JOURNAL OF VIROLOGY, Aug. 1996, p. 5548–5556 Vol. 70, No. 8
0022-538X/96/$04.00+0

A Critical Proteolytic Cleavage Site near the C Terminus of the Yeast Retrotransposon Ty1 Gag Protein

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Received 7 February 1996/Accepted 17 April 1996

Cleavage of the Gag and Gag-Pol polyprotein precursors is a critical step in proliferation of retroviruses and retroelements. The Ty1 retroelement of *Saccharomyces cerevisiae* forms virus-like particles (VLPs) made of the Gag protein. Ty1 Gag is not obviously homologous to the Gag proteins of retroviruses. The apparent molecular mass of Gag is reduced from 58 to 54 kDa during particle maturation. Antibodies raised against the C-terminal peptide of Gag react with the 58-kDa polypeptide but not with the 54-kDa one, indicating that Gag is proteolytically processed at the C terminus. A protease cleavage site between positions 401 and 402 of the Gag precursor was defined by carboxy-terminal sequencing of the processed form of Gag. Certain deletion and substitution mutations in the C terminus of the Gag precursor result in particles that are two-thirds the diameter of the wild-type VLPs. While the Ty1 protease is active in these mutants, their transposition rates are decreased 20-fold compared with that of wild-type Ty1. Thus, the Gag C-terminal portion, released in the course of particle maturation, probably plays a significant role in VLP morphogenesis and Ty1 transposition.

The Ty1 retroelement of *Saccharomyces cerevisiae* produces virus-like particles (VLPs) which never leave the cell (15, 23). There are about 25 copies of the transposon per genome, and it is not known how the abundance of Ty1 elements in the genome is regulated (3). Ty1 resides in the genome as a 6-kb-long piece of DNA flanked by two long terminal repeats (5, 11). The Ty1 RNA is then transported into the cytoplasm and translated to form two primary translation products, Gag (58 kDa) and Gag-Pol (190 kDa). These are assembled with Ty1 RNA into VLPs (15). There is a 7-bp frameshift signal located about 15 bp upstream of the Gag stop codon. The +1 frameshift occurs at the sequence CUU AGG C, resulting in the synthesis of the Gag-Pol readthrough protein (12, 17). Gag-Pol is a polyprotein containing Gag protein, protease (PR), integrase (IN), reverse transcriptase (RT), and RNase H. PR is presumably active as a part of the polyprotein. The pathway of proteolytic cleavage of Gag-Pol was studied by using antibodies raised against fragments of Gag-Pol. The Gag portion of Gag-Pol is probably released first in Gag-Pol processing, when the 190-kDa polyprotein is severed somewhere near the frameshift region. Released p160-Pol is normally cleaved to yield PR and p140-Pol. The 140-Pol intermediate is subsequently processed into 90-kDa IN and 60-kDa RT (16). RT (1, 27, 30) and IN (7) are also active in the form of the polyprotein. PR^- mutants do not transpose, implying that either Gag cleavage or Gag-Pol processing or both are critical for transposition (25, 30). The proteolytic cleavage pathway suggests at least three cleavage sites in the Gag-Pol polyprotein. Two of these sites determined by N-terminal sequencing of the purified IN and RT proteins have been reported elsewhere (6, 26).

The Gag protein is a major structural component of VLPs. It consists of 440 amino acids with a predicted molecular mass of 48,979 Da. However, the apparent molecular mass of unprocessed Gag is about 58 kDa as determined by electrophoresis in a denaturing polyacrylamide gel. The apparent molecular mass of Gag is reduced to 54 kDa during particle maturation, suggesting removal of a 4-kDa peptide.

Assembly of VLPs is a hallmark of Ty transposition (10). Although they never leave the cell, changes in their shape and size correlate with changes in transposition rates. The VLPs are spherical to ovoid particles with a mean diameter of 60 nm. Each particle is estimated to have a molecular mass of 14 to 17 MDa (9, 10). A mutation in Gag that affects morphogenesis eliminates transposition, providing direct evidence that VLP assembly is important for transposition (8, 25).

The RNA is believed to be embedded in the VLP shell (10). DNA intermediates may be found in the purified particles, suggesting that reverse transcription takes place in the VLPs (14). VLPs can be purified from the cells by fractionation in sucrose gradients. Since the VLPs prepared from cells expressing Ty1 PR^- mutants are more fragile and more diverse in size than the wild-type particles, Gag processing is probably important for transposition (8, 25).

We generated antibodies against the C-terminal portion of the 58-kDa Gag precursor and demonstrated that these interact only with the Gag precursor and not with the 54-kDa derivative, indicating the C-terminal cleavage of Gag. The C terminus of the 54-kDa Gag has been sequenced, and the PR cleavage site in the Gag precursor has been inferred from these data. Systematic mutagenesis of the Gag C terminus confirmed the location of this cleavage site and revealed short sequences critical for transposition and particle morphogenesis, suggesting that the Gag C terminus removed in the course of particle maturation plays an important role in Ty1 transposition. Finally, substitutions in the Gag cleavage site block both Gag and Gag-Pol processing as well as Ty1 transposition, suggesting that the cleavage near the N terminus of the PR domain is

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required to produce active PR able to cleave the other two sites in the Gag-Pol polyprotein.

MATERIALS AND METHODS

Yeast strains. All experiments except particle analysis were done using yeast strain YH8 (*MAT*α *ura3-167 his3Δ200 leu2Δ1 trp1Δ1*) (29). To prepare large amounts of particles, strains carrying plasmids that encode wild-type or mutant Ty1 were crossed with strain BY373 (*MAT***a** *ura3-52 his3Δ200*).

Vectors and plasmids. All the mutants were constructed on the basis of the pJEF1105 expression vector (see Fig. 1). Deletion mutant *d*1 was constructed by a three-piece ligation of pJEF1105 cleaved with *Hpa*I and *Bst*EII with two PCR fragments. The first PCR fragment was synthesized by using pJEF1105 vector as a template and oligonucleotides JB487 (5′ ACGGACACCCATCTATGATT CCG 3′) and JB899 (5′ GCGCGGCGCGCCGGATACATTGTGAGCCCT GGC 3′). The second fragment was synthesized by using pJEF1105 as well as oligonucleotides JB924 (5′GCGCGGCGCGCCTCTTAGGCCAGAAACTTAC TGAA 3′) and JB10 (5′ ATGTCGGATTGGAAGTC 3′). The first fragment was cleaved with endonucleases *Hpa*I and *Asc*I, the second one was digested with *Asc*I and *Bst*EII, and both fragments were ligated simultaneously into pJEF1105 precleaved with *Hpa*I and *Bst*EII. Thus, amino acids 405 through 434 were substituted by the peptide sequence GAP in the *d*1 mutant.

