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Ty3 Integrase Mutants Defective in Reverse Transcription or 3′-End Processing of Extrachromosomal Ty3 DNA

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Ty3, a retroviruslike element in *Saccharomyces cerevisiae*, encodes an integrase (IN) which is essential for position-specific transposition. The Ty3 integrase contains the highly conserved His-Xaa$_{3–7}$-His-Xaa$_{23–32}$-Cys-Xaa$_2$-Cys and Asp, Asp-Xaa$_{35}$-Glu [D,D(35)E] motifs found in retroviral integrases. Mutations were introduced into the coding region for the Ty3 integrase to determine the effects in vivo of changes in conserved residues of the putative catalytic triad D,D(35)E and the nonconserved carboxyl-terminal region. Ty3 viruslike particles were found to be associated with significant amounts of linear DNA of the approximate size expected for a full-length reverse transcription product and with plus-strand strong-stop DNA. The full-length, preintegrative DNA has at each 3′ end 2 bp that are removed prior to or during integration. Such 3′-end processing has not been observed for other retroviruslike elements. A mutation at either D-225 or E-261 of the Ty3 integrase blocked transposition and prevented processing of the 3′ ends of Ty3 DNA in vivo, suggesting that the D,D(35)E region is part of the catalytic domain of Ty3 IN. Carboxyl-terminal deletions of integrase caused a dramatic reduction in the amount of Ty3 DNA in vivo and a decrease in reverse transcriptase activity in vitro but did not affect the apparent size or amount of the 55-kDa reverse transcriptase in viruslike particles. The 115-kDa viruslike particle protein, previously shown to react with antibodies to Ty3 integrase, was shown to be a reverse transcriptase-IN fusion protein. These results are consistent with a role for the integrase domain either in proper folding of reverse transcriptase or as part of a heterodimeric reverse transcriptase molecule.

Retroviruses and retroviruslike elements (collectively referred to here as retroelements) reverse transcribe their RNA genomes into double-stranded DNA species and integrate the linear DNA copies into host cell genomic DNA (for reviews, see references 3 and 73). These functions are catalyzed by the retroelement-encoded enzymes reverse transcriptase (RT) and integrase (IN), respectively. For most retroviruses, the sequence of the proviral form suggests that the replicated extrachromosomal linear DNA will have at each end 2 bp which are not present at the ends of the provirus. Characterization of the ends of Moloney murine leukemia virus (MoMLV) extrachromosomal DNA from infected cells has shown that it is a combination of linear and circular molecules. The linear molecules are 2 nucleotides (nt) longer than the proviral DNA on each 5′ end, but the 3′ termini represent a mixture of species with either no extra nucleotides or 2 extra nt (8, 67). In vitro assays have shown that IN proteins from representative retroviruses have the predicted 3′-end processing activity which removes the 2 extra nt and the strand transfer activity necessary to join these ends to the host DNA at positions in the two target strands separated by 4 to 6 nt (9, 11, 18, 40, 41, 71, 79). Interestingly, although 3′-end processing is related mechanistically to strand transfer, preprocessed substrates can be used, indicating that processing and strand transfer are not concerted in vitro (18). In addition to 3′-end processing and strand transfer in vitro, IN can also catalyze the reversal of integration, disintegration (16).

Ty1 and Ty3 are members of the long terminal repeat (LTR) retrotransposon class of retroviruslike elements found in *Saccharomyces cerevisiae*. Investigation of the function of IN encoded by Ty1 and Ty3 has shown it to be essential for transposition. For Ty1, IN is necessary for integration in vivo (6) and necessary and sufficient for strand transfer in vitro (22, 58). For the yeast LTR retrotransposon Ty3, IN has been shown to be required for integration both in vivo and in vitro (35, 46). Until the description of Ty3, it appeared that a consistent distinction between retroviruses and LTR retrotransposons, primarily described in *S. cerevisiae* and *Drosophila melanogaster*, was the 2 extra bp at each end of replicated retroviral extrachromosomal DNA, which, on the basis of sequence analysis, was not predicted to be present at the ends of replicated extrachromosomal retrotransposon DNA (5). The Ty1 sequence (17), for example, predicts a terminus which is the same as the end of the integrated element. In the case of Ty1, blunt ends supplied from an exogenous DNA have been shown to act as donors in a strand transfer reaction mediated by viruslike particles (VLPs) or by recombinant IN protein (23, 58). The more recently described Ty3 sequence (35) predicts a retroviruslike 2 extra bp at least at one end. The 3′ ends of extrachromosomal DNA have not been directly examined for any LTR retrotransposon.

