

# A Long Terminal Repeat-Containing Retrotransposon of *Schizosaccharomyces pombe* Expresses a Gag-Like Protein That Assembles into Virus-Like Particles Which Mediate Reverse Transcription

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**The Tfl element of *Schizosaccharomyces pombe* is a long terminal repeat-containing retrotransposon that encodes functional protease, reverse transcriptase, and integrase proteins. Although these proteins are known to be necessary for protein processing, reverse transcription, and integration, respectively, the function of the protein thought to be Gag has not been determined. We present here the first electron microscopy of Tfl particles. We tested whether the putative Gag of Tfl was required for particle formation, packaging of RNA, and reverse transcription. We generated deletions of 10 amino acids in each of the four hydrophilic domains of the protein and found that all four mutations reduced transposition activity. The N-terminal deletion removed a nuclear localization signal and inhibited nuclear import of the transposon. The two mutations in the center of Gag destabilized the protein and resulted in no virus-like particles. The C-terminal deletion caused a defect in RNA packaging and, as a result, low levels of cDNA. The electron microscopy of cells expressing a truncated Tfl showed that Gag alone was sufficient for the formation of virus-like particles. Taken together, these results indicate that Tfl encodes a Gag protein that is a functional equivalent of the Gag proteins of retroviruses.**

Long terminal repeat (LTR)-containing retrotransposons are a diverse group of elements found in a broad variety of eukaryotic hosts. Their structures and mechanisms of propagation are closely related to those of retroviruses. Both retroviruses and LTR-containing retrotransposons encode Gag, reverse transcriptase (RT), and integrase (IN) proteins. The Gag proteins assemble into the coats of virus and virus-like particles (VLPs), and the formation of these particles is required for reverse transcription. Once RT produces the full-length double-stranded cDNA, IN inserts the cDNA into the genome of a host cell.

Most of what is known about the function of Gag proteins resulted from the study of retroviruses. With an estimated 1,500 Gag molecules per virion in the case of the Rous sarcoma virus (59), Gag constitutes the major component of viral particles. It is well established that Gag is able to form extracellular particles in the absence of the other viral proteins (10, 19, 23, 62). As a result of its role in particle formation, Gag is responsible for the size of virions, the process of budding, and the packaging of the other components of the virus, such as the genomic RNA, RT, IN, and envelope proteins (56).

Once the particles have assembled, their maturation results from the processing of Gag by the viral protease (PR) into at least three major products, matrix (MA), capsid (CA), and nucleocapsid (NC) (33, 64). MA is derived from the amino terminus of Gag and directs the location of particle formation (for a review, see reference 58). CA, the largest cleavage product of Gag, forms the shell surrounding the ribonucleoprotein complex that contains the genomic RNA. CA has several separate functions that contribute to the assembly of particles, the release of particles from infected cells, and the reverse transcription of viral RNA. Numerous mutations in the CA domains of the murine leukemia virus and of human immunodeficiency virus type 1 have been found to block assembly and particle release (11, 24, 43, 67). Certain mutations in the CA sequences of several retroviruses have been found to destroy infectivity without affecting assembly or budding (27, 43, 55, 60). Strong evidence for the role of the CA in postentry replication comes from experiments with the murine leukemia virus that showed that specific mutations inhibit the synthesis of viral cDNA (3). Each of the functions of Gag is thought to require the major homology region (MHR), a motif of CA that is about 20 amino acids long and is highly conserved among Gag proteins (49a, 64). Mutations in the MHR of the Rous sarcoma virus can be deleterious to particle release or have an effect on cDNA synthesis (9, 14).

Finally, the NC protein, located at the carboxy terminus of Gag, is tightly bound to the genomic RNA (18, 21). NC is basic and contains one or more zinc finger motifs involved in the packaging of the viral RNA (13). In biochemical experiments, NC appears to have chaperone activity resulting in the stimu-

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lation of template switching by RT (4, 17, 50, 65; for a review, see reference 51).

The spumaretroviruses are replication-competent retroviruses that express a Gag protein that has no sequence similarity to the MA, CA, and NC of other retroviruses (for a review, see references 31 and 41). This dissimilarity includes the lack of an MHR motif in CA and of Zn fingers in NC (44, 52). In place of the Zn fingers, a glycine-arginine-rich motif near the carboxy terminus of the spumaretroviral Gag has been shown to have nucleic acid binding properties (66).

Although less is known about the Gag proteins of LTR-containing retrotransposons, evidence from electron microscopy indicates that they form VLPs, as documented for Ty1 (22) and Ty3 (25) of *Saccharomyces cerevisiae* as well as for copia (46) of *Drosophila melanogaster*. Intracytoplasmic particles have also been detected for the endogenous retroviruses of *Drosophila*, *gypsy* (32) and *ZAM* (30), elements closely related to LTR-containing retrotransposons. The retrotransposon Ty3 encodes a CA protein that contains an MHR-like motif (48). However, no homologs of CA and no MHR motifs have been identified in elements such as Ty1 (12) or the Tf1 and Tf2 elements from *Schizosaccharomyces pombe* (37).

Tf1 and Tf2 are two closely related LTR-containing retrotransposons (37), and their sequences encode single primary products of translation that have homologies to PR, RT, and IN (36, 37, 61). Although the amino acid sequences encoded by Tf1 and Tf2 are virtually identical for RT and IN, the sequences of Gag diverge.

Tf1 and Tf2 have been studied as model retroviruses by using the techniques of yeast genetics. The expression of Tf1 RNA from an inducible promoter results in high levels of transposition (26, 34, 36). Mutations within the conserved residues of PR, RT, and IN greatly reduce the transposition of Tf1 and indicate that these enzymes are required for Tf1 activity (5, 6, 35). However, much less is known about whether a Gag-like protein is required for Tf1 transposition. A protein of 27 kDa, derived from the N terminus of the product of the open reading frame (ORF), cosediments in sucrose gradients with RT, IN, and cDNA of Tf1 (36). This protein also has a nuclear localization signal (NLS) that is required for nuclear localization and, as a result, for transposition activity (16). This evidence is consistent with the possibility that the 27-kDa protein may function as the Gag of Tf1. Nevertheless, its sequence has no similarity to that of any known Gag. It lacks the MHR motif, and the 27-kDa protein is not processed into smaller species, such as MA, CA, and NC. In addition, it is not known whether this putative Gag protein assembles to form the shell of a particle or even if it is required for transposition.

We tested the Gag of Tf1 for functions associated with Gag proteins. We generated deletions of 10 amino acids in each of the four hydrophilic domains of the protein and found that all four mutations reduced transposition activity. The characterization of these mutant transposons revealed that Gag expression was required for packaging of RNA, particle formation, and reverse transcription. These observations were possible because we report here for the first time that Tf1 is able to assemble into intracytoplasmic particles easily visible by electron microscopy. In fact, the expression of Gag alone was sufficient for the formation of VLPs. Taken together, these