Block substitution mutants were made by the same basic strategy. The first PCR fragment was synthesized by using pJEF1105 as a template and oligonucleotides JB487 and JB1093 (5′ GCGGATCCAGCAGCATTCCGAGCTATA ACTTTGGG 3′). The second fragment was synthesized by using pJEF1105 as well as oligonucleotides JB1094 (5′ GCGGATCCGCTGCTTCGAAATCGAA AACAGCCAGG 3′) and JB10. The first fragment was cleaved with *Hpa*I and *Bam*HI, the second one was digested with *Bam*HI and *Bst*EII, and both fragments were ligated simultaneously into pJEF1105 precleaved with *Hpa*I and *Bst*EII. Thus, amino acids 387 through 392 were substituted by the peptide sequence AAGSAA in the *s*1 mutant as shown in Fig. 3. Mutants *s*2 through *s*8 as well as *d*2 and *d*3 were assembled in a similar way.

To introduce single amino acid substitutions in the Gag cleavage site, the Ty1 fragment between the *Xho*I and *Sal*I sites was subcloned into the pBluescript KS+ vector (Stratagene) and site-directed mutagenesis was performed on this plasmid as described elsewhere (19). Oligonucleotide JB1116 (5′ TACATTGT GAGCAATGGCTGTTTTCGA 3′) was used to change two nucleotides (underlined), (R401I mutant). Oligonucleotide JB1117 (5′ GGATACATTGTGAATC CTGGCTGTTTT 3′) was used for the *s*3.2 mutant, JB1118 (5′ TGTGGATAC ATTGATAGCCCTGGCTGT 3′) was used for *s*3.3, JB1119 (5′ AGATGTGG ATACAATGTGAGCCCTGGC 3′) was used for *s*3.4, JB1120 (5′ ATTAGAT GTGGAAATATTGTGAGCCCT 3′) was used for *s*3.5, and JB1121 (5′ GTTA TTAGATGTGATTACATTGTGAGC 3′) used for *s*3.6. After mutants were identified by sequencing, the Ty1 fragments between the *Hpa*I and *Bst*EII sites were cloned back into the pJEF1105 vector. All six point mutations were verified by sequencing.

Immunoblotting. Cultures were grown at either 22 or 30°C. The starting density was always an A_{600} of 0.5, and the cells were collected when the density reached an A_{600} of 2 (about 16 h at 30°C and about 36 h at 22°C). Cultures (0.5 ml) were collected by centrifugation and resuspended in 40 μl of buffer B (10 mM *N*-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 8.0], 15 mM KCl, and 5 mM EDTA). Glass beads were added to the meniscus, and the tubes were vortexed at maximal speed at 4°C for 10 min. Forty microliters of 2× (sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer was added, and the tubes were shaken and boiled for 2 min. Cell debris was pelleted by centrifugation for 5 min at 12,000 rpm in an Eppendorf microcentrifuge, and 5 μl of the supernatant containing 5 μg of protein was loaded onto the gel.

Proteins were transferred onto Immobilon membranes (Millipore) at 900 mA for 1 h. Membranes were blocked in 15 ml of phosphate-buffered saline (PBS) buffer containing 5% milk, washed three times in PBS, and incubated with antibodies in PBS. Anti-Gag and anti-IN antibodies have been described earlier (14, 25). After three subsequent washes in PBS, filters were incubated with secondary antibodies and then with enhanced chemiluminescence fluorescent reagent (Amersham) and exposed to film.

To raise the antibodies against the Gag C terminus, peptide JB2 (KSTT EPIQLNNKHDLHLRPETY) was coupled to thyroglobulin by using glutaraldehyde. Two milliliters of 0.2% glutaradehyde in PBS was slowly added to 2 ml of a solution of 5-mg/ml JB2 and 12.5-mg/ml thyroglobulin in PBS. After 1 h of incubation at room temperature, 1 M glycine in PBS (pH 7.2) was added to a final concentration of 200 mM. This was incubated for 1 h at room temperature, after which the conjugate was separated from free peptide by dialysis against PBS.

Transposition assay. YH8 yeast cells were transformed to Ura^+ with plasmids carrying wild-type and mutant Ty1 retroelements. Transformants were patched on synthetic complete medium lacking uracil (SC-Ura) glucose plates, then replica plated onto SC-Ura galactose plates, and incubated at 22°C for 48 h to induce transposition. The patches were then replica plated onto yeast extract-peptone-dextrose (YPD) nonselective medium to allow loss of the donor plasmid. Cells that lost the donor plasmid were selected by replica plating onto SC–5-FOA glucose medium and finally plated onto YPD medium containing 75 μg of G418 per ml to select for the cells that acquired Ty1 in the genome.

VLP isolation. Cell pellets from 500-ml cultures grown at 22 or 30°C were resuspended in 5 ml of buffer B and lysed with glass beads at 4°C. The extract was clarified by centrifugation at 17,000 rpm in a Sorvall SS-34 rotor for 10 min. Supernatant (5 ml) was loaded on a preformed linear 20 to 70% sucrose gradient in buffer B and centrifuged for 18 h at 25,000 rpm at 4°C in a Beckman SW28 rotor. Gradients were fractionated as described previously (14).

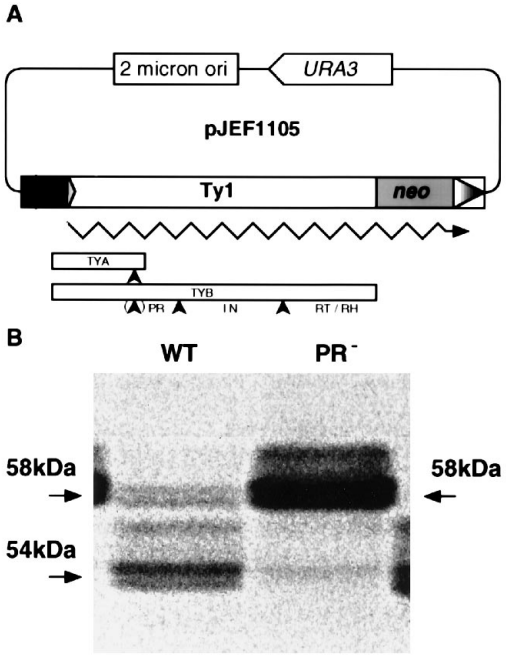
Electron microscopy. Freshly discharged carbon-coated grids were floated for 30 s on the surface of a 60% sucrose solution containing VLPs. The grids were then floated on a surface of buffer B solution and, finally, on the surface of a 1% solution of uranyl acetate in water. Grids were examined by transmission electron microscopy using a Zeiss electron microscope.

Protein sequencing. To purify the 54-kDa Gag, we first isolated VLPs from cells grown at 30°C. The VLP proteins were then separated on a 10% polyacrylamide gel and transferred onto a Teflon membrane. A band containing 54-kDa Gag was excised, and the membrane-bound protein was subjected to five cycles of sequential C-terminal degradation performed on a Hewlett-Packard model 1009A sequencer using the 2.0 routine chemistry. The chemistry of the C-terminal sequencing method used for Gag sequencing has been described elsewhere (2, 24).