Alignments of retroviral and LTR retrotransposon IN protein sequences (37, 45) led to identification of about 20 residues, positioned throughout the proteins, which are highly conserved. The amino-terminal region contains a highly conserved His-Xaa$_{3–7}$-His-Xaa$_{23–32}$-Cys-Xaa$_2$-Cys (HHCC) motif which has been shown to mediate zinc binding in vitro (10). Carboxyl terminal to the HHCC motif is an Asp, Asp-Xaa$_{35}$-Glu [D,D(35)E] motif which, on the basis of analogies with similar motifs in other proteins, is believed to be required for binding divalent metal cations (21, 45). A central region of about 120 residues of human immunodeficiency virus type 1 (HIV-1) IN containing the D,D(35)E motif (but not the HHCC motif) is resistant to mild proteolysis (27), suggesting that this region comprises a core structural domain. The region from residues 50 to 212 out of a total of 288 residues of HIV-1

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IN has been expressed in a variant which has elevated solubility in vitro, and its crystal structure has been determined (21). The final regions, the carboxyl-terminal domains of retroviral IN proteins, vary in length and are not highly conserved in sequence.

Extensive mutagenesis of several retroviral IN species and in vitro testing has led to a partial understanding of the contributions of the amino-terminal, core, and carboxyl-terminal domains. Mutations introduced into coding positions for conserved residues of the zinc binding domain reduce or abolish 3′-end processing and strand transfer activity but do not abolish disintegration activity (10, 12, 20, 27, 38, 45, 53, 75, 77). Although this region has not been shown to have independent DNA binding activity, it may contribute to recognition of viral LTRs (45, 77). The core region containing the D,D(35)E motif is competent to perform disintegration (10, 12, 26). Mutations introduced into conserved positions encoding the D,D(35)E motif result in inactivation of IN (27, 50, 53, 75). Thus, this region is considered to contain the catalytic active site. The core domain is also required for IN-IN dimer formation (39). IN protein binds DNA nonspecifically (48, 69). Deletion of the carboxyl-terminal domain results in a species which is much less active in DNA binding assays (29, 42, 60, 66, 78), and point mutations introduced into this domain have recently been shown to disrupt DNA binding activity (63). A nuclear magnetic resonance solution structure of the carboxyl-terminal dimer region has been determined recently; it contains a groove within which the substrate DNA could potentially rest (24, 56).

A subset of conserved IN residues have been mutated, and the effects on viral replication in tissue culture have been assessed. In general, mutations in conserved residues block production of infectious virus, although the extent of the effect is dependent on the nature of the mutation. Mutations in some residues and carboxyl-terminal deletions have been shown to disrupt 3′-end processing and/or viral maturation (13, 28, 53, 68, 72, 80). Conservative mutations in the catalytic triad result in the accumulation of circular, extrachromosomal DNA (28, 80).

Ty3 is a member of the gypsylike family of retroelements and is distantly related to the other yeast retrotransposons, Ty1, Ty2, Ty4, and Ty5, which belong to the copialike family. Ty3 is 5.4 kbp long and is transcribed to form a 5.2-kb RNA (33, 34). The two open reading frames of Ty3, *GAG3* and *POL3*, are analogous to the *gag* and *pol* genes of retroviruses (33, 34). *GAG3* encodes the major structural proteins capsid (CA) and nucleocapsid (NC). *POL3* encodes the enzymes protease (PR), RT, and IN. The latter set of proteins are expressed in this order as domains in a Gag3-Pol3 fusion polyprotein. The CA, NC, PR, RT, and IN encoded by *GAG3* and *POL3* have been identified by immunoblot analysis and are functionally equivalent to their retroviral counterparts (33, 35, 47, 61). Ty3 integrates within 1 to 3 nt of the transcription initiation site of genes transcribed by RNA polymerase III (14, 15). Although the Ty3 integration complex has not yet been defined, IN must certainly be a component and is a candidate for mediating target specificity. Ty3 has amino-terminal and carboxyl-terminal IN domains which are significantly larger than the comparable domains in retroviral IN, which are hypothesized to interact with substrate DNAs. Whether one or both of these domains specifically interacts with the target site is not yet known. It is of special interest to determine which regions of Ty3 IN are critical for particular aspects of integration including target site selection.

