gradient made from an extract of YHL1282, a Tf1-expressing *S. pombe* strain. Fractions were pooled in sets of two as indicated above each lane. The lane labeled 1:1 contained purified Gag and IN mixed to produce equal-intensity bands to indicate that the IN and Gag antisera were performing quantitatively as previously observed. The bacterial (Bact.) Gag and IN bands are indicated. The positions of the Tf1 VLP Gag and IN are labeled P. Gag and P. IN, respectively. (B) Immunoblot containing protein from the sucrose fractions 21 and 22 shown in panel A. The blot was probed with both anti-Gag and anti-IN antisera. The material was serially diluted to determine how many twofold dilutions were required to create a Gag band with the same intensity as the IN band in the first lane. The lane on the right marked with the asterisk has a Gag band with the same intensity as the IN band in the lane on the left labeled with an asterisk. The lane marked with 1:1 contained the purified Gag and IN mixed in the ratio to produce equal Gag and IN signals.

linear range of the assay (Fig. 5). A 16-fold dilution was required to reduce the Gag band to an intensity similar to that of the IN band seen in the first lane. Since the anti-IN antiserum was 1.6-fold more sensitive, we calculate that there was about 26 times more Gag than IN in this extract. We also examined partially purified Tf1 VLPs from stationary-phase YHL1282 by immunoblot analysis to determine if the low amounts of IN relative to Gag in the whole cell extracts would be reflected in the particles themselves. We induced expression of Tf1 proteins and prepared whole cell extracts that were fractionated by velocity sedimentation in a sucrose gradient. Protein from the fractions was subjected to immunoblot analysis using both the anti-Gag and anti-IN antisera. Figure 6A shows the particle profile of Gag protein, which is typical of that previously reported (28). By analyzing serial dilutions of the peak fractions 21 and 22, and taking into account the 1.6-fold greater sensitivity of the IN antiserum than of the Gag antiserum (Fig. 6B), we found that the level of IN in the particles was 26-fold lower than that of Gag.

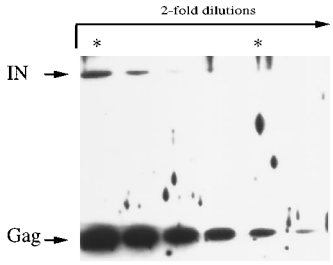

FIG. 5. Immunoblot of *S. pombe* extract serially diluted twofold. The immunoblot contained protein extracted from a Tf1-expressing strain (YHL1282). The positions of Gag and IN are indicated. The samples were diluted to determine how many twofold dilutions were required to produce a Gag band equal in intensity to the IN band in the first lane. The lanes marked with the asterisks indicate the dilutions required for this determination.

Specific degradation leads to high ratios of Gag to IN. Our findings that Tf1 proteins were synthesized in one long precursor and that whole cell extracts contained 26-fold more Gag than IN led us to predict that IN and presumably Pol were specifically degraded. In an effort to detect intermediates in this degradation process, we examined proteins at earlier times after inducing transcription of Tf1 when cells were still in log-phase growth. Figure 7A is an immunoblot of cells induced for Tf1 expression and harvested at increasing times after inoculation. Equal numbers of cells were extracted for all of these samples, and all lanes on the blot contained equal levels of total protein, as determined by Coomassie blue staining (data not shown). We found that log-phase cells contained approximately equal levels of Gag and IN until they reached an OD_{600} of 6.0, after which the amount of IN dropped precipitously to the levels characteristic of stationary-phase cells. Since all lanes in this blot contained equivalent levels of total