RESULTS

Multiple Gag species detected in the cells expressing wild-type Ty1. To study Gag processing and its role in transposition, we expressed a wild-type Ty1 and 18 mutant Ty1 retrotransposons in yeast cells using the pJEF1105 expression vector (Fig. 1). This plasmid consists of the Ty1 retroelement fused to the inducible *GAL1* promoter. Since the 5′ long terminal repeat of this Ty1 is truncated, the Ty1 lacks its own promoter and transcription is driven entirely by the *GAL1* promoter. When cells carrying pJEF1105 are grown on galactose-containing media, transcription from the *GAL* promoter is induced and transposition of the plasmid-encoded Ty1 element can occur at an average frequency of more than one event per cell. The frequency of genomic Ty1 transposition is only about 10^{-4} to 10^{-6} per Ty element per cell division (13). Although Ty1 RNA is abundant in a yeast cell, Ty1 proteins and VLPs are present at disproportionally low levels in normal cells. Hence, overexpression of the Ty1 element in the cell enhances the frequency of transposition and therefore facilitates the study of Ty1 transposition. Both 58- and 54-kDa forms of Gag protein were detected by immunoblot analysis in the cells expressing wild-type Ty1, while only the 58-kDa species was detected in the cells expressing PR^- mutant Ty1 (Fig. 1B), indicating that the Gag protein is proteolytically processed by the Ty1 PR.

Antibodies raised against a C-terminal peptide react with 58- but not 54-kDa Gag. To prove that the 54-kDa form of Gag is a product of the C-terminal proteolytic cleavage of the 58-kDa Gag precursor, we synthesized a peptide with a sequence corresponding to amino acids 420 through 440 of the Gag precursor and raised rabbit antibodies against the synthetic peptide, referred to as the JH695 serum. The JH695 serum detects only the 58-kDa form of Gag in the extracts prepared either from the cells expressing wild-type Ty1 or from the cells expressing PR^- mutant Ty1 (Fig. 2B). To ensure that the reaction between the JH695 antibodies and the Gag precursor was specific, we also incubated the membrane-bound proteins with the pre-immune-phase serum (Fig. 2C). The pre-immune-phase serum detected no proteins in the cell extracts, proving that the JH695 antibodies bind the Gag precursor specifically. In contrast, the anti-Gag antibody R1-1 detects both 58- and 54-kDa forms of Gag in the extracts prepared from the cells expressing wild-type Ty1 (Fig. 2A). Thus, the JH695 antiserum detects only the 58-kDa form of Gag in the cells expressing wild-type or PR^- mutant Ty1, while R1-1 antiserum detects both Gag species in the cells expressing wild-type Ty1 but only the 58-kDa form in the cells expressing mutant Ty1 (Fig. 2A and B). Taken together with earlier results (4), these results



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Mutant	Plasmid	Strain
wt	pJEF1105	MY198
PR⁻	pJEF1105 PR⁻	MY199
d1	pJEF1105 d1	MY201
d2	pJEF1105 d2	MY202
d3	pJEF1105 d3	MY203
s1	pJEF1105 s1	MY204
s2	pJEF1105 s2	MY205
s3	pJEF1105 s3	MY206
s4	pJEF1105 s4	MY207
s5	pJEF1105 s5	MY208
s6	pJEF1105 s6	MY209
s7	pJEF1105 s7	MY210
s8	pJEF1105 s8	MY211
s3.1	pJEF1105 s3.1	MY212
s3.2	pJEF1105 s3.2	MY213
s3.3	pJEF1105 s3.3	MY214
s3.4	pJEF1105 s3.4	MY215
s3.5	pJEF1105 s3.5	MY216
s3.6	pJEF1105 s3.6	MY217

FIG. 1. (A) Genetic map of plasmid pJEF1105. Mutants were constructed on the basis of plasmid pJEF1105. Black box, *GAL1* promoter; boxed triangles, LTR sequences; *neo*, Ty1 marker; *URA3*, vector selectable marker; 2 micron ori, yeast 2 μm plasmid origin of replication; PR, IN, and RT/RH, regions of homology with retroviruses (RH, RNase H); arrowheads, PR cleavage sites. Plasmid backbones are not drawn to scale. (B) Gag species in the cells expressing wild-type Ty1 (WT) or Ty1 PR$^-$ mutant. (C) Plasmids and strains.

provide strong evidence that the 54-kDa species of Gag results from proteolytic cleavage of the Gag precursor at the C terminus and not from proteolytic cleavage at its N terminus or from posttranslational modification.

C-terminal sequencing of the 54-kDa Gag protein. The scissile bond of the PR cleavage site in the Gag protein could be identified either by C-terminal sequencing of the 54-kDa product or by N-terminal sequencing of the putative C-terminal peptide released upon proteolysis. Since such a 4-kDa peptide has not yet been detected in cells expressing wild-type Ty1, we sequenced the C terminus of purified 54-kDa protein. To purify the 54-kDa Gag, we first isolated VLPs. The 54-kDa form of Gag normally represents 80 to 90% of a typical VLP preparation. The VLP proteins were then separated on an SDS–10% polyacrylamide gel and transferred onto a Teflon membrane. A band containing 54-kDa Gag was excised, and the membrane-bound protein was subjected to five cycles of automated C-terminal protein sequencing (24). The amino acid derivatives released in every cycle of sequential degradation were determined by high-performance liquid chromatography. Histidine was released in the first cycle and was followed by alanine, arginine, and, once again, alanine (Fig. 3A). The ARAH peptide occurs only once in the Gag sequence at an appropriate position, and therefore a PR cleavage site in the Gag precursor could be unambiguously inferred from the position of the C-terminal sequence of processed Gag. The scissile bond is located between amino acids 401 and 402 of the Gag precursor. Since the amino acid sequence corresponding to the Gag cleavage site is located upstream of the frameshift signal of Ty1, it occurs in both Gag and Gag-Pol and therefore could be recognized and cleaved by Ty1 PR not only in Gag but also in Gag-Pol. Cleavage of the Gag protein is an intermolecular reaction, while cleavage at this site in Gag-Pol could be either intramolecular or intermolecular. Two other PR cleavage sites had been identified in the Ty1 Gag-Pol polyprotein previously, one between PR and IN and another between IN and RT. Although each processing site sequence is unique, all the PR sites found in Gag-Pol have a Val residue at the P2′ position. Position P2 is occupied by isoleucine in two PR cleavage sites or by alanine in one site. The P1′ position is occupied either by asparagine in two PR cleavage sites or by alanine in one of them (Fig. 3A).

The hydrophobicity index of each amino acid in positions P10 through P10′ was determined for each of the three cleavage sites by method of Kyte and Doolittle (20), summed, di-

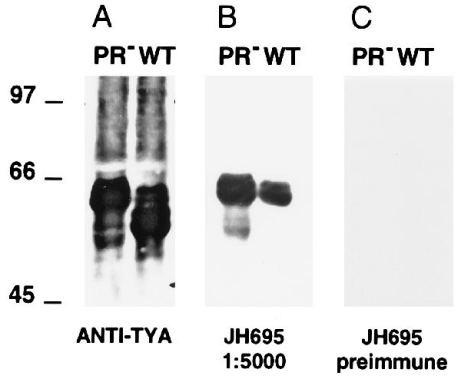

FIG. 2. Immunoblot analysis of Gag processing products with antibodies derived against the C terminus of the Gag precursor. Antiserum JH695 was raised against synthetic peptide JB2, the sequence of which corresponds to the 22 C-terminal amino acids of the Ty1 Gag precursor. Cultures carrying wild-type Ty1 (wt) or mutant Ty1 with the PR$^-$ mutation were grown in liquid SC-Ura galactose medium at 22°C. Cells were pelleted, lysed, and subjected to SDS-PAGE. Proteins were transferred onto Immobilon membranes, and the membranes were incubated with either anti-Gag antiserum diluted 1,000 times (A), JH695 antiserum diluted 5,000 times (B), or JH695 pre-immune-phase serum diluted 5,000 times (C).