TABLE 1. Oligonucleotides

Oligo-nucleotide	Description
HL38	5'-GGAAGAGGAATCCTGGC-3': 5'-flanking primer, upstream of <i>Xho</i> I site, used to generate $\Delta$ A, $\Delta$ B, and $\Delta$ C mutations
HL39	5'-CCAATTGCTCCAGTCTTTG-3': 3'-flanking primer, downstream of <i>Avr</i> II site, used to generate $\Delta$ A, $\Delta$ B, and $\Delta$ C mutations
HL87	5'-CGATAATTGAACGCTACACC-3': 5'-flanking primer, upstream of <i>Avr</i> II site, used to generate $\Delta$ D mutation
HL88	5'-TTGTTTATTTGACATGTATGGC-3': 3'-flanking primer, downstream of <i>Bsr</i> GI site, used to generate $\Delta$ D mutation
HL205	5'-CAAGTCGTTCTATAGCGTAGCCGGCATCGG AATGTTACCAAAAAA-3': bottom-strand primer used to create $\Delta$ B deletion <sup>a</sup>
HL206	5'-TTTTTTGGTGAACATTCGATGCCGGCTACG CTATAGAACGACTTG-3': top-strand primer used to create $\Delta$ B deletion
HL207	5'-CAATATCCACCATTTCCATCGCCGGCCATTCT TGGAAATAGCGAGT-3': bottom-strand primer used to create $\Delta$ A deletion
HL208	5'-ACTCGCTATTTCAAGAATGGCCGGCGATGG AAATGGTGGATATTG-3': top-strand primer used to create $\Delta$ A deletion
HL209	5'-AAAGTTAAAAATCTTGTCTATATTGCCGGCC TGTTTAATCAGATAGGGTG-3': bottom-strand primer used to create $\Delta$ C deletion
HL210	5'-CACCTATCTGATTAACAGCCGGCAATAT AGACAAGATTTTTAACTTT-3': top-strand primer used to create $\Delta$ C deletion
HL211	5'-TGAAACATTTGTTTTCTTTGGCCGGCAAAC TTATTGTTTTCCAATTG-3': bottom-strand primer used to create $\Delta$ D deletion
HL212	5'-CAATTGGAAAAACAATAAGTTTGCCGGCCC AAAGAAAACAATGTTTCA-3': top-strand primer used to create $\Delta$ D deletion

<sup>a</sup> Top- and bottom-strand primers were used to introduce an *Ngo*MI site, as well as to delete amino acids. Deletion constructs were created by fusion PCR, including amplification with HL38 (for bottom strands corresponding to HL205, HL207, and HL209), HL39 (for top strands corresponding to HL206, HL208, and HL210), HL87 (for a bottom strand corresponding to HL211), and HL88 (for a top strand corresponding to HL212).

results indicate that the Gag of Tf1 functions similarly to the Gag proteins of retroviruses.

#### MATERIALS AND METHODS

**Media.** The *S. pombe* minimal liquid and plate media were composed of Edinburgh minimal medium (EMM). Ten micromolar vitamin B<sub>1</sub> (thiamine) was added to EMM when indicated to repress the *nmt1* promoter. 5-Fluoro-orotic acid (5-FOA; U. S. Biological, Swampscott, Mass.) was used at 1 mg/ml in EMM or in yeast extract plus supplements (YES) with 500  $\mu$ g of Geneticin (GIBCO)/ml.

**Plasmid construction.** Most of the plasmids used for this study were constructed with techniques of PCR cloning. The oligonucleotides and plasmids used are listed in Tables 1 and 2.

The plasmids pHL891-19 and pHL449-1 were used to create mutant versions of the Tf1 Gag protein. The mutations corresponded to deletions of 10 amino acids and the additions of the dipeptide alanine-glycine. The sequence of the introduced DNA contained an *Ngo*MI restriction site that was unique within the plasmids. The constructs are hereafter referred to as Tf1- $\Delta$ A, Tf1- $\Delta$ B, Tf1- $\Delta$ C, and Tf1- $\Delta$ D, and two independently generated plasmids were created for each, pHL1260 and pHL1261, pHL1262 and pHL1263, pHL1264 and pHL1265, and pHL1258 and pHL1259, respectively (Table 2).

The constructs were created by fusion PCR as described earlier (34). Briefly, two overlapping oligonucleotides containing a specific deletion were used to

TABLE 2. Plasmids

Plasmid(s)	Description	Source or reference
pHL167-1	<i>EcoRI</i> fragment containing Tf1 Gag sequence	38
pHL449-1	<i>neoAI</i> -marked version of Tf1 in a <i>ura4</i> selectable plasmid	34
pHL490-80	Tf1- <i>neoAI</i> with frameshift in PR	6
pHL476-3	Tf1- <i>neoAI</i> with frameshift in IN	34
pFL20	<i>S. pombe</i> vector with autonomous replicating sequence and stabilization fragment	42
pHL891-19	<i>neoAI</i> -marked Tf1 in a <i>ura4</i> selectable plasmid; <i>XhoI</i> -less <i>neo</i> without intron; <i>SpeI</i> site just 3' downstream of hairpin after Tf1 primer binding site	40
pHL1260 and pHL1261	Tf1- <i>neoAI</i> with deletion of amino acids 2 to 11, ( $\Delta A$ )	This study
pHL1262 and pHL1263	Tf1- <i>neoAI</i> with deletion of amino acids 64 to 73 ( $\Delta B$ )	This study
pHL1264 and pHL1265	Tf1- <i>neoAI</i> with deletion of amino acids 165 to 174 ( $\Delta C$ )	This study
pHL1258 and pHL1259	Tf1- <i>neoAI</i> with deletion of amino acids 215 to 224 ( $\Delta D$ )	This study

amplify by PCR two fragments that overlap at the site of the mutation. Each of these fragments extends to a unique restriction site that can be used to subclone the final fusion product. The fusion PCR products were produced by mixing both overlapping PCR products with an oligonucleotide that hybridizes to the extreme end of the top strand of one product and another oligonucleotide that hybridizes to the extreme end of the bottom strand of the other fragment.

The two overlapping oligonucleotides were HL207 and HL208 for Tf1- $\Delta A$ , HL205 and HL206 for Tf1- $\Delta B$ , HL209 and HL210 for Tf1- $\Delta C$ , and HL211 and HL212 for Tf1- $\Delta D$ ; the flanking oligonucleotides were HL38 and HL39 for the Tf1- $\Delta A$ , Tf1- $\Delta B$ , and Tf1- $\Delta C$  constructs and HL87 and HL88 for the Tf1- $\Delta D$  construct (Table 1).

The fusion PCR products were then digested by *XhoI* and *AvrII* and cloned into pHL891-19, also digested by *XhoI* and *AvrII*, for the Tf1- $\Delta A$ , Tf1- $\Delta B$ , and Tf1- $\Delta C$  constructs or digested by *BsrGI* and *AvrII* and cloned into pHL449-1, also digested by *BsrGI* and *AvrII*, for the Tf1- $\Delta D$  construct.

The *neo* gene interrupted by an intron was introduced in the transposons with  $\Delta A$ ,  $\Delta B$ , and  $\Delta C$  by replacing the *NarI/BamHI* fragment of 2.0 kb with that of pHL449-1. Although the mutations introduced by PCR were verified by sequencing, the plasmids were created in duplicate from independent PCRs and the properties of each plasmid were studied in parallel. Since no differences were seen between the duplicate plasmids, we present here the results for one duplicate. Plasmids were introduced into *S. pombe* strains (Table 3) by lithium acetate transformation (47).

**Transposition and homologous recombination assays.** Tf1 transposition and homologous recombination frequencies were determined as described previously (35). Tf1 transposition was monitored by placing a *neoAI*-marked Tf1 element under the control of an inducible *nmI1* promoter. The *neo* gene allowed cells to grow in the presence of 500  $\mu$ g of G418/ml. *S. pombe* strains that contained a Tf1-*neoAI* plasmid were grown as patches on EMM-uracil dropout agar plates in the absence of thiamine to induce transcription of the *nmI1* promoter. After 4 days of incubation at 32°C, the plates were replica printed to medium containing 5-FOA to eliminate the *URA3*-marked plasmid with Tf1-*neoAI* (8). Finally, 5-FOA-resistant patches were printed to YES medium containing both 5-FOA

and G418 and incubated at 32°C for 2 days to detect strains that became resistant to G418 as the result of insertions of the Tf1-*neo* element into the genome. Wild-type Tf1 produced confluent patches of G418 resistance, while mutations in the element reduced the growth on the G418 plates.

Homologous recombinations between cDNA and plasmid sequences were assayed by using a protocol similar to the transposition assay (5). The strains with the Tf1-*neoAI* plasmids were first grown as patches on agar plates that contained EMM (plus 10  $\mu$ M thiamine and dropout powder) and then replica printed to similar EMM plates that lacked thiamine. After 4 days of incubation at 32°C, the plates were replica printed directly to YES medium that contained 500  $\mu$ g of G418/ml. cDNA recombination was scored on the G418 plates after 48 h of growth at 32°C.

