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Ten-Kilodalton Domain in Ty3 Gag3-Pol3p between PR and RT Is Dispensable for Ty3 Transposition

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Ty3 is a gypsy-type, retrovirus-like element found in the budding yeast *Saccharomyces cerevisiae*. In cells overexpressing Ty3 under the *GALI* upstream activation sequence, Ty3 RNA, proteins, and DNA are made. Elucidation of the molecular masses and amino-terminal sequences of protease and reverse transcriptase indicated the existence of an additional intervening domain, designated J, in the Ty3 Gag3-Pol3p polyprotein. A region analogous to J can be found in many retrotransposable elements closely related to Ty3; however, J does not correspond to any of the highly conserved retroviral protein domains. Ty3 mutants deleted for the J-coding region showed moderately reduced transposition frequency but greatly reduced levels of Ty3 DNA. These results show that under galactose regulation, the Ty3 J domain is not absolutely essential.

Ty3 is a gypsy-type, retrovirus-like element found in the budding yeast *Saccharomyces cerevisiae*. It consists of a 4.7-kb internal domain flanked by 340-bp long terminal repeats (LTRs) (2). Ty3 contains two open reading frames (ORFs) that encode Gag3p and Gag3-Pol3p polyproteins (5, 6). These proteins assemble into virus-like particles (VLPs), which are analogous to retroviral core particles (5). The Ty3 aspartyl protease (PR) is required for processing of the polyprotein into mature species (9). Homologs of retroviral capsid (CA), nucleocapsid (NC), PR, reverse transcriptase (RT), and integrase (IN) have been characterized by sequence alignment, mutagenesis, immunoblot analysis, and, with the exception of CA, amino-terminal sequence analysis (reviewed in reference 12). Amino-terminal sequence analysis of the 16-kDa PR and the 55-kDa RT and inspection of the relative positions where these ends are encoded in the Ty3 *POL3* ORF showed that the predicted carboxyl-terminal end of PR and the known amino-terminal end of the major RT are separated by a domain of approximately 10 kDa (8).

As shown in Fig. 1, the predicted carboxyl-terminal end of PR corresponds to a position 35 amino acids (aa) carboxyl terminal to a conserved hhG motif (where h = a hydrophobic residue), while the experimentally determined (8) amino-terminal end of Ty3 RT begins 20 aa amino terminal to a pair of hP motifs. In the transpositionally active Ty3-1, this region is comprised of 93 aa and will be referred to here as the J domain. A domain of similar size can be found in those other LTR retrotransposable elements that are phylogenetically most closely related to Ty3 (10). The only exception is Skipper, where this region corresponds to a region containing a frame-shift between the *gag* and *pol* ORFs. Sequence comparisons between these various elements reveals that sequence conser-

vation within the J region is significantly less than within the PR or RT protein domains. For example, sequence similarity (identical and similar amino acids) between Ty3 and Skipper is 40% for PR, 54% for RT, and only 15% for the J region. Similarly, sequence conservation between the more closely related Skipper and CfI is 48% for PR, 69% for RT, and 21% for J. In retroviruses, particularly lentiviruses and spumaviruses, small auxiliary domains are encoded in *pol* and in ORFs expressed from differentially spliced transcripts (3). The current study was undertaken to determine whether the J domain between PR and RT is essential for transposition of Ty3 expressed under control of the *GALI* upstream activation sequence (UAS).

The ability of a Ty3 mutant containing a deletion of the J region to transpose was examined. The amino-terminal sequence of Ty3 RT was known, and the predicted carboxyl-terminal sequence of J was inferred to have been created from the same cleavage. The position of the amino terminus of the mature J protein was predicted based on the mass of PR as estimated from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (4); this position was in agreement with the typical distance from a conserved motif in retroviral PR to the carboxyl terminus of retroviral PR. The major determinants for retroviral PR processing sites are contained within a hydrophobic region of ca. 7 aa, including 4 aa amino terminal to the scissile site and three residues carboxyl terminal to the site. The known Ty3 PR cleavage sites are similar to retroviral PR processing sites (8). Inspection of the region at the carboxyl terminus of PR for cleavage sites that resemble the known Ty3 PR preferences showed that there were two sites that were compatible with the conserved features of other Ty3 PR processing sites. These were contained within the sequence ETVN*NVR**TYS* (indicated by asterisks) encoded by *POL3* codons 162 to 171. The phagemid carrying wild-type (wt) Ty3 expressed under control of the *GALI*-UAS, pEGTy3-1 (6), was prepared as single-stranded DNA and an oligonucleotide (5'-CCGAAACCGTTAATAACGTTAGAAACGTTGTCTC