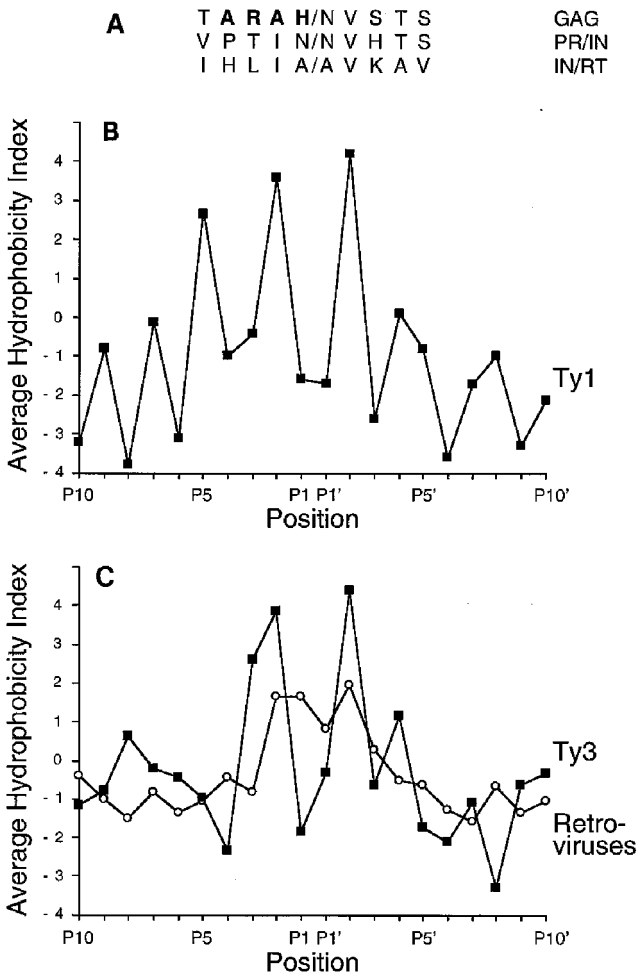

FIG. 3. Hydrophobicity profiles of the Ty1 protease cleavage sites. Hydrophobicity was determined by the method of Kyte and Doolittle for each amino acid in positions P10 through P10′, summed at each position, and divided by the number of sites. (A) C-terminal sequence of the processed Gag protein as determined by chemical sequencing (boldface, Ty1 PR cleavage sites); (B) analysis of the three Ty1 PR cleavage sites; (C) a similar analysis of five Ty3 PR cleavage sites and 46 retroviral PR sites according to previously published data. Squares, Ty3 sites; circles, retroviral sites.

vided by three, and plotted (Fig. 3B). Apparently, the P2 and P2′ residues are significantly more hydrophobic than the P1 and P1′ residues or amino acids flanking P2 and P2′. Interestingly, the hydrophobicity profile of the Ty1 cleavage sites is similar to that of the Ty3 cleavage sites (18) and markedly different from the hydrophobicity profile of retroviral cleavage sites (28) (Fig. 3B and C). Whereas the retroviral P1 and P1′ residues are hydrophobic, the retrotransposon P1 and P1′ residues are hydrophilic.

The Gag C terminus, released during particle maturation, may play a significant role in VLP morphogenesis and Ty1 transposition. To determine whether the C-terminal peptide removed from the Gag precursor is required for transposition, we have constructed 11 mutant Ty1 elements in which the C-terminal sequence of Gag has been altered. These include three deletion mutants, *d*1, *d*2, and *d*3, and eight block substitution mutants, *s*1 through *s*8, that were assembled by the strategy described in Materials and Methods. Amino acids 405 through 434 are substituted by the sequence GAP in the *d*1 mutant, amino acids 390 through 404 are substituted by the sequence AAGSAA in the *d*2 mutant, and amino acids 405 through 422 are substituted by the sequence AAGSAA in the *d*3 mutant. Substitution mutant *s*1 has amino acids 387 through 392 of Gag substituted by the peptide sequence AAGSAA. Substitution mutants *s*2, *s*3, *s*4, *s*5, *s*6, *s*7, and *s*8 have amino acids 393 to 398, 399 to 404, 405 to 410, 411 by 416, 417 to 422, and 423 to 428, and 429 to 434, respectively, substituted by AAGSAA. Thus, these 11 mutations cover the C-terminal region of Gag between amino acids 387 and 434 (Fig. 4). None of the mutations overlap with the 7-bp-long frameshift signal between the *gag* and *gag-pol* genes, so the Gag-Pol protein is expressed in all these mutants. If the cleavage between the Gag and PR domains in fact occurs at the same sequence as in the GAG precursor, then the N-terminal portion of PR would be altered in mutants *d*1, *d*2, *d*3, and *s*3 through *s*8. The proposed cleavage site between the Gag and PR domains would be altered in mutants *s*3 and *d*2.

Interestingly, the phenotypes associated with these 11 mutations can be divided into three groups. Mutations *d*3, *s*1, *s*2, *s*4, *s*5, and *s*6 do not block Gag processing (Fig. 5A and C) and do not lower Ty1 transposition rates more than twofold (Fig. 4D and 6). Mutations *d*1, *s*7, and *s*8 do not block PR cleavage (Fig. 5) but lower Ty1 transposition rates either more than 100-fold (*d*1) or 20-fold (*s*7 and *s*8) (Fig. 4D and 6). Mutations *d*2 and *s*3 block Gag processing (Fig. 5A and C) and lower Ty1 transposition rates more than 100-fold (Fig. 4D and 6).

The 90-kDa IN was detected in the cells expressing the *d*1, *s*7, or *s*8 mutant (Fig. 5B and D), while the 190-kDa Gag-Pol precursor was identified in these cells grown at either 22 or 30°C (Fig. 5B and D). The Gag protein was converted almost completely into the 54-kDa product in the cells expressing these mutants at both temperatures (Fig. 5A and C). Thus, no difference between the PR activity of these mutants and the PR activity of the wild-type Ty1 is revealed by immunoblot assays. Furthermore, Gag and Gag-Pol are processed more slowly in *s*2 and *s*4 than in *d*1, *s*7, or *s*8 (Fig. 5A and C), but the transposition was blocked in *d*1, *s*7, and *s*8 and not in *s*2 and *s*4 (Fig. 4D and 6). Therefore, changes in Gag structure rather than changes in PR activity are likely to account for the dramatic decrease of transposition rates in the mutants *d*1, *s*7, and *s*8.