**Preparation and analysis of nucleic acid.** cDNA preparations were performed as described previously (6). Total DNA was phenol extracted from stationary-phase cells grown under induction conditions (absence of thiamine) for 36 h. These preparations were then digested with *BsrXI* prior to analysis by DNA blotting. The probe consisted of a 1-kb *BamHI* fragment with the sequence of *neo* (6). The DNA blot analyses were done by using the DIG High Prime DNA labeling and detection kit (Roche).

**Protein extraction and detection of Tf1 Gag, IN, and RT by immunoblot analysis.** Total proteins were extracted from cells grown under inducing conditions (absence of thiamine) by a previously published protocol (6). Cultures at the optical densities (ODs) of 1 and 10 were harvested and resuspended in 400  $\mu$ l of buffer B (15 mM NaCl, 10 mM HEPES-KOH [pH 7.8], 5 mM EDTA). Cells were subjected to a vortex in the presence of glass beads (acid washed) with a diameter of 0.4 mm. Protein extracts were then recovered, and an equal volume of 2 $\times$  sample buffer (6) was added. The mixture was boiled for 3 min, and 10  $\mu$ g of total protein from each sample was loaded on sodium dodecyl sulfate-10% polyacrylamide gels for immunoblot analysis. Standard electrophoresis and transfer techniques were used with Immobilon-P membranes (Millipore). The detection method used was the ECL system as described by the manufacturer (Amersham). Horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin was used at a 1:10,000 dilution. The previously described primary polyclonal antisera used for each filter were from production bleeds 660 (anti-Gag), 657 (anti-IN) (36), and 1571 (anti-RT) (26). Other bleeds (63085-4 and 63085-5) were prepared by immunizing rabbits with a peptide containing the first 15 amino acids of the Gag protein (see Fig. 1A). These bleeds were pooled and then affinity purified.

**Computational analyses of Gag.** ClustalX (default parameters) (57) was used to construct an amino acid alignment of the Gag portions of Tf1 and Tf2 polyproteins. We used Se-AI (courtesy of Andrew Rambant) to construct a DNA alignment of the corresponding codons. DnaSP version 3.51 (53) was used to calculate the Ks (the number of synonymous changes over the number of potential sites of synonymous changes) and Ka (the number of nonsynonymous changes over the number of potential sites of nonsynonymous changes) values. Statistical analysis of negative selection was done by using the Z test as implemented in Mega version 2.1 (29).

**Electron microscopy.** After 36 h of culture at 32°C in EMM lacking thiamine to induce Tf1 transposition, 6 OD units of cells was washed once in 10 ml of PEM [0.1 M piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES; pH 6.9), 2 mM EGTA, 1 mM MgCl<sub>2</sub>] and fixed in 2% glutaraldehyde (Tousimis, Rockville, Md.) in PEM for 2 h at room temperature. Cells were then washed and resuspended in 5 ml of PEM containing 0.2% tannic acid (catalog no. 21710; Electron Microscopy Sciences, Ft. Washington, Pa.) and incubated for 15 min at room

TABLE 3. Yeast strains

Strain <sup>a</sup>	Plasmid	Source or reference
YHL 912	No plasmid	J. Boeke, 21X52
YHL 1282	pHL449-1	34
YHL 1836	pHL490-80	6
YHL 1554	pHL476-3	34
YHL 5889	pHL1260	This study
YHL 5890	pHL1261	This study
YHL 5891	pHL1262	This study
YHL 5892	pHL1263	This study
YHL 5893	pHL1264	This study
YHL 5894	pHL1265	This study
YHL 5826	pHL1258	This study
YHL 5827	pHL1259	This study
YHL 6566	pFL20	7

<sup>a</sup> All strains had the genotype *h<sup>-</sup> ura4-294 leu1-32* and YHL 912 as the parent strain.





sequence of the Gag of Tf1 with the sequence of the corresponding protein of Tf2, a closely related and active transposon. The best alignments of the Gags were obtained when the second methionine of the sequence corresponding to the Tf2 ORF was aligned with the first methionine of the sequence corresponding to the Tf1 ORF (Fig. 1A). Based on this alignment, we propose that the second methionine of the Tf2 Gag initiates translation, contrary to what has been previously proposed (61).

Out of the 245 amino acids from the Gag of Tf1 and the 244 amino acids from that of Tf2, 36% were identical. Of the 89 pairs of identical amino acids, 45% corresponded to codons with synonymous nucleotide changes. To test whether the identical amino acids were being maintained due to natural selection, we determined the Ks/Ka ratio. This was calculated independently for two regions where the two sequences align unambiguously. The sequences used were amino acids 46 through 170 for Tf1 Gag and 46 through 170 for Tf2 Gag and, separately, amino acids 216 through 241 for Tf1 Gag and 211 through 236 for Tf2 Gag (Fig. 1A). The Ks/Ka ratios were 2.6 ( $P = 0.0005$ ) and 3.0 ( $P = 0.0183$ ), respectively. Both values were well above 1.0, indicating that the amino acid identities between the Gag of Tf1 and that of Tf2 resulted from negative or purifying selection. The evidence for this selective pressure in these regions demonstrates that Gag has recently had an important function that was or is needed for the activity of the transposon.

To examine the sequences of the Tf1 and Tf2 Gags for evidence of domain structure, we compared their hydropathy profiles. A positive value indicates local hydrophobicity and a negative value suggests a water-exposed region on the face of a protein. Figure 1B shows four regions of Tf1 Gag with strong hydrophilic scores. Interestingly, the positions of these hydrophilic domains were conserved in Tf2 Gag, suggesting that these domains possess an important function (Fig. 1C). In addition, there were several amino acid conservations within each hydrophilic domain (Fig. 1A).

**Mutations in Gag resulted in reduced frequencies of transposition.** To determine whether Gag was required for transposition and whether domains with independent functions exist, we generated deletions of 10 amino acids in each of the four regions of hydrophilicity (Fig. 1A). For the purposes of future studies, an *Ngo*MI site was introduced at the deletion positions, and these nucleotides resulted in the insertion of an alanine followed by a glycine. Thus, 10 residues of Gag were replaced with alanine-glycine.

We tested the transposition activities of the Tf1 elements with the deletions, referred to as Tf1- $\Delta$ A, Tf1- $\Delta$ B, Tf1- $\Delta$ C, and Tf1- $\Delta$ D, by using an assay based on the expression of Tf1 RNA from a strong promoter on a multicopy plasmid (Fig. 2A) (34). The sequence of Tf1 in the plasmid contained a *neo* gene as an indicator for transposition events. For reasons described below, the *neo* gene was disrupted by an artificial intron that can be spliced out of the Tf1 RNA. Patches of cells in which transcription of Tf1-*neoAI* was induced were subsequently exposed to 5-FOA to select for cells that no longer possessed the Tf1-*neoAI* plasmid (see Materials and Methods). The presence of transposed copies of Tf1-*neoAI* was identified by replica printing the cells from the 5-FOA medium to medium with G418. Figure 2B (left panel) shows that patches of cells that