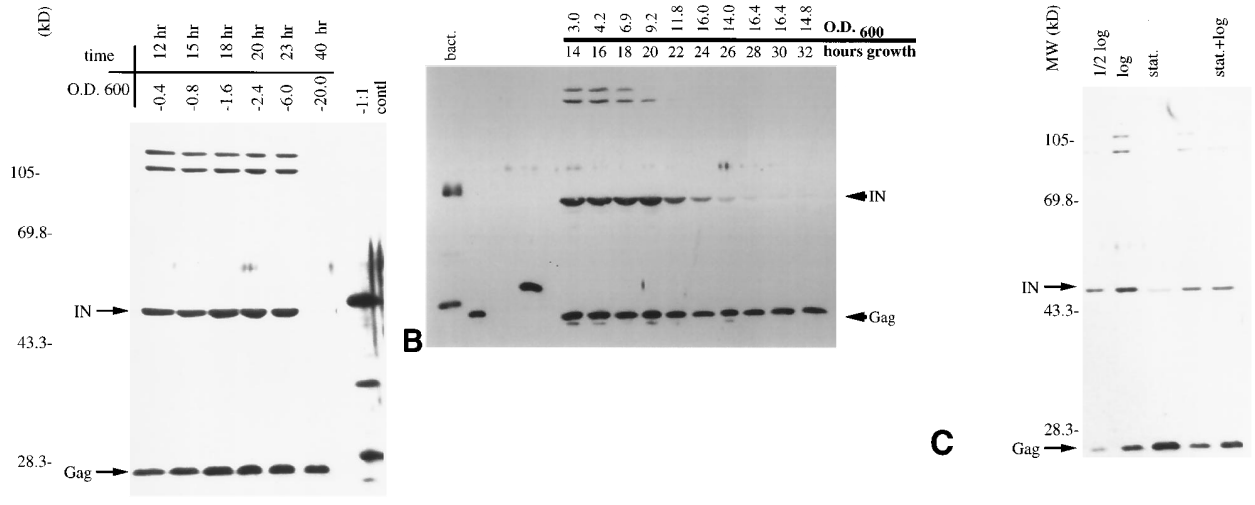

FIG. 7. Time course of Tf1 proteins from different stages of culture growth. (A) Immunoblot of *S. pombe* extracts made from cells harvested at different times of culture growth. The times and OD_{600} values are indicated above the lanes. The filter was probed with anti-Gag and anti-IN antisera. The lane marked 1:1 contl is the purified Gag and IN mixed in the ratio to produce equal Gag and IN signals. The positions of molecular mass standards are shown. The arrows labeled Gag and IN indicate the positions of the *S. pombe*-expressed Gag and IN. (B) Similar to panel A except that the protein was extract from a separate *S. pombe* YHL1282 culture that was sampled every 2 h starting from an OD_{600} of 3.0. The filter was probed with both the anti-Gag and anti-IN antisera. bact., bacterial cell extract. (C) Log- and stationary-phase cells were harvested, extracted, and subjected to immunoblot analysis. The anti-Gag and anti-IN antisera were used as probes. The lane marked stat.contained material from the stationary-phase cells; the lane marked log was produced by using half as many cells, but in this case they came from the log-phase culture. The lane labeled stat.+log contained protein extracted after stationary- and log-phase cells were mixed together so that twice as many stationary-phase cells were used. The lane labeled 1/2 log contained a twofold dilution of the material in the log lane.

protein, the drop in IN that we detected was due to an increase in the degradation rate of IN relative not only to Gag but also to the bulk of cellular proteins. The equivalent levels of Gag and IN at the early time points confirmed our conclusions that the two proteins are synthesized at equal levels. Because the sharp drop in IN levels occurred between samples separated by a 17-h period, we initiated another time course experiment in which samples were collected every 2 h (Fig. 7B). Most of the IN degradation occurred within a 6-h period between the OD_{600}s of 9.2 and 14.0. The final Gag-to-IN ratio observed in this experiment was 40:1.

We considered the possibility that stationary-phase cells appeared to have relatively low levels of IN only because most of the IN was selectively degraded in the extracts by cellular proteases released upon cell lysis. Cultures of Tf1-expressing cells were grown to log phase or stationary phase. The lane labeled stat. in Fig. 7C contained material from the stationary-phase cells, while the lane marked log was produced by using half as many cells from a log-phase culture. The lane labeled stat.+log contained protein extracted after stationary- and log-phase cells were mixed in a 2:1 ratio. The IN band in the stat.+log lane was found to be comparable in intensity to the IN band in the lane labeled 1/2 log, which contained half the material in the log lane. These results indicated that the IN from stationary-phase cells was not being degraded in the cell extracts.