VLPs purified from cells expressing the *d*1 or *s*7 mutant are considerably smaller than VLPs isolated from cells expressing wild-type Ty1. Electron microscopy of negatively stained wild-type particles shows VLPs as stain-excluding spherical shells of variable size (Fig. 7b). Diameters of 39 individual particles were measured and plotted as a histogram (Fig. 7b). Diameters of the wild-type particles varied from 30 to 70 nm, while the majority are 60 nm across. Particles prepared from *d*1 or *s*7 mutants are more homogeneous, with a mean diameter of 40 nm (Fig. 7b). Interestingly, the peaks of the *d*1, *s*7, and *s*8 particles are found nearer the top of sucrose gradients used in VLP preparation, suggesting that density of the *d*1 and *s*7 particles differs from that of wild-type VLPs (Fig. 7a). Compared to wild-type VLPs, *s*7 and *s*8 particles contain reduced amounts of Ty1 double-stranded DNA (data not shown). We also examined VLPs from mutants PR^-, *s*3, and *s*3.5, in which Gag and Gag-Pol do not undergo proteolytic processing. These VLPs exhibited a size distribution similar to that of wild-type VLPs. VLPs from PR^-, *s*3, and *s*3.5 are probably more fragile than the wild-type VLPs, since many more broken particles were found in the preparations from cells expressing PR^-, *s*3, and *s*3.5 mutants (data not shown). Thus, substitutions in the 17 C-terminal amino acids of the VLPs' major component correlate with the size reduction of the corresponding VLPs.

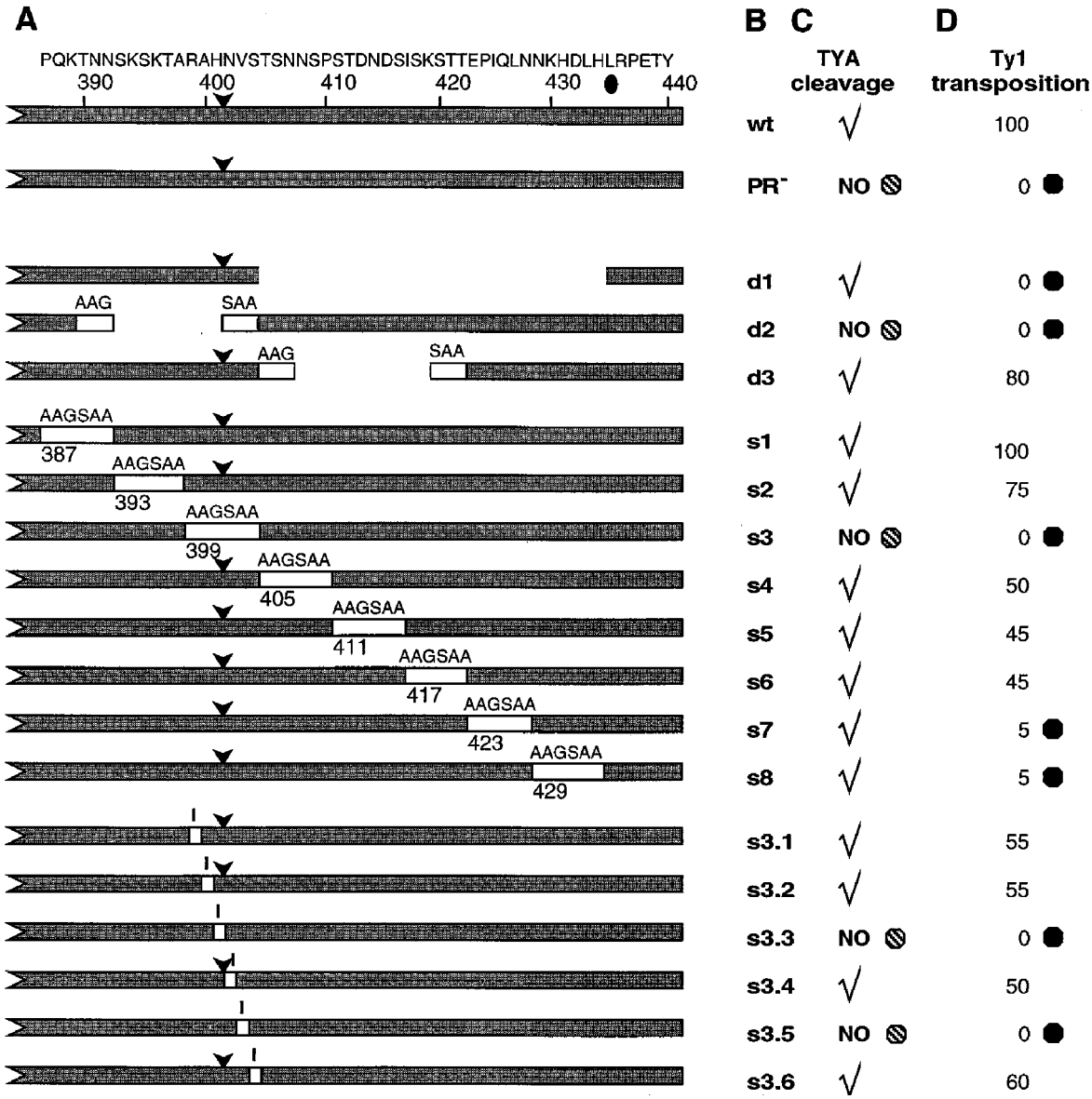

FIG. 4. Map of the mutations analyzed in the C terminus of Ty1 Gag. The amino acid sequence of the Ty1 Gag C terminus subjected to mutagenesis is shown on the top. (A) Long shaded boxes, Ty1 Gag C termini; white boxes, 6-amino-acid substitutions in mutants *s*1 through *s*8 and single-amino-acid substitutions in mutants *s*3.1 through *s*3.6. (B) Names of the mutants. wt, wild-type (C) Ty1 Gag processing in the mutants according to the immunoblot assay. Grey dots, mutants with no Ty1 Gag processing. (D) Transposition rates of mutant Ty1 retroelements. Black dots, mutants with transposition rates of 5% and lower.

Mutagenesis of amino acid residues in the Gag cleavage site. From 11 mutants analyzed, the only 2 showing no Gag processing were the *d*2 and *s*3 mutants, in which the Gag cleavage site had been altered (Fig. 5A and C). Surprisingly, the Gag-Pol protein was not processed in these mutants either (Fig. 5B and D). These data support our hypothesis that the Gag and the Gag-PR cleavage sites have the same sequence and are located at the same distance from the amino termini of their precursors. Studies on kinetics and mechanism of autoprocessing of human immunodeficiency virus type 1 (HIV-1) PR from an analog of the Gag-Pol polyprotein suggest that cleavage of the peptide scissile bond at the N terminus of the PR domain is required to activate HIV PR from its relatively inactive precursor (21). This may be a common feature of aspartic PRs. The amino acid sequence normally recognized and cleaved by Ty1 PR has been eliminated in the *d*2 and *s*3 mutants; therefore, the Ty1 PR could not liberate its N terminus. We propose that this mutant PR was unable to cleave Gag-Pol at the PR-IN and IN-RT sites. Since there was a chance that the foreign sequence AAGSAA could alter the secondary structure of Gag-Pol, Gag-Pol processing was also examined in cells expressing mutant Ty1 elements with single amino acid substitutions introduced into positions P3 through P3′ of the Gag cleavage site. Amino acids P3 through P3′ of the Gag cleavage site were substituted by isoleucine, because amino acids branched at the β-carbon are excluded from the P1 position in all PR cleavage sites found in retroviruses (28), Ty3 (18), or Ty1. Moreover, isoleucine placed in the P1 position of the retroviral PR cleavage site located at the N terminus of the PR encoding domain in the HIV-1 Pol precursor rendered this site