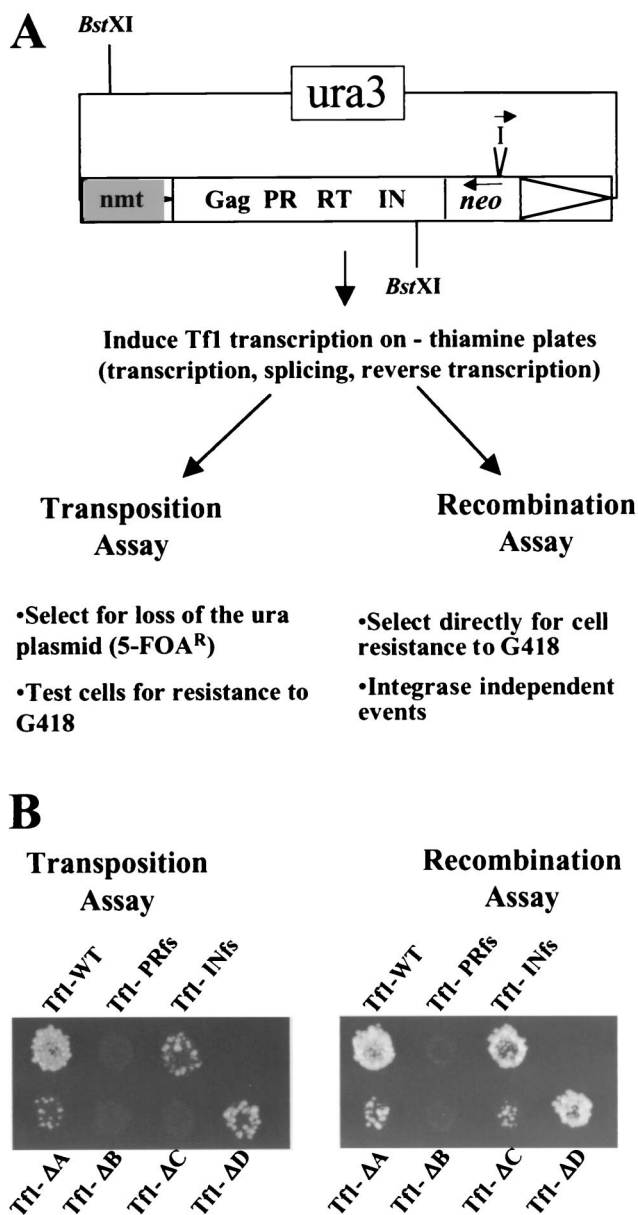


FIG. 2. Effects of Gag deletions on transposition and recombination activity. (A) Schematic of the genetic assay for measuring the levels of transposition and homologous recombination. The restriction sites (*Bst*XI) used for the cDNA blot are shown. I indicates the artificial intron placed in *neo*, -thiamine indicates the absence of thiamine, and 5-FOA<sup>R</sup> indicates resistance to 5-FOA. (B) Results of the transposition and recombination assays for the four transposons containing deletions in Gag (Tf1- $\Delta$ A, Tf1- $\Delta$ B, Tf1- $\Delta$ C, and Tf1- $\Delta$ D) as well as for the control strains (Tf1-WT, Tf1-PRfs, and Tf1-INfs). Patches of cells in which the expression of Tf1 was induced were replica printed to plates containing G418 to measure recombination activity (right panel). To measure transposition, cells were replica printed to plates with 5-FOA, then printed to plates with 5-FOA and G418.

contained wild-type Tf1-*neoA* (Tf1-WT) exhibited sufficient transposition to produce confluent growth on the plate containing G418. A strain that contained a copy of Tf1-*neoAI* that, due to a frameshift mutation, lacked IN expression (Tf1-INfs)

exhibited significantly reduced resistance to G418. This low level of resistance to G418 from Tf1-INfs was due to homologous recombination between Tf1 cDNA and endogenous copies of the related element, Tf2 (data not shown). A transposon that lacked PR, RT, and IN, due to a frameshift in PR (Tf1-PRfs), exhibited no residual transposition activity.

The transposition assays showed that Tf1- $\Delta$ B and Tf1- $\Delta$ C exhibited no transposition at all. Tf1- $\Delta$ A generated low levels of G418-resistant papillae that were below those observed for Tf1-INfs. The Tf1- $\Delta$ D element also exhibited a significant defect in transposition, albeit less than that of the other constructs.

To identify which aspects of the transposition pathway were affected by the mutations, we employed an assay for homologous recombination that detected the presence of Tf1 cDNA in the nucleus (5). This method used the cDNA produced by the same Tf1 expression plasmid described above. The configuration of the *neo* gene in reverse orientation to Tf1 and the orientation of the artificial intron allowed the intron to be spliced only from the Tf1 RNA. Once transcription of Tf1-*neoAI* was induced and without selecting against the expression plasmid, we replica printed cells directly to medium containing G418 (see Materials and Methods). This allowed us to detect homologous recombination events between Tf1 cDNA and copies of the Tf1 plasmid. Reduced levels of homologous recombination would be caused by mutations that either reduced reverse transcription or inhibited the import of Tf1 cDNA into the nucleus. The level of recombination that was generated by a wild-type copy of Tf1-*neoAI* is shown in Fig. 2B (right panel). The Tf1-INfs element showed that these recombination events were independent of IN. The lack of any G418 resistance generated by the Tf1-PRfs element indicated that reverse transcription was necessary for any homologous recombination to be detected. The mutants Tf1- $\Delta$ A, Tf1- $\Delta$ B, and Tf1- $\Delta$ C showed significant reductions in recombination. While Tf1- $\Delta$ B appeared to be totally defective in this assay, Tf1- $\Delta$ C showed residual levels of recombination activity. Tf1- $\Delta$ A appeared to generate slightly more G418 resistance than Tf1- $\Delta$ C, and the Tf1- $\Delta$ D element showed more activity than Tf1- $\Delta$ A and Tf1- $\Delta$ C. These defects in homologous recombination could reflect a reduction in reverse transcription or a defect in the nuclear import pathway of the preintegration complex containing cDNA, IN, and Gag.

**Three of the deletion mutations caused reduced levels of cDNA synthesis.** To test whether the deletions in Gag caused defects in the production of cDNA, we used DNA blot analysis (39). The strains of *S. pombe* expressing the different versions of Tf1-*neoAI* were grown to stationary phase. Extracts of total DNA were digested with *Bst*XI, subjected to agarose gel electrophoresis, and blotted with a probe specific for the *neo* sequence. The restriction enzyme *Bst*XI was chosen because there is only one site within the mature cDNA (Fig. 2A), and its use resulted in a 2.1-kb fragment from the cDNA and a 9.5-kb band from the plasmid. The plasmid expressing Tf1-INfs produced wild-type levels of cDNA as expected for an element that expressed RT (39) (Fig. 3). Also as expected, Tf1-PRfs did not produce cDNA because it did not express RT. The DNA blot also showed that Tf1- $\Delta$ A generated a wild-type level of cDNA. Tf1- $\Delta$ D produced significantly lower levels of cDNA, while repeated examination of Tf1- $\Delta$ B and

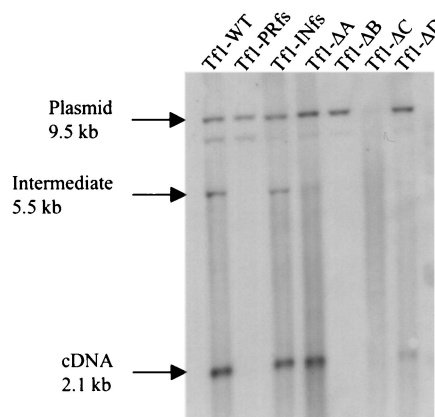


FIG. 3. Effects of mutations in Gag on the synthesis of cDNA. Total DNA was extracted from *S. pombe* strains in which Tf1 expression had been induced. The DNA was digested with *Bst*XI (Fig. 2) and probed with a *neo*-specific sequence. The 9.5-kb band was produced by a plasmid sequence, and the 2.1-kb band was generated by Tf1 cDNA. The additional band is likely derived from single-LTR circles; however, this identification is not definite. The reduced level of the plasmid band from Tf1- $\Delta$ C was reproducible with independent transformants and may be due to degradation during extraction that is mediated by RT or IN.

Tf1- $\Delta$ C detected no cDNA band. These data indicated that the reduced levels of transposition observed for Tf1- $\Delta$ B, Tf1- $\Delta$ C, and Tf1- $\Delta$ D were due to significantly reduced levels of reverse transcription. On the other hand, the reduced transposition and homologous recombination caused by Tf1- $\Delta$ A were not the result of defects in reverse transcription. Instead, the mutation likely reduced transport of Tf1 into the nucleus. This conclusion is based on previous work that identified an NLS within the sequence deleted from Tf1- $\Delta$ A (16).