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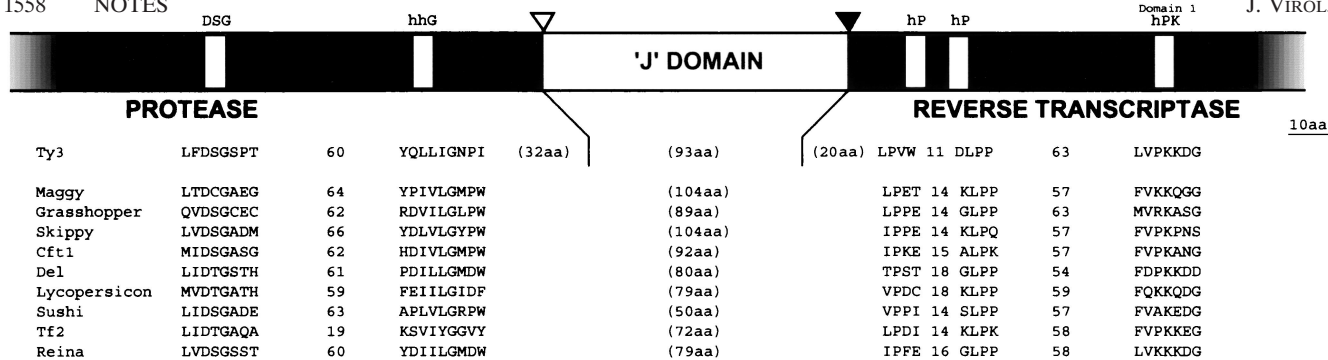


FIG. 1. Comparison of J region domains of retrotransposons. The region spanning the conserved domain of PR through domain 1 of RT is shown. The J region was defined as the domain spanning the region from the inferred the carboxyl terminus of PR (as described in the text) to the biochemically determined amino terminus of RT (8). The elements (accession numbers) are Maggy (g522302) and Grasshopper (M77661) (*Magnaporthe grisea*), Skippy (g2133292) (*Fusarium oxysporum*), Cft1 (g2564) (*Cladosporium fulvum*), Del (g226407) (*Lilium henryi*), Lycopersicon (g4235644), Sushi (6425168) (*Fugu rubripes*), Tf2 (g173439) (*Schizosaccharomyces pombe*), and Reina (U69258) (*Zea mays*).

AACCATTCAG-3') was used to loop out *POL3* codons 169 to 255, inclusive (9). The mutant Gag3-Pol3p was deleted for the J region but retained putative PR and RT processing sites together with flanking residues. The plasmid carrying this Ty3 deletion mutant (Ty3ΔJ) was designated pJC936. Thus, cleavage at either the putative PR site or the inferred RT site would result in PR and RT species differing by only a few terminal residues from the proteins expressed in the wt element. Recombinant DNA techniques and yeast and *Escherichia coli* manipulations were as described in *Current Protocols in Molecular Biology* (1), except where specifically indicated.