It was previously shown that a Ty3 IN mutant was not capable of transposition in vivo (35). However, these studies did not identify the step in transposition that was blocked as a result of the IN mutation. Here, we analyze particle formation, polyprotein processing, reverse transcription, 3′-end processing, and transposition for Ty3 elements with mutations in conserved residues in the putative catalytic core domain of the IN protein and with deletions of the nonconserved carboxyl-terminal region of IN.

The Ty3 sequence predicts that the extrachromosomal DNA will have 2 extra bp. It contains an 8-nt putative primer binding site (PBS) sequence, complementary to the 3′ end of initiator $tRNA^{Met}$ (43, 44), located 2 nt downstream of the U5 region of the upstream LTR. Thus, the 5′ end of the minus strand primed from the tRNA would begin with these 2 extra nt and continue in LTR sequence, and the plus strand copied from this template would have 2 extra nt at its 3′ end. In the case of retroviruses, a polypurine tract (PPT) which is located immediately upstream of the U3 region of the downstream LTR functions similarly, priming from 2 nt within the PPT and resulting in addition of 2 nt to the 5′ end of the plus-strand species (which is copied in minus-strand completion). Because the PPT is continuous with the LTR in Ty3, whether this occurs cannot be deduced from the sequence. The presence of extra nucleotides would require terminal processing of the Ty3 DNA ends, a function anticipated to be performed by Ty3 IN, on the basis of analogies with retroviruses. In the present study, the termini of Ty3 VLP DNA and a candidate plus-strand strong-stop DNA were identified. The preintegrative DNA is 2 bp longer at each end than the integrated Ty3 element. Removal of the two bases from each 3′ terminus does not occur for Ty3 elements carrying mutations in the IN catalytic triad.

MATERIALS AND METHODS

Yeast and bacterial strains and culture conditions. Culturing and transformation of *Escherichia coli* and *S. cerevisiae* strains were performed according to standard methodology (2). *S. cerevisiae* yTM441 (57) (*MAT***a** *trp1-H3 ura3-52 his3-Δ200 ade2-101 lys2-1 leu1-12 can1-100* ΔTy3 *GAL3*$^+$), a derivative of yVB110 which contains no endogenous Ty3 elements (34), was used for experiments in which Ty3 transposition was monitored. VLP protein and DNA analyses were performed with strain AGY-9 (*MAT***a** *ura3-52 his4-539 lys2-801 trp1-Δ63 leu2-Δ1 spt3*) (a gift from A. Gabriel and J. Boeke, The Johns Hopkins University). AGY-9 transformed with plasmids expressing Ty3 was used for VLP production because the mutation in *SPT3* reduces Ty1 expression and because this strain produces higher levels of Ty3 VLPs than the Ty3 null strain yTM441. *E. coli* RZ1032 (*lysA*[*61-62*] *thi-1 relA1 spoT1 dut-1 ung-1* [Tet^r] *supE44*) was used for production of single-stranded DNA for site-directed mutagenesis by the method of Kunkel (51). Plasmids were amplified in HB101 (F^- *hsd-20* [r_B^- m_B^-] *recA13 leuB6 ara-14 proA2 lacY1 galK2 rpsL20* [Sm^r] *xyl-5 mtl-1 supE44* λ^-).

Recombinant DNA manipulations. All recombinant DNA procedures were performed essentially as described in *Current Protocols in Molecular Biology* (2). Galactose-inducible Ty3 elements were used for transposition studies and VLP analyses. Fusion of the *GAL1-10* upstream activation sequence upstream of the putative Ty3 TATA element was previously described for the construction of pEGTy3-1 (pDLC201) (34). The helper plasmid, pJK312AC (pJK723), and the donor plasmid, pJK422, were described previously (47). The yeast selectable marker for pEGTy3-1 and pJK422 is the *URA3* gene, and that for pJK312AC is the *TRP1* gene. Both pEGTy3-1 and pJK422 have the 2 μm sequence for maintenance at high copy number in *S. cerevisiae*. Plasmid pJK312AC has the *ARS1* and *CEN4* sequences for maintenance at low copy number in *S. cerevisiae*. A pIBI20 plasmid containing the 3′ half of Ty3 on a 2.2-kb *Sal*I-*Eco*RI fragment (pVB193 [4]) was used for mutagenesis. Four oligonucleotides were used to mutagenize sequences within the Ty3 IN coding region of *POL3*. Oligonucleotide 65 (5′GTCTTGTGTTTCGGGATCCTAGAGTTGTC3′) was used to change a tyrosine codon to a stop codon at codon position 510 in the IN-coding sequence. This Ty3 mutant is referred to as CΔ27, to indicate that IN has been truncated at the carboxyl terminus by 27 residues. Oligonucleotide 19 (5′CT GAAAGTGTTGGATCCACGTCTTGCATG3′) was used to introduce a *Bam*HI site at nucleotide position 4884. The plasmid DNA containing this mutation was digested with *Bam*HI, the overhanging ends were filled in with Klenow polymerase, and the resulting duplex blunt ends were ligated. These manipulations resulted in a frameshift mutation which caused changes in codons from positions 480 to 490 and introduced a stop codon at position 491 in the IN-coding sequence. This Ty3 mutant is referred to as FSΔ46, which indicates that the frameshift mutation resulted in deletion of 46 residues from the C terminus of IN. Oligonucleotide 263 (5′GACATCTCTTTCACTGGTTA3′) was