**Deletion mutants Tf1- $\Delta$ B and Tf1- $\Delta$ C produced reduced levels of Gag.** To investigate whether the transposition and recombination defects were due to decreased stability of Tf1 proteins, we performed immunoblot analysis with antibodies raised against the Tf1 Gag, IN, and RT. Total cell proteins were extracted from log-phase and stationary-phase cultures of strains expressing Tf1-WT, Tf1-INfs, Tf1-PRfs, and the four versions of Tf1 with the deletions. From previous results, it is known that the Tf1 Gag and IN proteins are expressed at equal levels within a single primary translation product (Fig. 4A) and that during the log-to-stationary phase transition of the culture, the IN and RT proteins are specifically targeted for degradation (6).

In cultures grown to log phase, only Tf1- $\Delta$ A and Tf1- $\Delta$ D were able to produce normal levels of Gag protein relative to Tf1-WT (Fig. 4B, left panel). No Gag proteins were detected for the Tf1- $\Delta$ B and Tf1- $\Delta$ C elements by using polyclonal antibodies raised against residues 21 through 245 of Gag. The Gag proteins produced by Tf1- $\Delta$ A and Tf1- $\Delta$ D were also stable in stationary-phase cells (Fig. 4B, right panel). To test whether the Gag proteins produced by Tf1- $\Delta$ B and Tf1- $\Delta$ C were not detected with this antiserum because the deletions removed key epitopes, we raised new antibodies against the first 15 amino acids of the Gag protein and used them for immunoblotting of the same extracts. The results show that in log-phase cells (Fig. 4C, left panel), Tf1- $\Delta$ B and Tf1- $\Delta$ C expressed



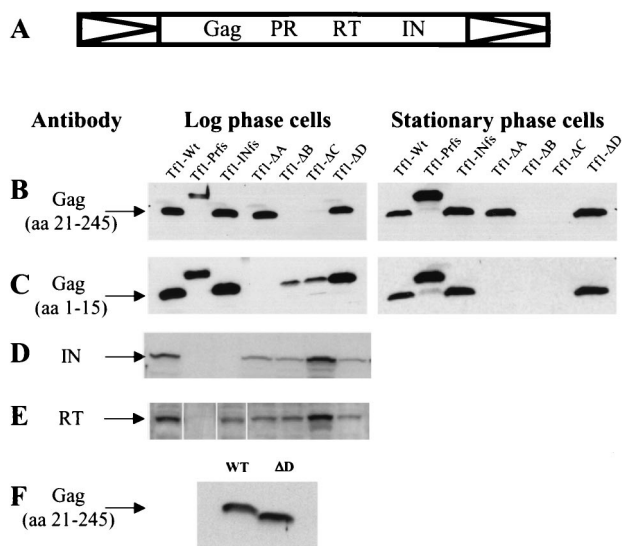


FIG. 4. Immunoblot of extracts made from *S. pombe* expressing different mutants of Tf1. (A) Schematic of the Tf1 polyprotein. (B to F) Immunoblots were performed by using polyclonal antibodies directed against Gag peptide amino acids (aa) 20 through 245 (bleed 660) (B and F) and 1 through 15 (bleed 63085-4+5) (C), against IN peptide (bleed 657) (D), and against RT peptide (bleed 1571) (E). The predicted sizes of the proteins are 27, 56, and 60 kDa, respectively, for Gag, IN, and RT. WT, wild type.

unstable Gag proteins which were no longer detectable in stationary-phase cells (Fig. 4C, right panel). The fact that the Gag protein of Tf1-ΔA was not recognized by this set of antibodies was expected since the ΔA deletion removed the majority of the epitope recognized by the antibodies. In log-phase cultures, all the mutants except for Tf1-ΔC produced slightly lower levels of IN than the wild type (Fig. 4D). Tf1-ΔC accumulated wild-type amounts of IN. The extracts from cells grown to stationary phase showed that none of the deletions affected the degradation of IN that was observed with Tf1-WT (data not shown). Antibodies that recognized RT showed that all the mutants except for Tf1-ΔC produced slightly lower levels of the 60-kDa species of RT seen in log-phase cultures (Fig. 4E).

The fact that the Gag protein of Tf1-ΔD showed a faster mobility than the wild type (Fig. 4F) was consistent with the assumption that the residues removed from Tf1-ΔD exist in the Gag protein. This confirmation is important because the position of the PR cleavages in Tf1 are not known but are predicted based on alignments with Ty3, a retrotransposon for which the cleavage sites were sequenced (28).

**The particles of Tf1 can be observed by electron microscopy, and expression of Gag is sufficient for their formation.** Previous work established that Gag, RT, IN, and cDNA assemble into large macromolecular complexes that cosediment in sucrose gradients (36). Here we tested whether Tf1 assembled spherical VLPs that can be observed with electron microscopy. Figure 5A shows typical structures of particles easily detectable in the cytoplasm of cells expressing the Tf1-WT element. They were observed in 3.7% of 269 cells sampled with a single section and were never observed in sections of 530 cells not expressing Tf1. They appear as clusters of well-defined parti-

cles or aggregates. The value 3.7% is not surprising given that the particles were tightly clustered and a single section samples only a small portion of each cell. A fraction of the particles were electron-dense rings with electron-lucent centers, while the majority had dense centers (Fig. 5A, A', and C). Associated with the particles were electron-dense aggregates (Fig. 5A and D). The maximum diameter of these intracytoplasmic particles was 50 nm. Surprisingly, the cytoplasmic particles and dense aggregates were often associated with arrays of membranes of unknown origin (Fig. 5B).

The same techniques of electron microscopy were used to determine whether the forms of Tf1 with deletions in Gag produced spherical particles. No particles or dense aggregates were found in the strains with Tf1-ΔC (data not shown). Since the Gag proteins of Tf1-ΔC were unstable (Fig. 4B), these results indicate that Gag was required for particle formation. In the case of Tf1-ΔA, no spherical VLPs were observed. However, dense aggregates in the cytoplasm were found in 2.0% of 300 cells (Fig. 5F). In the case of Tf1-ΔD (Fig. 5E and E'), the size and the number of particles were similar to those for wild-type Tf1. Of 350 cells, 7.1% contained particles. The presence of cytoplasmic membranes in the vicinity of Tf1-ΔD particles was also seen (data not shown).

The finding that the expression of Gag correlated with the formation of spherical particles is consistent with a role for Gag in particle structure. Many Gag proteins of retroviruses and LTR-containing retrotransposons are able to form particles in the absence of any other retroelement proteins (1, 10, 19, 23, 62). We tested whether the expression of the Tf1 Gag was sufficient for the formation of spherical particles. The version of Tf1 with the frameshift at the beginning of PR, Tf1-PRfs, does not express RT or IN (Fig. 4B, D, and E). The sole protein produced was a version of Gag fused to the first approximately 30 amino acids of PR followed by 20 residues encoded out of frame. The additional sequence results in an increase in molecular weight that can be observed in Fig. 4B. The electron microscopy revealed that a large percentage of cells expressing Tf1-PRfs contained particles and dense aggregates (Fig. 5G). Although the particles were more dense and less uniform than those of the wild type, their shapes and sizes resembled those of normal particles (Fig. 5G'). Of 300 cells, 5.3% contained structures related to particles. Since the fraction of cells with particles was approximately similar to that with Tf1-WT, these data indicate that Tf1 Gag is sufficient for the assembly of spherical particles. However, it was surprising that particles produced by Tf1-PRfs were often found in the nucleus whereas wild-type particles were observed only in the cytoplasm. Even more interesting was the observation that the nuclear particles appeared to associate with heterochromatin (Fig. 5G).