Transposition of the Ty3ΔJ mutant was compared to that of wt Ty3 in a genetic assay that takes advantage of Ty3 integra-

tion specificity for genes transcribed by RNA polymerase III. Yeast strain YTM443 (*MATa ura3-52 trp1-H3 his3-Δ200 ade2-101 lys2-1 leu1-12 can1-100 bar1::hisG Ty3 null*) was transformed (13, 14) with either pEGTy3-1 or pJC936 and with the target plasmid pCH2bo19v (7; J. A. Claypool and S. B. Sandmeyer, unpublished data). The target plasmid contains two divergent tRNA genes, a nonexpressed suppressor *sup2bo* and a tRNA^{Val} gene. Transposition into the intergenic space activates expression of the suppressor tRNA so that *ade2-101* and *lys2-1* ochre nonsense mutations are suppressed. Cells in which transposition has occurred are detected as colonies on minimal medium supplemented with tryptophan and leucine and lacking adenine and lysine. Transformants were grown in raffinose-containing synthetic medium (SR; 0.67% yeast nitrogen base, 2% raffinose) lacking uracil, histidine, and arginine and containing canavanine to early logarithmic phase ($A_{600} = 0.2$).

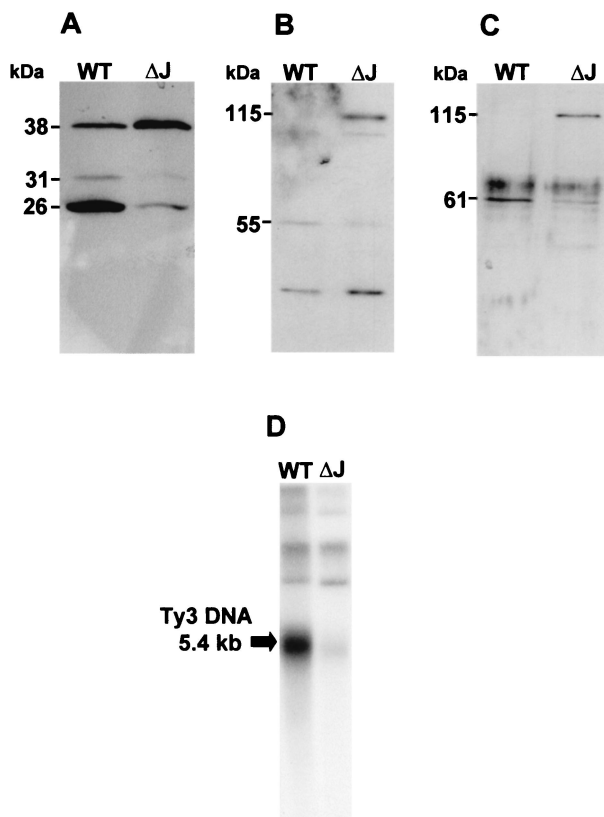
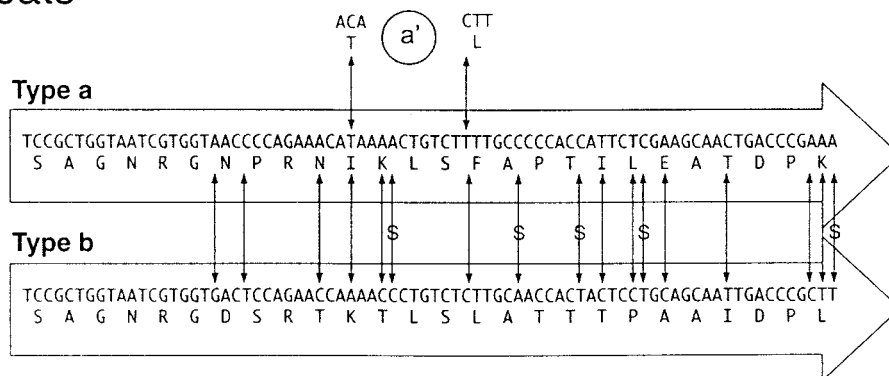