used to change the Asp codon at position 225 to a Glu codon in the IN-coding sequence. This Ty3 mutant is referred to as D225E. Oligonucleotide 264 (5′GTATCGTTCGATCGGATTGTC3′) was used to change the Glu codon to an Asp codon at position 261 in the IN-coding sequence. This Ty3 mutant is referred to as E261D. The plasmid DNA containing the mutation engineered with oligonucleotide 263 was also subjected to mutagenesis with oligonucleotide 264. The Ty3 mutant with both substitutions is referred to as D225E/E261D. The *Sal*I-*Eco*RI fragments each containing one of the five mutant sequences were then cloned separately into plasmids pJK312AC and pEGTy3-1 by replacing the analogous fragment of wild-type Ty3 in each. The Ty3 elements in pJK312AC served as helper elements in the genetic assay for transposition. The pJK312AC plasmids carrying the mutant Ty3 elements, designated according to the mutating oligonucleotide, are pJK312ACmut65 (pJK735), pJK312ACmut19 (pJK736), pJK312ACmut263 (pJK785), pJK312ACmut264 (pJK786), and pJK312ACmut 263/264 (pJK787). The Ty3 elements in pEGTy3-1 and its derivatives were used for high-level Ty3 expression to produce VLPs for protein and DNA analyses. The pEGTy3-1 derivatives carrying mutant Ty3 elements are pEGTy3mut65 (pJK731), pEGTy3mut19 (pJK732), pEGTy3mut263 (pJK782), pEGTy3mut264 (pJK783), and pEGTy3mut263/264 (pJK784). Plasmid pEGTy3-2 (pDLC202) has been described previously (35) and is similar to pEGTy3-1 except that it carries a mutant Ty3. The Ty3 element in this construct, Ty3-2, contains a frameshift mutation in the IN-coding sequence which changes the codons from positions 432 to 461 and results in a stop codon at position 462. This Ty3 mutant is referred to as FSΔ75, to indicate that it has a frameshift deletion resulting in truncation of the C terminus by 75 residues.

Transposition assays. Transposition assays were performed as previously described (47), using a helper Ty3 carried on plasmid pJK312AC. The helper element was either a wild-type Ty3 or one of five mutant derivatives (D225E, E261D, D225E/E261D, CΔ27, or FSΔ46). The pJK312AC Ty3 from which helper Ty3 elements were derived has a deletion which removes the putative PBS, rendering it incapable of transposition. The donor Ty3 was marked with the yeast *HIS3* gene, which replaced the majority of the IN-coding sequence, rendering it incapable of autonomous transposition. Yeast strain yTM441 was transformed with the donor plasmid and then was transformed separately with each of the six helper plasmids. It was also transformed with the donor plasmid alone. Two independent transformants for each donor or donor-helper combination were grown for 5 days on synthetic medium lacking uracil or uracil and tryptophan, respectively, to select for the retention of these plasmids. The medium also contained an appropriate carbon source to induce Ty3 transcription (galactose) or to repress Ty3 transcription (glucose). Five purified colonies representing each original transformant were patched onto complete medium (YPD) and grown for 1 day to allow loss of the marked donor plasmid. Patches were replica plated from YPD onto synthetic medium containing 5-fluoro-orotic acid (5-FOA) and lacking histidine to select for cells which had lost the *URA3*-marked donor plasmid but retained the *HIS3*-marked Ty3.