**Gag is required for packaging of the Tf1 RNA.** One principal role of Gag proteins is to assemble into particles that package the RNA of the retroelement. We therefore tested whether Tf1 Gag was required for packaging of the Tf1 RNA. Since retrotransposons do not release their VLPs into culture medium, measuring packaged mRNA in purified VLPs has been hindered by the difficulties of isolating particles free of cellular transcripts. The approach taken here to address whether Gag is required for packaging of Tf1 mRNA was to use a previously developed assay that detects the amount of Tf1 RNA that is

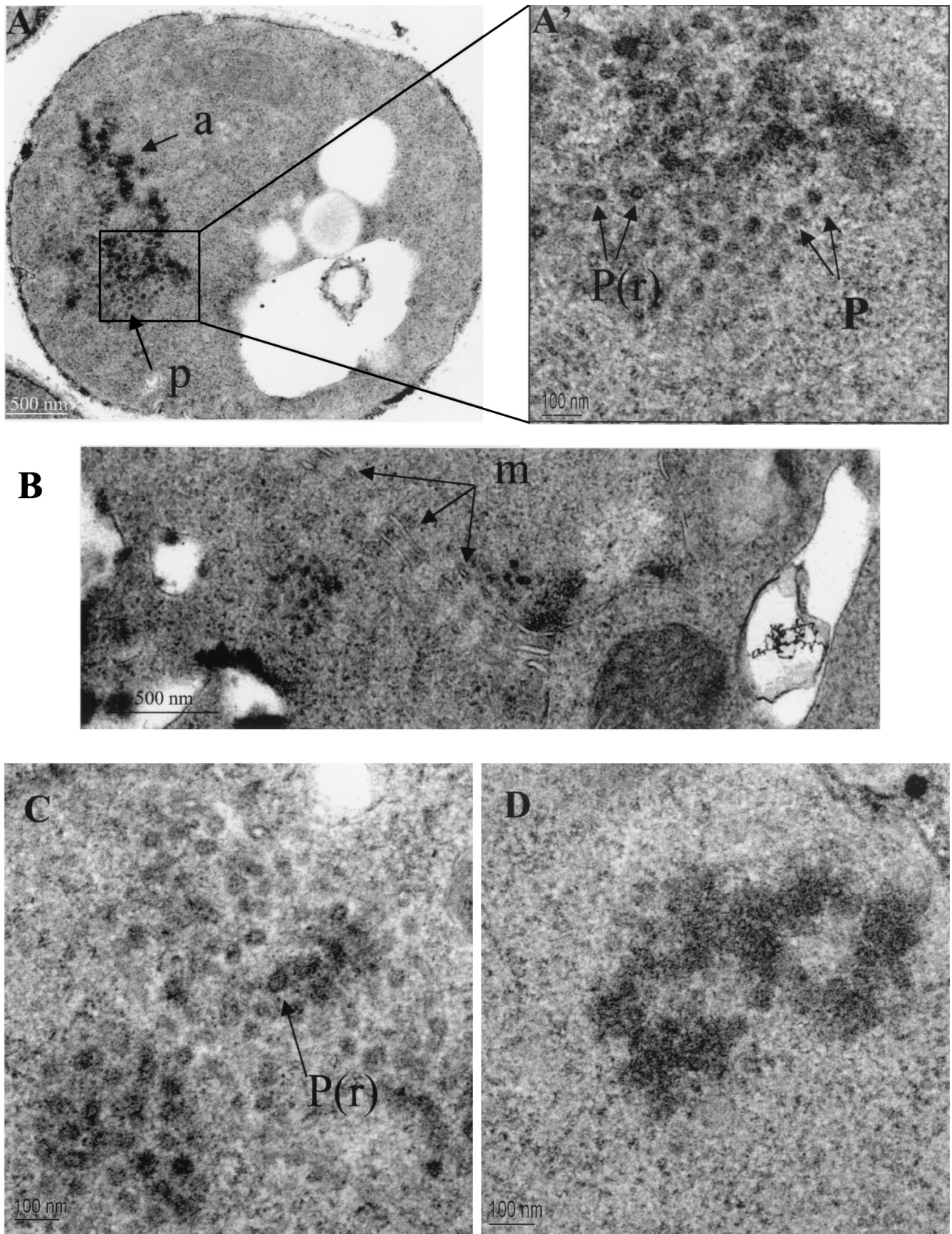


FIG. 5. Electron micrographs of *S. pombe* cells expressing Tf1-WT (A, A', B, C, and D), Tf1-PRfs (G and G'), Tf1- $\Delta$ A (F), and Tf1- $\Delta$ D (E and E'). A', E', and G' are higher magnifications of the images shown in A, E, and G, respectively. a, aggregates of Tf1 material; P(r), particles with dense rings; P, particles without rings; m, membranes; n, heterochromatin; nm, nuclear membrane.



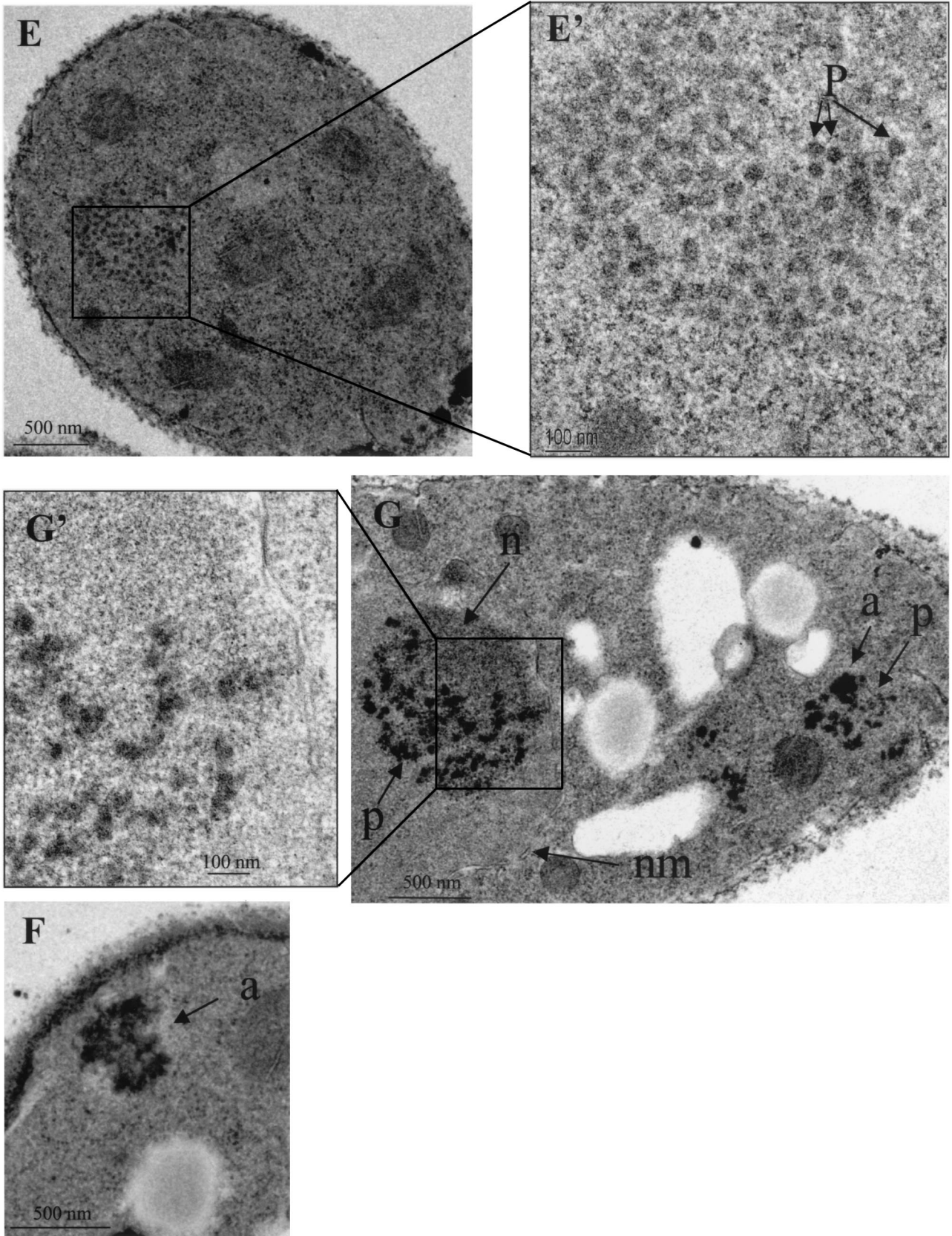


FIG. 5—Continued.