FIG. 2. Ty3 proteins and DNA in WCEs of cells expressing Ty3 ΔJ mutant. (A) Immunoblot of Ty3 CA. YTM443 was grown to mid-logarithmic phase in Ura⁻ SG, WCEs were prepared, and 10 μg of protein/sample was fractionated in a sodium dodecyl sulfate-polyacrylamide gel, and transferred to Hybond-ECL membrane (Amersham) as described previously (17). Membranes were incubated with 1:1,000 dilution of rabbit αCA immunoglobulin G (IgG), followed by incubation with anti-rabbit IgG F(ab)'₂-linked fragments conjugated to horseradish peroxidase, which was detected with enhanced chemiluminescence reagent (Amersham). Proteins were visualized by exposure to X-ray film. Ty3 VLPs were fractionated as size markers, and the positions of the 39-kDa Gag3-Pol3p-derived precursor, 38-kDa Gag3p, 31-kDa Gag3p-derived species, and 26-kDa Gag3p-derived CA species are shown. WT, WCE from cells expressing wt Ty3; ΔJ, WCE from cells expressing Ty3 ΔJ mutant. (B) Immunoblot of Ty3 RT protein. Samples were the same as those described in panel A. Membranes were incubated with a 1:1,000 dilution of rabbit αRT IgG and were processed for immunoblots as described in panel A. Positions of 115-kDa RT-IN fusion species and the 55-kDa RT species are shown. (C) Immunoblot of Ty3 IN protein. Samples were the same as those described in panel A. Membranes were incubated with 1:1,000 dilution of rabbit αIN IgG. The positions of the 115-kDa RT-IN fusion species and 61-kDa IN are indicated. (D) Southern blot of DNA from cells described in panel A. Total nucleic acid was extracted, and samples were processed for Southern blot analysis as described previously. Twenty micrograms of DNA was fractionated on an agarose gel, transferred to nitrocellulose, and probed with a Ty3 internal-domain-specific probe. Ty3 DNA was visualized by exposure of the membrane-bound DNA to x-ray film. The position of the full-length, extrachromosomal Ty3 DNA is indicated.

J repeats



J repeats in two Ty3 elements

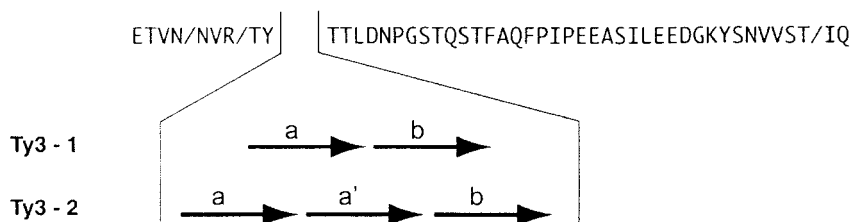


FIG. 3. Alignment of repeated region of J domain. Shaded arrows show DNA sequence encoding repeated sequences in Ty3-1 (a and b) and Ty3-2 (a, a', and b). Changes from a to b indicated are replacement and synonymous (S). The overall structures of a and b repeats in Ty3-1-type (Z72894 and Z72895) and Ty3-2-type (Z46728) elements are indicated below at the point of insertion in Gag3p.

Approximately 10^6 cells were plated to synthetic medium (0.67% yeast nitrogen base) containing 2% dextrose (SD) or 2% galactose (SG) and grown at 30°C for 29 and 48 h, respectively, to allow for equal numbers of generations. Cells were replica plated to minimal medium plus leucine and tryptophan and grown for 5 days at 30°C to determine the transposition frequency. The ΔJ Ty3 element transposed at a frequency approximately 61% of that of the wt Ty3 element, indicating that the Ty3 J domain does not perform an essential function in *GALI-UAS* inducible transposition.

In order to determine the basis of the reduction in Ty3 transposition observed in the Ty3 ΔJ mutant, the levels of mature Ty3 proteins and replicated, full-length DNA were measured. The plasmids pEGTy3-1 and pJC936 were transformed independently into YTM443. Transformants were grown in SR lacking uracil to early logarithmic phase at 30°C. Galactose was added (2%) to each culture to induce expression of Ty3. Cultures were grown for an additional 6 h and harvested.