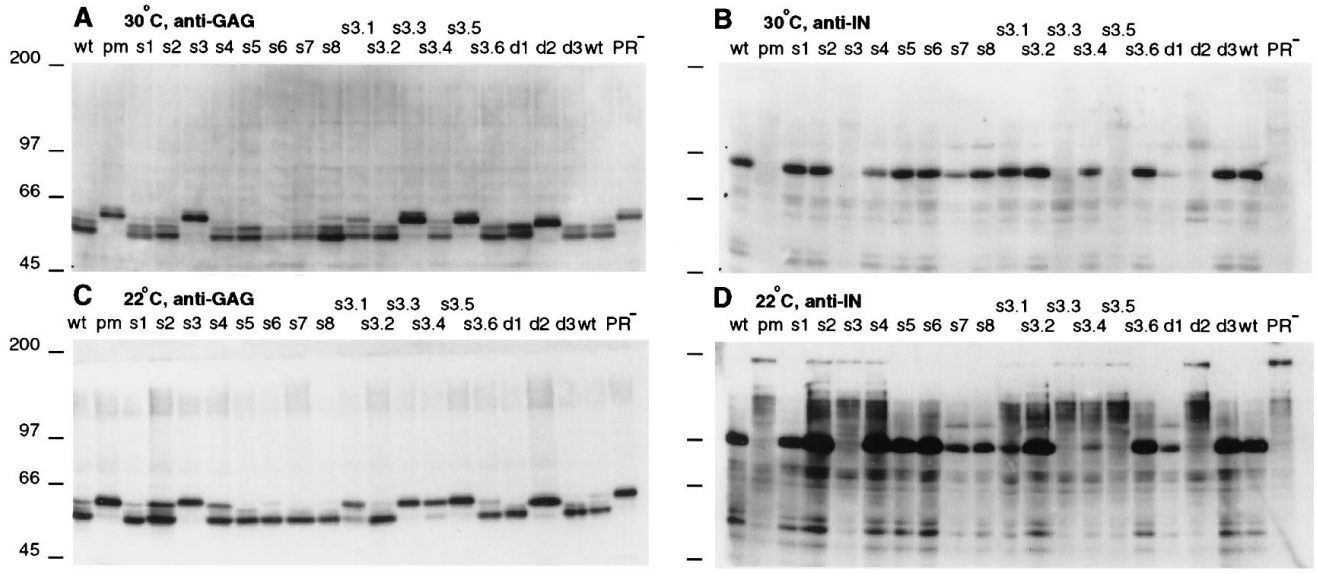

FIG. 5. Immunoblots of mutant Gag and Gag-Pol expressed in *S. cerevisiae*. Cultures containing plasmids with mutations in the Gag C terminus were grown in liquid SC-Ura galactose medium at 22°C (C and D) or 30°C (A and B). Cells were pelleted, lysed, subjected to SDS-PAGE, transferred onto an Immobilon membrane, and incubated with anti-Gag (A and C) or anti-Integrase antiserum 8B11 (B and D). Numbers on the left show molecular masses in kilodaltons.

uncleavable but did not eliminate PR activity in the in vitro experiments. The six residues surrounding the scissile bond were substituted by isoleucine in mutants *s*3.1 through *s*3.6 (Fig. 4). Only the unprocessed Gag and Gag-Pol proteins were detected in the cells expressing mutants *s*3.3 and *s*3.5. As in the case of *s*3, the transposition rates of *s*3.3 and *s*3.5 mutants of Ty1 were at least 100 times lower than those of wild-type Ty1. The Gag and Gag-Pol cleavage occurred more slowly in cells expressing the *s*3.1 and *s*3.4 mutants than in the cells expressing *s*3.2 or *s*3.6 or wild-type Ty1 at 22°C, although the transposition rates of all four mutants differ less than twofold from those of wild-type Ty1. We suggest that slowing down the processing of the VLP components to a certain degree does not significantly affect transposition rates. These results support the hypothesis that the same cleavage site is cleaved by Ty1 PR in the Gag and Gag-Pol proteins.

DISCUSSION

The experiments reported here reveal a Ty1 PR cleavage site in the C terminus of the Gag protein, a major component of Ty1 VLPs, and demonstrate that the Gag C-terminal portion, removed during particle maturation, may play an important role in transposition. The scissile bond of the Gag cleavage site has been located between amino acid residues 401 and 402 of the 440-amino-acid Gag protein, suggesting that a 39-residue C-terminal peptide is removed from the C terminus of the Ty1 Gag precursor. As we and others demonstrated earlier, no

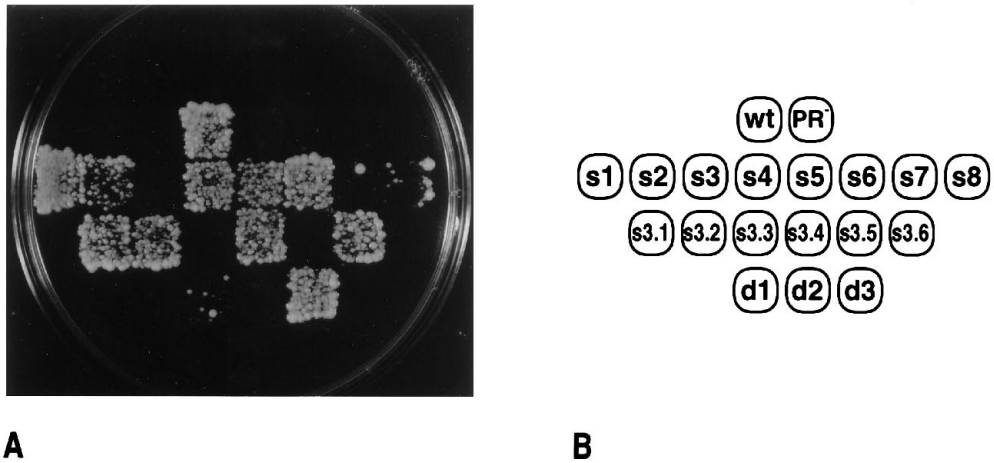

FIG. 6. The transposition assay. YH8 yeast cells were transformed to Ura^+ with plasmids carrying wild-type and mutant Ty1 retroelements. Transformants were patched on SC-Ura glucose plates, then replica plated onto SC-Ura galactose plates, and incubated at 22°C for 48 h to induce transposition. The patches were then replica plated onto YPD nonselective medium to allow loss of the donor plasmid. Cells that lost the donor plasmid were selected by replica plating to SC–5-FOA glucose medium and finally plated onto YPD medium containing 75 mg of G418 per ml to select for the cells that acquired Ty1 in the genome. (A) Growth on YPD medium containing G418; (B) a diagram showing positions of mutant strains on the plate.