VLPs from log-phase cells contain large amounts of IN. We examined VLPs from exponentially growing cells to evaluate their ratio of Gag to IN to see if the high level of IN detected in whole cell extracts was reflected in particles. An extract from a log-phase culture of Tf1-expressing cells was subjected to sucrose gradient centrifugation, and these fractions were immunoblotted (Fig. 8). The simultaneous probing of this blot with anti-Gag and anti-IN antisera revealed that much of the IN in this extract cosedimented with the Gag, suggesting an association between equivalent amounts of Gag and IN in a different type of particle structure. These log-phase particles may be composed of a heterogeneous population wherein each particle contained different amounts of IN. We noted that the position of the Gag peak in the gradient that contained log-phase particles was a few fractions lower than the Gag peak in the gradient that contained stationary-phase particles. This difference in sedimentation rates may have been caused by the association of large amounts of IN with the Gag in the log-phase particles. Gag and IN in fractions from different positions in gradients of extracts of log-phase cells sedimented to their original positions when resedimented, indicating that Gag and IN are stability associated and that the broad trail of IN was not due to a rapid dissociation (29).

Time course of reverse transcript production. To evaluate the functional role in transposition of the Tf1 particles that

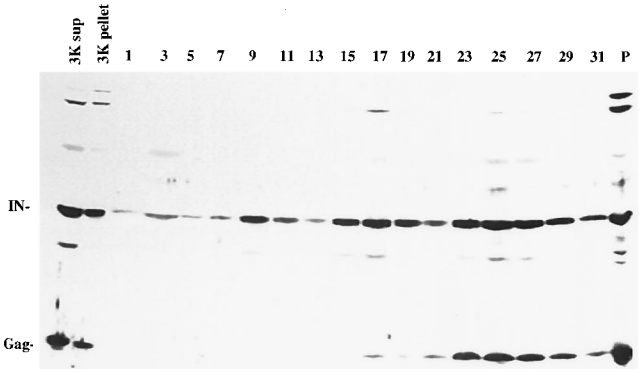

FIG. 8. Immunoblot of a sucrose gradient that contained Tf1 VLPs isolated from log-phase cells. This blot contained material from a sucrose gradient made from a log-phase extract of YHL1282, a Tf1-expressing *S. pombe* strain. Fractions were pooled in sets of two as indicated above the lanes. The lane labeled P contained the protein pelleted during sucrose gradient centrifugation. The 3K sup and 3K pellet lanes were loaded with material from the initial 3,000 rpm spin in an SS34 rotor to remove cell debris. The positions of Gag and IN are shown.