VLP preparation. To study Ty3 proteins and DNA, yeast strain AGY-9 was transformed separately with pEGTy3-1 carrying a wild-type Ty3 or one of six mutant derivatives: D225E, E261D, D225E/E261D, CΔ27, FSΔ46, or FSΔ75. Transformed cells were pregrown in medium containing raffinose (a nonrepressing carbon source) and lacking uracil. Cells were transferred to synthetic medium containing galactose to induce transposition and lacking uracil. VLPs were partially purified from whole cell extracts as previously described (33). Briefly, cells were grown to late log phase, washed in buffer, digested with zymolyase, lysed with glass beads, and fractionated over a 25-ml 70/30/20% sucrose step gradient of 5, 5, and 15 ml, respectively, by centrifugation in an SW28 rotor at 83,000 × *g* for 3 h at 4°C. A total of 4 ml from the 70/30% interface of each gradient was collected and divided into two portions. One portion was extracted with phenol-chloroform (1:1), and the nucleic acid was precipitated with ethanol. The other portion was concentrated by centrifugation in a Ti50 rotor at 100,000 × *g* for 1 h at 4°C and resuspended in 50 μl of buffer (9 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.8], 13.5 mM KCl, 4.5 mM $MgCl_2$, 10% glycerol).

Southern analysis of VLP DNA. The concentration of nucleic acid isolated from VLP preparations was measured by A_{260}. To detect full-length replicated DNA, 10 μg of nucleic acid was treated with 2 μg of RNase A in a total volume of 15 μl for 20 min at 37°C, separated on a 0.8% agarose gel by electrophoresis, transferred to a nitrocellulose membrane (Duralon UV; Stratagene), immobilized by cross-linking with UV light in a Stratalinker 1800 (Stratagene) at 1,200 × 10^2 μJ/cm^2 for 30 s, and probed with a DNA fragment from the internal (non-LTR) portion of Ty3.

For the analysis of Ty3 DNA termini, 20 μg of nucleic acid from each VLP preparation was digested with 60 U of *Hin*fI and 2 μg of RNase A for 2.5 h at 37°C. The DNA was then extracted with phenol-chloroform (1:1) and precipitated with ethanol in the presence of 5 μg of carrier DNA. Two micrograms of plasmid pEGTy3-1 was manipulated in the same manner and used as a control for the presence of this plasmid in VLP preparations. Twenty micrograms of VLP nucleic acid and 5 μg of carrier DNA were also digested with 2 μg of RNase A for 2.5 h at 37°C without the addition of *Hin*fI. After ethanol precipitation, the samples were resuspended in the same volumes of buffer and dye as the sequencing reactions (described below). Samples were then divided into four aliquots and separated on individual 8% polyacrylamide gels containing 6 M urea. Equal volumes of digested VLP nucleic acid samples and sequence reaction samples were loaded. Sequence ladders were run in adjacent lanes to serve as molecular standards for determining the size of the Ty3 DNA fragments (described below). Each gel was electrophoresed at 1,350 V for 5 h and 10 min and exposed to X-ray film (Hyperfilm; Amersham) at −70°C to determine the appropriate portion for use in the Southern analysis. For the analysis of plus-strand strong-stop DNA, the VLP nucleic acid samples were heated to 80°C for 3 min, quickly cooled on ice, and digested with RNase A as described above. The samples were loaded on 8% polyacrylamide gels with 6 M urea and electrophoresed at 1,350 V for 11.5 h. For each experiment, fragments in the appropriate size range were transferred electrophoretically to a nylon membrane (GeneScreen, DuPont) in 1× TBE (0.45 M borate, 1.32 M trizma base, 0.045 M EDTA) for 30 min at 60 V and then for 2 h at 40 V. The DNA was immobilized by UV cross-linking (as described above) and baking at 80°C for 2 h. The blots were then probed separately with one of four oligonucleotides ^{32}P labeled at the 5′ end, which were complementary to sequences in the LTR: oligonucleotide 175 (5′CAGGGTGACGTATT GTC3′; probe A) is complementary to LTR nt 32 to 48 on the plus strand; oligonucleotide 201 (5′TGACAACTGGTTACTTCC3′; probe B) is complementary to LTR nt 260 to 277 on the minus strand; oligonucleotide 202 (5′TACGGGCTCGAGTAATCTCGGAGTGTCTTGACA3′; probe C) is complementary to LTR nt 300 to 332 on the plus strand; and oligonucleotide 2 (5′CCTAGCTGAACTACC3′; probe D) is complementary to LTR nt 118 to 132 on the minus strand. Two hundred nanograms of each oligonucleotide was 5′-end labeled with 10 U of T4 polynucleotide kinase (Boehringer Mannheim) and 500 μCi of [γ-^{32}P]ATP (7,000 Ci/mmol; ICN) in a 20-μl reaction for 1 h at 37°C. The free label was removed by purifying the oligonucleotide over a Nuc-Trap push column (Stratagene). Hybridization was performed with 2 × 10^6 cpm of end-labeled oligonucleotide at 50 to 52°C for 12 h. After hybridization and washing, the blots were exposed either to X-ray film (Hyperfilm; Amersham) or to a PhosphorImager screen and scanned with a PhosphorImager (Molecular Dynamics).