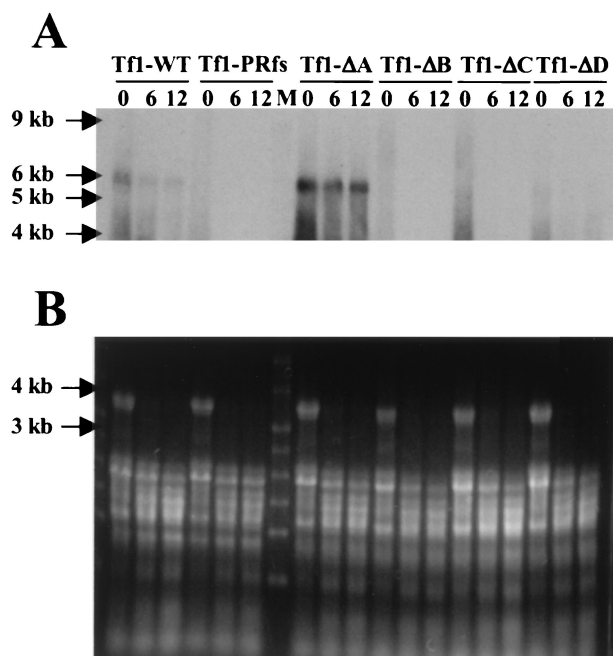


FIG. 6. RNase protection assay to detect packaging of Tf1 RNA in VLPs. (A) Cellular extracts containing VLPs were treated with various amounts of Benzonase (0, 6, or 12 U, lanes 0, 6, and 12, respectively) for 6 min at room temperature. RNA was then transferred to membranes and subjected to RNA blot analysis with a probe specific for the Gag region of Tf1. (B) The total RNA was visualized by ethidium bromide staining of the agarose gel under UV light.

protected from degradation by the nuclease Benzonase (40). Cellular extracts containing VLPs were subjected to digestion with various concentrations of Benzonase for 6 min. The resistant RNA was isolated from each digested sample by phenol extraction and analyzed by RNA blot analysis. An ethidium bromide stain shows that for each condition, equal amounts of digested RNA were loaded (Fig. 6A). The RNA was transferred to a membrane that was hybridized with a probe for the Tf1 RNA. As previously described, the Tf1 RNA from Tf1-WT shows significant resistance to Benzonase treatment (Fig. 6A) (40). It was also shown previously that this protection from degradation was specific for the Tf1 RNA, since the ribosomal and actin RNAs were degraded. Here too, we found that the ribosomal RNAs were not protected from degradation (Fig. 6B). Although the electron microscopy indicated that Tf1-PRfs formed spherical particles, the RNA was not packaged (Fig. 6A). This suggests that Tf1 Gag is not sufficient for the packaging of Tf1 RNA. The strains expressing Tf1-ΔB and Tf1-ΔC were unable to protect RNA from degradation, and this corresponded to the reduced levels of Gag (Fig. 4) and the lack of particles detected by electron microscopy. The Gag protein of Tf1-ΔD also exhibited a significant defect in RNA packaging. This lack of RNA packaging correlated well with the reduced levels of cDNA produced by Tf1-ΔD (Fig. 3). The packaging of RNA by the Gag protein from Tf1-ΔA was perhaps the most surprising. Since we identified a defect in particle assembly, we expected that the Tf1 RNA would not be packaged. Instead, the result obtained demonstrated that the deletion in Tf1-ΔA significantly increased the protection and stability of the RNA.

## DISCUSSION

In this study, we addressed whether the 27-kDa protein of Tf1 encoded by the segment of the ORF corresponding to the N terminus of the polyprotein was the functional equivalent of the Gag proteins of retroviruses and LTR-containing retrotransposons. Although the position of the Tf1 protein at the N terminus of the polyprotein is similar to that of Gag proteins, very little was known about its function. Our experiments tested the putative Gag of Tf1 for typical functions of Gag, such as roles in reverse transcription, the assembly of spherical particles, and the packaging of RNA.

Our initial characterization of Tf1 Gag was to determine whether the protein was required for transposition. The comparison of sequences from Tf1 and the related element, Tf2, revealed that 36% of the amino acids in the Tf1 and Tf2 Gags were identical. That 45% of the conserved residues were encoded by synonymous codons suggested strongly that the proteins possessed a critical function. This was supported by the high  $K_s/K_a$  ratio.

The indications that the Gag of Tf1 possessed an important function were supported by our study of four Tf1 elements with deletions in the conserved hydrophilic domains of Gag. Each of these deletions significantly reduced the ability of Tf1 to transpose and indicated that Gag possessed activity necessary for the process of transposition. The most dramatic defects in transposition were observed in the cases of Tf1-ΔA, Tf1-ΔB, and Tf1-ΔC. Tf1-ΔD showed trace levels of transposition activity above that of Tf1-INfs.

We explored the individual functions of Gag by characterizing the nature of the defects caused by each deletion. For each of the four Tf1 elements with deletions in Gag, we measured the production of Gag, RT, IN, and cDNA (Table 4). Tf1-ΔA produced wild-type levels of cDNA. As indicated above, the defect in transposition despite the wild-type levels of cDNA correlated well with the likely possibility that Tf1-ΔA had a defect in nuclear import due to the deletion of the critical NLS sequence (16). The critical residues of the NLS were KRIR, and when these were removed by a deletion of the first 10 amino acids of Gag, the frequency of transposition was significantly reduced. In addition, it was shown that the deletion of the first 10 residues and the replacement of them with a Flag tag caused a defect in the nuclear localization of Gag (16). As was found with Tf1-ΔA, no reduction in cDNA or Tf1 proteins was observed in this case.

Our study of protein levels identified severe defects in the expression of Gag by Tf1-ΔB and Tf1-ΔC. This lack of Gag correlated with the significant reduction in cDNA levels for Tf1-ΔB and Tf1-ΔC. The elements Tf1-ΔA, Tf1-ΔB, and Tf1-ΔD accumulated slightly smaller amounts of RT and IN (Table 4). The reduced levels of RT were not sufficient to cause the defects in cDNA levels since Tf1-ΔA had reduced RT levels but nevertheless made wild-type amounts of cDNA. In addition, we know that the reduced levels of IN could not have caused defects in reverse transcription since Tf1-INfs produced normal levels of cDNA.