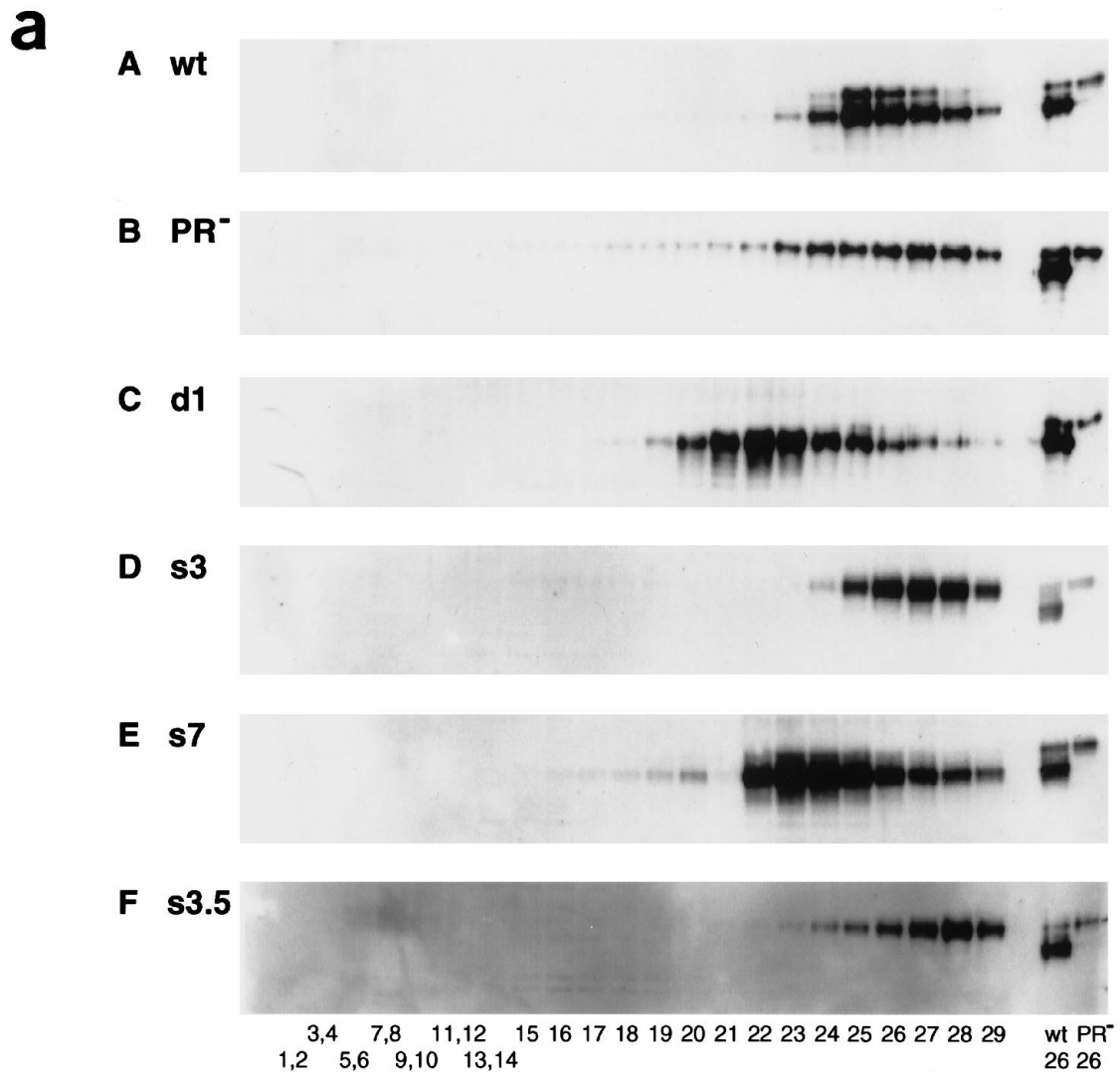

FIG. 7. Wild-type (wt) and mutant VLPs. (a) Western blots (immunoblots) of Ty1 Gag proteins from Ty1 VLPs. Particles were purified on the 20 to 70% sucrose gradients. Thirty fractions were collected from each gradient following centrifugation. Approximately 5-μl aliquots from each fraction were subjected to immunoblot analysis with anti-Ty1 Gag antibodies. Lanes 26 in rows A and B, the peak fractions from the wild-type and protease mutant particle preparations, used as markers. (b) Ultrastructure and size distributions of the wild-type and mutant particles. (Left) Transmission electron microscopy images of negatively stained Ty1 VLP particles; (right) histograms of the particle diameter distributions.

54-kDa form is detected in cells expressing the Ty1 element with a PR^- mutation, suggesting that conversion of the 58-kDa Gag into the 54-kDa form results from cleavage of Gag rather than from posttranslational modification (1, 27, 30). Ty1 Gag protein is phosphorylated; therefore, the 58- and 54-kDa forms may be detected as doublets (22, 29). In this study we show that antibodies raised against the 22 C-terminal amino acids of Gag react with the 58-kDa form of Gag but not with the 54-kDa form, indicating that the C-terminal but not the N-terminal end of Gag is processed. Since the amino acid sequence corresponding to the Gag cleavage site is located upstream of the frameshift signal of Ty1, it could be recognized and cleaved by Ty1 PR not only in Gag but also in Gag-Pol.

Features of the newly mapped Ty1 PR cleavage site are consistent with those of two other PR cleavage sites identified in the Gag-Pol polyprotein previously (Fig. 3). Although the amino acid sequences surrounding the scissile bonds in the three PR cleavage sites are quite different, some remarkable similarities were found in their hydrophobicity profiles. Only valine occurs in position P2′ in all three PR sites found in Gag-Pol. Position P2 is occupied by isoleucine in two PR cleavage sites or alanine in one site. The P1′ position is occupied either by asparagine in two PR cleavage sites or by alanine in one of them. Hydrophobicity profiles appear to be a better measure for similarities and differences in retroviral PR cleavage sites (28). The P2 and P2′ amino acids of Ty1 PR cleavage sites have higher hydrophobicity indices than P1, P1′, or flanking amino acids (Fig. 3A). Hydrophobicity profiles of Ty1 and Ty3 cleavage sites are strikingly similar (Fig. 3). On the other hand, the HIV-1 PR cleavage sites exhibit notably different hydrophobicity profiles, with amino acids P3 through P3′ being more hydrophobic than the flanking amino acids (28) (Fig. 3B). An interesting feature of all identified Ty1, Ty3, and retroviral PR cleavage sites is that amino acids with the side chain branched at the β-carbon are absent from the P1 position (28). Isoleucine at the P1 position of the cleavage site