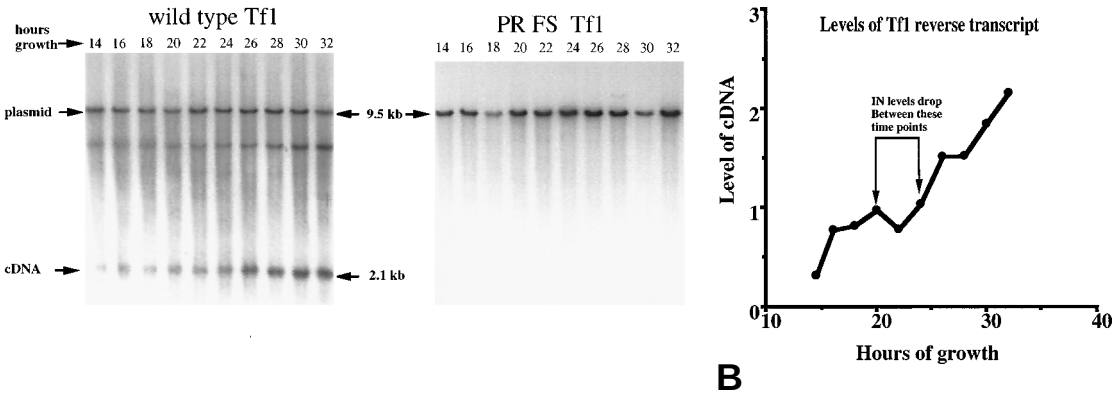

FIG. 9. Time course of Tf1 cDNA production. (A) DNA blot containing nucleic acid extracted from the cells used for the protein extractions shown in Fig. 7B. The DNA was digested with *Bst*XI and loaded onto a 0.6% agarose gel, which was transferred to a filter that was probed with a 1-kb *neo* fragment. The panel on the left was produced from a culture that expressed wild-type Tf1 protein (YHL1282), and the panel on the right was made from a culture that expressed Tf1 with a frameshift (FS) mutation at the end of PR (YHL1836). The number above each lane represents the number of hours after inoculation the samples were harvested. The 9.5-kb band was produced by the plasmid *Bst*XI fragment, while the 2.1-kb band resulted from Tf1 cDNA. The PR frameshift produced no cDNA since it expressed no RT. (B) Plot of the signal strengths of the 2.1-kb cDNA bands seen in panel A. The *x* axis is the time when samples were harvested, and the *y* axis is the amount of signal measured on a PhosphorImager (Molecular Dynamics). The cDNA band strength was divided by the plasmid signal to normalize each lane to the total DNA extracted. The time points marked with arrows bracket the time when the IN protein was degraded.

contain excess Gag, we examined the cells used for Fig. 7B for reverse transcription products. Figure 9A is a blot of DNA extracted from whole cells, cut with *Bst*XI, and hybridized with a *neo* probe shown in Fig. 3. The restriction cut allowed us to distinguish between *neo* sequences derived from reverse transcripts and plasmids because Tf1 has a single *Bst*XI site 2 kb from the 3′ end of the element. Additional evidence that the 2-kb band was derived from the Tf1 cDNA came from examination of an isogenic strain with a Tf1 plasmid that bears a frameshift mutation in PR and therefore produced no RT. This mutant strain contained the plasmid band but not the 2-kb cDNA band. The 2-kb cDNA band in Fig. 9A, derived from the double-stranded Tf1 cDNA, was observed to increase greatly in the cells that contained reduced IN protein. The levels of the 2-kb band were quantitated and normalized to the amount of plasmid-derived signal in each lane. The increase in Tf1 cDNA content observed during the entire time course was eightfold, and the bulk of this increase occurred after the Gag-to-IN ratios increased to the levels observed in stationary phase (Fig. 9B). The fact that most of the cDNA is produced following degradation of the majority of IN protein was consistent with the idea that particles containing a large molar excess of Gag to IN are functional in producing double-stranded cDNA. We cannot determine whether the small amounts of cDNA produced in log-phase cells are produced by particles of this type or by particles containing equal molar amounts of Gag and IN.

DISCUSSION

The sequence of Tf1 indicated that all of the transposon proteins are encoded within a single primary translation product (27). Subsequent analysis of Tf1 protein expressed from a Tf1 PR mutant bearing an epitope tag at the N terminus of Gag indicated that the sole primary translation product was the full-length polypeptide (28). We sought an independent means of examining the primary translation products encoded by a more natural version of Tf1 because the epitope-tagged construct contained a heterologous mRNA leader and a three-codon deletion in PR near the position in the element where a translational frameshift would be expected to occur if Tf1 resembled other retroelements. The Tf1 construct described here with a single base mutation 18 codons from the active site of PR was completely defective for processing activity and produced only the full-length polypeptide. This finding confirmed our earlier conclusion that Tf1 synthesizes all of its proteins in a single primary translation product, which provided additional evidence against the possibility of a transcriptional or translational mechanism for expressing a molar excess of Gag protein.

Once the full-length product is translated, it is cleaved into the mature-sized transposon proteins by an ordered process that requires PR. Our pulse-chase experiments indicated that the first cleavage of the primary translation product resulted in a 125-kDa species that originated from the removal of the Gag domain. Because of the early processing of the 150-kDa primary product into the 125-kDa protein, we suggest the cleavage of Gag occurred before any of the other processing events. Perhaps the rapid production of mature Gag reflects an immediate need for this protein in the assembly of particles, considering that other LTR-containing retroelements produce high levels of Gag as a primary translation product. The 110-kDa species precipitated by the anti-IN antiserum resulted from the removal of about 15 kDa from the 125-kDa species. We predict that the 15-kDa species was PR, whereas the 110-kDa product was composed of IN and RT.