The impact of the reduced levels of protein on particle formation was examined by electron microscopy. A deletion that greatly reduced levels of Gag, ΔC, also resulted in the absence of spherical particles and aggregates, even though IN



TABLE 4. Summary of Gag mutant phenotypes<sup>a</sup>

Tf1 element	Transposition	cDNA synthesis	Gag	RT	IN	Particle formation	RNA packaging
Tf1-WT	+++	+++	+++	+++	+++	Cytoplasmic particles and aggregates	+++
Tf1-PRfs	-	-	+++	-	-	Cytoplasmic and nuclear particles and aggregates	-
Tf1-ΔA	-	+++	+++	++	++	Cytoplasmic aggregates	++++
Tf1-ΔB	-	-	-	++	++	Not done	-
Tf1-ΔC	-	-	-	+++	+++	No particles	-
Tf1-ΔD	+	+	+++	++	++	Cytoplasmic particles and aggregates	-

<sup>a</sup> +, very little; ++, less than the wild type; +++, wild type; +++++, more than the wild type; -, not detected.

and RT were expressed at wild-type levels. The results of RNA packaging assays for Tf1-ΔB and Tf1-ΔC indicate that Gag expression was required for particle formation. Tf1-ΔB and Tf1-ΔC produced no cDNA, indicating that reverse transcription was dependent on particle formation. Tf1-ΔD made spherical particles clearly visible by electron microscopy. It was therefore surprising that the particles were unable to package the Tf1 RNA. In their entirety, these data demonstrate that Tf1-ΔB and Tf1-ΔC are defective for transposition because the reduced levels of Gag resulted in the absence of particles. Without particles, the Tf1 RNA could not be packaged or reverse transcribed. Although the proteolytic processing of the Tf1 proteins produced by Tf1-ΔB and Tf1-ΔC suggests that Gag-Gag associations occur, the reduced levels of Gag indicate that any particles that did form were unstable. In addition, the data demonstrate that Tf1-ΔD was defective for transposition not because of a lack of particles but because the deletion in Gag blocked the packaging of the RNA.

The mutation in Tf1-ΔD caused a defect in reverse transcription and in the packaging of RNA. This indicates that Gag plays an important role in reverse transcription by packaging the Tf1 RNA into the particles. The deletions in Tf1-ΔB and Tf1-ΔC resulted in low levels of Gag expression, and taken together with the lack of spherical particles, these data demonstrate that Gag is required for the formation of VLPs. More importantly, the expression of Tf1-PRfs, the element that does not produce RT or IN, revealed that the accumulation of Gag is sufficient for the assembly of spherical particles. Our findings that Gag is required and sufficient for particle formation and that it participates in the packaging of its RNA correlate well with the functions of the Gag proteins of retroviruses. As a result, it is likely that the Gag of Tf1 is a functional equivalent of the Gags encoded by other LTR-containing retroelements.

The characterization of Tf1 with deletions in Gag also contributed to our understanding of how Tf1 enters the nucleus. The Tf1-ΔA protein lacks the N-terminal Gag residues whose replacement with the Flag epitope resulted in a defect in entry into the nucleus (16). The deletion in Tf1-ΔA also caused a defect in transposition, and this too was likely due to a block of import into the nucleus. The DNA blot demonstrated that Tf1-ΔA produced normal levels of cDNA, and the homologous recombination assay indicated that the cDNA was not transported into the nucleus.

The results of the electron microscopy demonstrated that spherical particles produced by Tf1-WT in stationary-phase cells were observed only in the cytoplasm. Previous results of immunofluorescence microscopy revealed that under these

conditions, the bulk of Gag is localized in the nucleus (7, 15). These observations are consistent with a process of particle disassembly that obscures the visualization by electron microscopy of Gag in the nucleus. The disassembly of particles could occur before or after transit through the nuclear pores. This is an important question because the size of Tf1 particles, 50 nm, is larger than the maximum diameter of the channel, 20 to 25 nm, as measured by testing nucleoplasm-coated gold particles of various sizes for the ability to pass through the nuclear pore (20, 45). However, the mutation in Tf1-PRfs resulted in large numbers of spherical and aggregated particles within the nucleus, suggesting that particles were able to pass through the pores. It is therefore possible that the particles reorganize to fit through the pore or that the pore itself has the capability to accommodate the particles. Nevertheless, the presence of particles in the nucleus indicates that Tf1 particles have the ability to pass through the nuclear pore without first being disassembled. The ability of Tf1 particles to pass through the nuclear pore intact is not surprising since evidence indicates that the 50-nm particles of simian virus 40 and the 39-nm particles of human hepatitis B virus pass intact through nuclear pores (49, 63).

The electron micrographs of cells expressing Tf1-PRfs not only showed particles in the nucleus but also revealed a surprising association between the particles in the nucleus and heterochromatin. This association of the particles with heterochromatin correlates well with results of immunofluorescence microscopy that show Gag localized in the nucleolus (M. Kim and H. Levin, unpublished data). Although the biological significance of this is not clear, it indicates that Gag possesses sequences that either target the protein to the nucleolus or cause its retention there. In addition, the recent finding that Tf1 integration exhibits a preference for the third chromosome, which contains the rRNA gene repeats, suggests that the localization of Tf1 protein in the nucleolus may have mechanistic importance (54; N. Bowen, unpublished data).

The deletion in Tf1-ΔA resulted in a surprising combination of effects. This deletion removed the NLS and should have blocked import into the nucleus. The decrease in transposition activity and the drop in homologous recombination between cDNA and plasmid sequences correlate well with a defect in import. Tf1-ΔA produced normal levels of Gag and cDNA, suggesting that the lack of import is the only defect caused by the mutation. However, the electron microscopy demonstrated that cells expressing Tf1-ΔA contained only aggregates and not spherical particles (Table 4). This defect in particle structure may not be the result of removing the NLS but may instead be



due to alterations in Gag-Gag and/or Gag-Pol interactions. One unexpected result is that despite the disruption of the particle structure, the Gag of Tf1- $\Delta$ A was able to package dramatically higher levels of Tf1 RNA than the wild type (Fig. 6). Although we have no clear explanation for this, one possibility is that the deletion of residues 2 through 11 results in a Gag that assembles with an increase in affinity for RNA. Another hypothesis is that Tf1- $\Delta$ A packages more RNA per particle than wild-type Tf1.

The assay for packaging of Tf1 RNA revealed additional information about the role of Gag in protecting the RNA. Although the expression of Tf1-PRfs produced some spherical particles, no packaging was observed. This indicates that the formation of particles is not sufficient for packaging of Tf1 RNA. It also suggests that the expression of Gag alone is not sufficient and that RT and/or IN may be required for packaging to occur. Alternatively, the frameshift mutation in PR caused approximately 50 residues to be fused to the C terminus of Gag, and these could have perturbed the packaging of RNA. More important is the result that Tf1- $\Delta$ D allows the formation of spherical particles that are indistinguishable from those of the wild type. Surprisingly, these particles are severely defective for packaging of Tf1 RNA. Thus, the formation of highly ordered particles is not sufficient for packaging of Tf1 RNA. Instead, it is likely that residues of Gag interact with the RNA and protect it from degradation. The residues removed in the Gag of Tf1- $\Delta$ D are the first amino acids found to participate in the packaging of the RNA. Given the lack of Zn fingers and NC in Tf1 Gag, the residues removed in Tf1- $\Delta$ D are the best candidates for domains of interaction with Tf1 RNA. It is also possible that the deletion in Tf1- $\Delta$ D reduced interactions with the Tf1 cDNA. This may explain the surprising result that despite low levels of transposition activity, Tf1- $\Delta$ D produced significant levels of homologous recombination between cDNA and plasmid sequences. Reduced association of the cDNA with Gag could increase exposure of the cDNA to the recombination machinery.

The presence of several basic amino acids in the region deleted in Tf1- $\Delta$ D and their location at the C terminus are two significant similarities to the unusual nucleic acid binding site in the Gag of spumavirus (66). The presence of basic residues in the C terminus of Gag has also been observed in *gypsy*, and because this endogenous retrovirus also lacks Zn fingers, these basic residues have been proposed to function in nucleic acid binding (2). It is compelling to think that spumavirus and Tf1 represent two LTR-containing retroelements that have not adopted Zn finger structures to bind RNA but instead may have convergently evolved charged structures that mediate RNA interactions.

The unusual features of Tf1, such as the single ORF, the self-priming mechanism of reverse transcription, and the lack of sequence similarity to the Gags of LTR-containing elements, has led to the speculation that Tf1 may transpose by a pathway significantly different from that of the other well-studied elements in yeast, Ty1 and Ty3. The results of the electron microscopy used here to examine VLPs in *S. pombe* represent the first report that Tf1 does produce highly ordered, spherical particles. This is strong evidence that Tf1 transposition does indeed occur via intermediates similar to those of other LTR-containing retroelements.

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