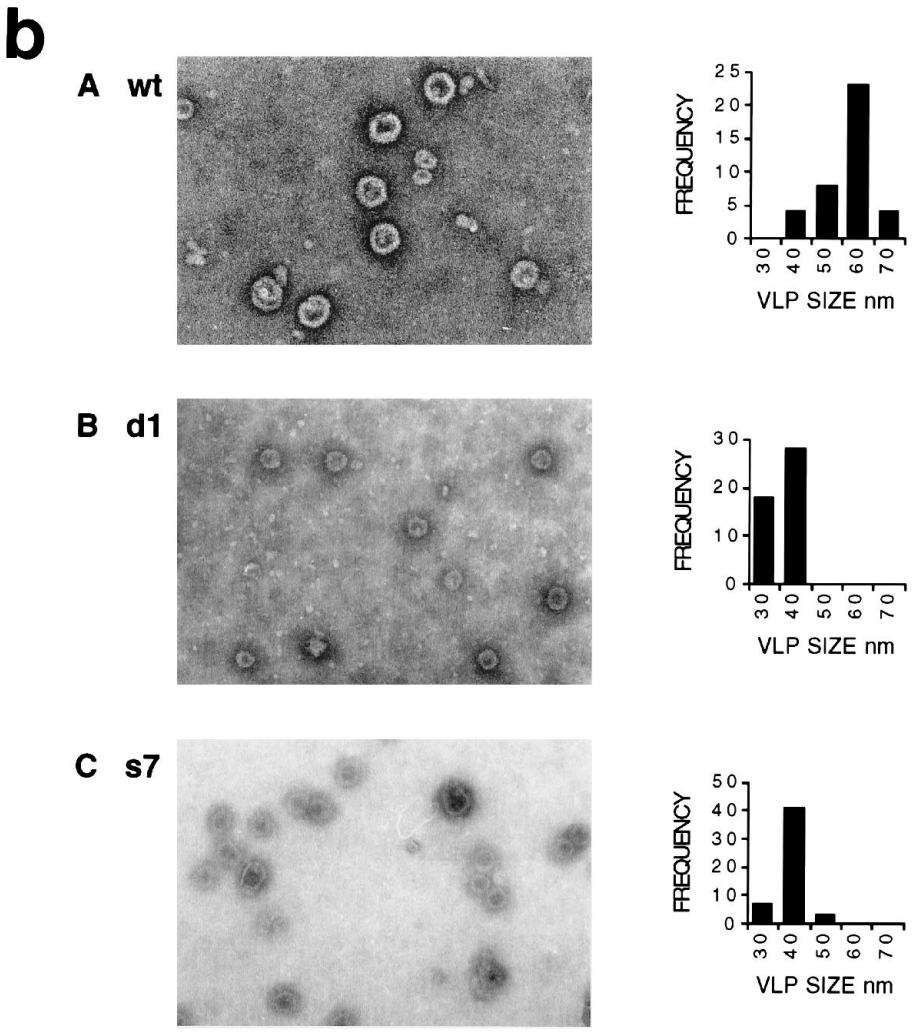

FIG. 7—*Continued.*

located at the N terminus of the PR encoding domain in the HIV-1 Pol precursor blocks cleavage at this site but does not eliminate PR activity (31). We show that isoleucine at the P1 position of the Gag cleavage site also renders this site uncleavable. Our results indicate that only six amino acids, P3 through P3′, in the Gag cleavage site may be sufficient for cleavage of Gag by PR, since block substitutions of the flanking sequences did not block the cleavage at this site. The sequence required for interaction with retroviral PR most probably resides between amino acids P4 and P3′, since amino acids in positions P4 through P3′ were found to be least variable and amino acids P3′ through P3 exhibit a higher hydrophobicity index than the flanking amino acids in retroviral PR cleavage sites (28).

Although Gag and Gag-Pol proteins are processed in mutants *d*1, *s*7, and *s*8, transposition rates of these mutants are at least 20 times lower than those of the wild-type Ty1. Moreover, particles prepared from the cells carrying mutants *d*1 and *s*7 were found to be two-thirds the diameter of the particles prepared from the cells carrying wild-type Ty1, which cannot be accounted for by the change in subunit molecular mass. Therefore, the Gag C-terminal portion, released in the course of particle maturation, may play a significant role in VLP morphogenesis and Ty1 transposition. Although the function of the Gag C-terminal peptide remains to be determined, we propose that it could be implicated either in the binding of Ty1 RNA or in assembly of the VLP shell, because the peptides resulting from the processing of Ty3 Gag and retroviral Gag proteins are involved in these processes. Unfortunately, the function of this peptide cannot be deduced simply from sequence comparison, because Ty1 Gag has no obvious homology with Gag proteins from retroviruses and Ty3 elements. The C-terminal peptide of Gag is rich in serine and threonine and contains a significant number of hydrophilic and charged amino acids, which might form contacts with RNA. Interestingly, nucleocapsid proteins of retroviruses and Ty3 element are significantly smaller than the capsid proteins. The Ty3 Gag is processed into two peptides, CA (capsid protein, 26 kDa) and NC (nucleocapsid protein, 7 kDa). The HIV-1 Gag polyprotein is processed into four polypeptides, MA (matrix protein, 17 kDa), CA (24 kDa), NC (7 kDa), and p6 (6 kDa). However, unlike retroviral and Ty3 NC proteins, the 4-kDa Ty1 peptide lacks a Zn-finger-like motif. The C-terminal portion of Gag could direct particle assembly in a number of ways. For instance, it could interact with molecular chaperones or it could modulate proteolytic cleavage at the Gag-Pol sites. However, the presence of Ty1 DNA in the *s*7 and *s*8 mutant VLPs

could also be consistent with a role in a late step in the transposition process for the 4-kDa peptide.

We show that substitutions of the P3 through P3′ amino acid residues of the Gag cleavage site by the sequence AAGSAA block processing of both Gag and Gag-Pol proteins. Furthermore, substitution of either the P1 or P2′ amino acid by isoleucine blocks Gag and Gag-Pol processing as well. These data are consistent with our hypothesis that the Ty1 PR cleavage site of the same sequence is located in both Gag and Gag-Pol upstream of the frameshift point. Studies of the Gag-Pol processing pathway clearly indicate that the Gag portion is released first from the Gag-Pol polyprotein and are consistent with a single cleavage site between the Gag and PR domains (16). Studies on kinetics and mechanism of autoprocessing of HIV-1 PR from an analog of the Gag-Pol polyprotein indicate that cleavage of the peptide scissile bond at the N terminus of the PR domain is required to activate HIV PR from its relatively inactive precursor (21). Blocking the HIV-1 PR cleavage site nearest to the PR active site did not block the PR activity, but the PR utilized other sites located farther upstream in the Gag-Pol polyprotein (31). Since there is only one cleavage site upstream of the Ty1 PR active site, blocking this site renders the PR incapable of autoactivation and therefore the Gag-Pol protein is not processed in this mutant. Alternatively, although less probably, the altered Gag cleavage site could bind the PR active site tightly enough to inhibit the PR activity. Structural study of the PR protein will be the best approach to clarify this question. The PR N-terminal sequence proposed in this paper can be used to heterologously express Ty1 PR, purify the enzyme, and study its mechanism in detail.

The evidence that the Gag protein is divided into two functional domains separated by a PR cleavage site may provide helpful information in further studies of VLP assembly. It will be interesting to compare the functional organization of the Ty1 Gag with its analogs from other elements in this widespread retrotransposon family, with members in fungi, insects, plants, and vertebrates.

ACKNOWLEDGMENTS

We thank Qinghua Feng and Vit Lauermann for helpful discussions, Clark Riley for synthesis of the synthetic peptide, and Mike Delannoy for assistance with electron microscopy.

This work was supported in part by N.I.H. grants U01AI35282 and GM36481 to J.D.B.

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