The sequence reactions used to generate size standards for analysis of the Ty3 DNA termini were primed with oligonucleotide 355 (5′AATCTCGGATCTA AACTAAT3′) or oligonucleotide 356 (5′ATTCATATTTTATATAATAT3′). Oligonucleotide 355 represents the 5′ endpoint in the minus strand of *Hin*fI digestion at the terminus-proximal site of two restriction sites in the LTR. Oligonucleotide 356 represents the 5′ endpoint in the plus strand of *Hin*fI digestion at the terminus-proximal restriction site in the LTR. A pIBI20 plasmid with either the 5′ half (pVB192 [4]) or the 3′ half (pVB193 [4]) of Ty3 cloned into the polylinker was used as a template for sequencing. Oligonucleotide 355 was annealed to the LTR in pVB193 to prime synthesis of minus-strand DNA through the U3 sequence of the LTR. Oligonucleotide 356 was annealed to the LTR in pVB192 to prime synthesis of plus-strand DNA through the U5 sequence of the LTR. The sequence ladder for the analysis of plus-strand strong-stop DNA was primed with oligonucleotide 73 (5′GATGTTGTATCTCAAAATGAG3′). Oligonucleotide 73 was annealed to the LTR in pVB193 to prime synthesis of DNA equivalent to plus-strand strong-stop DNA primed 2 bp upstream of U3. Each oligonucleotide was labeled at the 5′ end as described above for the oligonucleotide probes, and ~1 pmol (10^6 cpm/pmol) was annealed to 1 pmol of template DNA. Sequencing was performed under standard reaction conditions but without the addition of [α-^{32}P]dGTP.

Immunoblot analysis. Proteins fractionated on sodium dodecyl sulfate (SDS)-polyacrylamide gels were transferred electrophoretically to nitrocellulose membranes (Hybond ECL; Amersham) and probed with an antibody to RT or IN. The purification and use of these antibodies have been described previously (57). Secondary antibodies to rabbit immunoglobulin G were detected by chemiluminescence, using the ECL system (Amersham).

RT assays. Exogenous RT activity was measured under conditions optimized for Ty3 RT (33). VLPs were prepared as described above from galactose-induced AGY-9 cells transformed with pEGTy3-1 containing either wild-type Ty3 or one of six IN mutants (D225E, E261D, D225E/E261D, CΔ27, FSΔ46, or FSΔ75). A VLP preparation was also made by using yeast strain AGY-9 cells that were not transformed with a Ty3 expression plasmid. Five micrograms of protein for each sample was analyzed by SDS-polyacrylamide gel electrophoresis followed by Coomassie blue staining and scanning of the 26-kDa CA protein with a densitometer (Pharmacia LKB Ultrascan II). On the basis of the scanning measurement, each sample was assigned a value in CA units (an arbitrary designation to reflect the amount of VLP, rather than total, protein). RT activity was calculated as the percentage incorporation of [α-^{32}P]dGTP at 26°C in 2.0 h, using a poly(C) template and an oligo(dG) primer. Incorporation of radioactivity was linear during this time for the amount of protein used (33, 47). Datum points represent averages of at least two experiments performed in duplicate. The percentage incorporation was plotted as a function of CA units.

RESULTS

Ty3 elements with mutations in IN fail to complement transposition of a marked donor. Several laboratories have demonstrated the necessity of preserving the invariant residues of the D,D(35)E motif for the activity of IN proteins from several types of retroviruses in vitro and for HIV replication in vivo.