Our quantitative immunoblot analysis of the Gag-to-IN ratio revealed that about 26-fold excess levels of Gag accumulated in stationary-phase cells, and this was reflected in the 26-fold excess Gag seen in the particles. This observation was in contrast to the equal amounts of Gag and IN translated, indicating that a posttranslational mechanism, previously unknown to exist in retroelements, was responsible for the reduction of IN. Our time course analysis of *S. pombe* cultures allowed us to document the degradation process that is responsible for the excess levels of Gag observed in stationary-phase cells. Although unlikely, we have not directly ruled out the possibility that the high ratio of Gag to IN is due to a sharp increase in Gag stability in stationary-phase cells. However, pulse-chase experiments indicate that Gag is stable in log-phase cells and could not therefore increase its stability in stationary-phase cells (1).

The biogenesis of particles composed of mature Tf1 proteins must in some fashion differ greatly from that of retroviruses

and other retrotransposons because Gag and Pol proteins are translated in equal amounts and assembled into particles that contained equivalent amounts of Gag and IN. In cases in which retroviruses and LTR-containing retrotransposons have been modified by fusing the Gag ORF to Pol to synthesize equal amounts of these proteins, few particles were formed (11, 18, 19, 21). One possible difference may be that the PR in Tf1 is better than those of other elements at cleaving all primary translation products into mature protein.

The sucrose gradients of particles from log-phase cells indicated that much of the IN degraded was associated with an unusual type of particle that contained similar levels of Gag and IN. These particles may represent an intermediate in the formation of VLPs that initially accommodated the equal molar amounts of Gag and IN proteins. In addition, these data showed that protein processing was virtually complete before the IN degradation began, indicating that mature IN is the substrate for the proteolysis that occurred in stationary phase. Unpublished data showing that the primary translation product with the mutant PR assembled into particles suggested that Tf1 protein processing may occur after the initial association of primary product (1). We also know that these particles from log-phase cells undergo the early steps of reverse transcription because minus-strand strong-stop DNA was isolated from just these kind particles (25). We propose that the degradation of IN shown to occur as cells reach stationary phase represents another step in the development of mature Tf1 particles that must take place before the bulk of reverse transcription can occur. Our observation that the majority of Tf1 reverse transcription occurred after IN degradation indicated that the particles with excess Gag are functional and represent real intermediates in the transposition process. The idea that IN degradation is a programmed aspect of particle development is also consistent with our observation that the majority of the RT activity present in log-phase cells is absent in stationary-phase cells (29). Although we propose that the reduction in IN levels is required for the final stages of assembly of Tf1 particles, the possibility exists that assembly is completed before IN degradation and excess IN not incorporated into the VLPs is degraded simply because of greater exposure to cellular proteases. In this case, IN degradation would not be required for transposition. The question of how important the degradation of Pol protein is to the assembly process can be resolved only after we develop direct genetic means of manipulating the Gag-to-Pol ratios.

We found that the IN degradation began during the last doubling of the *S. pombe* culture, which coincides with the time in the growth of *Saccharomyces cerevisiae* cultures that several physiological changes occur. Our recent finding that cells starved for glucose produce normal Tf1 proteins but do not degrade IN provides additional evidence that the reduction of IN levels is not just a time-dependent process but is initiated by a change in culture conditions (1). Prior to the entry into stationary phase, *S. cerevisiae* cells grown in YPD (1% yeast extract, 2% peptone, 2% glucose) undergo the diauxic shift when their glucose supply is exhausted, and they adapt to respiratory metabolism (36). Also at the beginning of stationary phase, cells are starved for nutrients including nitrogen, phosphorus, and sulfur. In addition, cellular proteases accumulate in various subcellular locations as *S. cerevisiae* cells approach stationary phase (16). These physiological changes that occur as cells approach stationary phase represent a few examples of the types of processes that may directly or indirectly regulate the degradation of Tf1 IN. The assembly of Tf1 particles and the synthesis of Tf1 cDNA may occur during the time when cells are most stressed because transposition may result in recombination events that provide selective advantages for the host cells.

Tf1 is one of several LTR-containing retrotransposons that possess a single ORF. Some of these are members of the *copia* family and may overexpress Gag from a spliced mRNA, a mechanism that has been shown for *copia* itself (3). Other single-ORF LTR-containing retrotransposons not directly related to *copia* include *SURL*, Micropia, and *Cer1* (4, 24, 33). The mechanisms of particle formation used by these elements could very well be related to our proposed model of Pol degradation.

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