In order to evaluate Ty3 proteins, whole cell extracts (WCEs) and VLPs were prepared as described previously (11). Ty3 proteins from WCEs were visualized by immunoblot analysis with αCA , αRT , and αIN antibodies (Fig. 2A to C). Ty3 Gag3p is essential for particle formation (4). The 38-kDa precursor is processed by PR into a 26-kDa CA species and a 9-kDa NC species. A 39-kDa Gag3-Pol3p derived species and a 31-kDa protein are also observed at lower levels. The extracts

of cells expressing the ΔJ mutant contained 31- and 26-kDa CA species. Nevertheless, comparison of the patterns obtained from ΔJ and wt showed that the ΔJ mutant had a higher ratio of 38-kDa species to the 26-kDa species, suggesting that processing was less efficient. Inspection of the pattern of proteins visualized using αIN and αRT antibodies showed reduced amounts of 55-kDa RT and 61-kDa IN species but increased amounts of a previously identified 115-kDa RT-IN fusion protein in WCEs. However, in ΔJ VLPs, the ratio of 38-kDa precursor to 26-kDa CA and the profile of *POL3*-derived proteins were similar to those observed in WCEs (data not shown). Thus, the levels of different protein species in WCEs suggested that processing is less efficient for the Ty3 ΔJ mutant but that the protein composition of mutant VLPs is similar to that of wt VLPs.

Ty3 replicates its almost full-length genomic RNA into a full-length, 5.4-kDa DNA species that is subsequently integrated into the chromosome. Total nucleic acid was isolated from cells expressing wt and Ty3 ΔJ , as described previously (4), and DNA was examined by Southern blot analysis. An internal Ty3 *Bgl*III fragment was used as the Ty3-specific probe (Fig. 2D) to visualize replicated, extrachromosomal Ty3 5.4-kb DNA. Cells expressing the ΔJ mutant contained one-fifth to one-third of the amount of Ty3 DNA that was present in cells expressing wt Ty3. This result indicated that the ΔJ mutation interfered with reverse transcription of the Ty3 element or with

stability of the Ty3 VLP DNA. The Ty3 Δ J VLP fraction showed a similar reduction in 5.4-kb DNA compared to the wt fraction (data not shown). Thus, despite the fact that mutant VLP proteins approximate the distribution of proteins in the wt VLPs, there is less reverse transcription product in the mutant VLPs. This suggests that reverse transcription may be less efficient or that the product may be less stable.

The J domain between the PR and RT domains of the Ty3 Gag3-Pol3p polyprotein is not essential for *GALI*-UAS-regulated Ty3 transposition. A deletion of the entire domain did not abolish transposition, although it did result in a 40% decrease in transposition frequency. Because deletion of the J domain constitutes a relatively drastic change in the Gag3-Pol3p primary sequence, it is possible that the effects on transposition, protein, and DNA that we observed resulted solely from the disrupted tertiary structure of the VLP or of the PR or RT proteins themselves. On the basis of these data, we cannot distinguish between reduced levels of transposition-competent particles and normal levels of particles with reduced transposition competence.

While these experiments suggest that the J domain may not play an essential protein-coding function in retrotransposition, it may play another noncoding role. Inspection of the J sequence of Ty3-1 revealed that it contained a 78-nucleotide (corresponding to 26 aa) tandem duplication (Fig. 3). This duplication is relatively old because 18 nucleotide differences have accumulated between the two repeats (referred to as "a" and "b"). Consistent with the lack of selective pressure on the encoded protein, these substitutions correspond to 12 replacement and 5 synonymous changes, similar to that predicted based on a random accumulation of mutations. Comparison of the two Ty3 sequences present in the sequenced yeast genome revealed that one of the elements is similar to Ty3-1 (accession no. Z72894 and Z72895, YGR109c) (6). The other element is similar to Ty3-2, which contains an additional duplication of the 78-nucleotide sequence (Accession no. Z46728; YIL082w) (5). The additional Ty3-2 duplication occurred more recently, since these repeats differ by only two base substitutions (Fig. 3, a and a'). The recovery of these expansions suggests that this region is relatively tolerant of recombination events resulting in sequence duplication. Collectively, these results demonstrate that retrotransposons carry domains that at least under some conditions are nonessential. These domains occur in

retroviruses (15). Such nonessential domains could represent the precursors of auxiliary domains, such as those in retroviruses, which are not essential under all conditions.

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