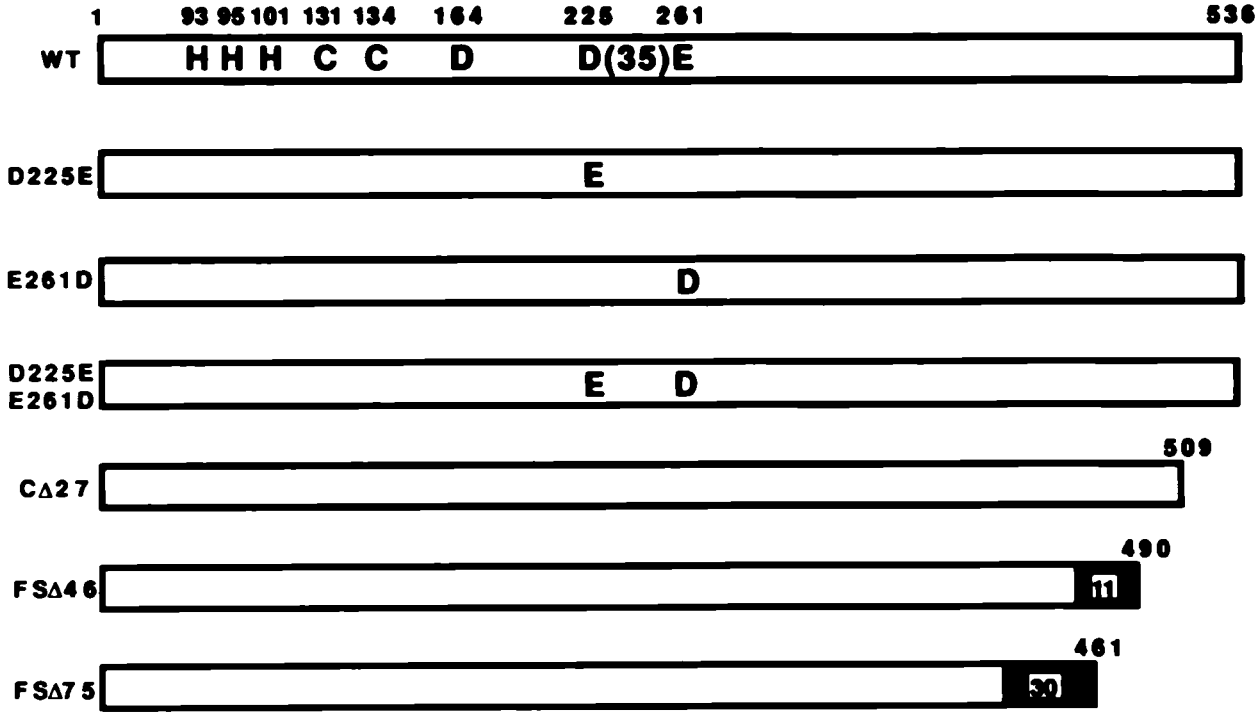

FIG. 1. Schematic diagram of mutations in Ty3 IN. The 61-kDa IN protein is shown as an open box. Position 1 is the first amino acid, as determined from the known N-terminal sequence of IN. The conserved residues of the HHCC and D,D(35)E motifs and their positions in the 536-amino-acid protein are shown. Functional studies have not yet been performed to determine the requirement for each of the closely spaced His residues. Therefore, all three were included in the diagram. The positions of the mutations in the D225E, E261D, D225E/E261D, CΔ27, FSΔ46, and FSΔ75 IN mutants are shown. Each shaded region indicates residues differing from the wild-type sequence, with the length of the sequence indicated in the shaded box.

These residues constitute a catalytic triad which is critical for IN nucleotidyl transfer activity. Ty3 IN has the D,D(35)E motif. The effects of conservative changes in the second Asp and the Glu residues (Fig. 1) of helper elements were tested in the genetic assay for transposition (Fig. 2). These mutants contained a single substitution of Glu for Asp at position 225 (D225E), a single substitution of Asp for Glu at position 261 (E261D), or the substitutions in combination (D225E/E261D) (Fig. 1). These mutations were introduced into a helper Ty3. The mutant helpers were used in a genetic assay to determine whether they could complement transposition of a *HIS3*-marked donor Ty3 (Fig. 2). Cells containing a particular helper-donor combination were grown on medium containing galactose to induce Ty3 transcription or glucose to repress Ty3 transcription. Cells in which wild-type Ty3 transcription was induced grew on medium containing 5-FOA and lacking histidine, indicating that transposition occurred. None of these three mutants was able to complement transposition of the marked donor Ty3. Thus, these two residues in Ty3 IN are critical for transposition and probably for IN function.

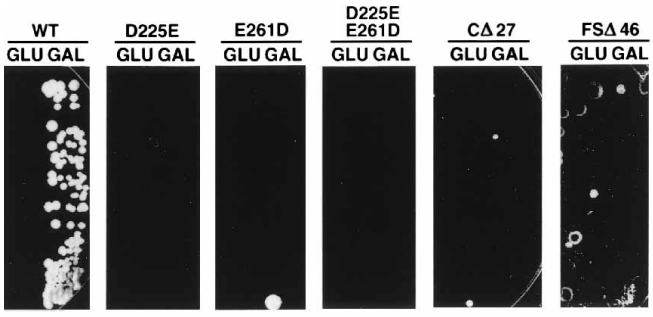

FIG. 2. Transposition assays of Ty3 IN mutants. A genetic helper-donor assay for transposition was performed with each of the Ty3 IN mutants. Strain yTM441 cells were transformed with each mutant Ty3 helper element and a donor Ty3 marked with the yeast *HIS3* gene, carried on independent plasmids. Ty3 transcription was induced by streaking the cells for growth on galactose (GAL)-containing medium or repressed by growth on glucose (GLU)-containing medium. Five independent colonies were patched onto rich medium to allow plasmid loss and then replica plated to medium containing 5-FOA and lacking histidine. The ability of each helper element to complement transposition of a marked donor was assessed by growth of cells that had been induced on GAL-containing medium on medium containing 5-FOA and lacking histidine. The IN mutants encoded by helper Ty3 elements were D225E, E261D, D225E/E261D, CΔ27, and FSΔ46. A wild-type (WT) Ty3 was used as a control in each experiment but is shown for only one.

Previous studies in our laboratory with a naturally occurring mutant of Ty3, Ty3-2, showed that the carboxyl-terminal region of Ty3 was important for transposition (35). The frameshift mutation in Ty3-2 causes the protein to be truncated by 75 residues, with the last 30 residues of this protein differing from the wild-type IN amino acid sequence. While this study clearly showed that intact IN was required for transposition, it did not determine which portion of the 105 carboxyl-terminal residues was important for function, nor did it identify the precise step in transposition that was blocked. Shorter carboxyl-terminal regions of IN were deleted to determine whether less dramatic changes in the protein would be tolerated. Stop codons were introduced into the IN-coding sequence either directly (mutant CΔ27) or by virtue of a frameshift mutation (mutant FSΔ46), using oligonucleotide site-directed mutagenesis (Fig. 1). The IN protein of CΔ27 ends at residue 509, resulting in a protein truncated by 27 residues. The IN protein of FSΔ46 ends at

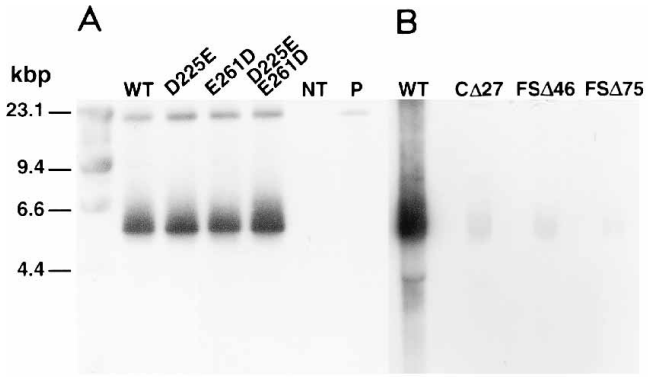

FIG. 3. Southern blot analysis of VLP nucleic acid from cells expressing Ty3 IN mutants. Ten micrograms of total VLP DNA was processed as described in Materials and Methods and separated on a 0.8% agarose gel at 80 V for 10 h. The DNA was blotted to a nitrocellulose membrane and probed with a fragment of Ty3 spanning the majority of the internal, non-LTR sequence. (A) VLP samples from AGY-9 cells transformed with pEGTy3-1 carrying a wild-type Ty3 (WT) or one of the IN mutants (D225E, E261D, or D225E/E261D). Nucleic acid from AGY-9 cells that were not transformed with a plasmid (NT) and the expression plasmid pEGTy3-1 DNA (P) were included as controls. (B) Wild-type Ty3 (WT) or one of the IN carboxyl-terminal truncation mutants (CΔ27, FSΔ46, or FSΔ75). Molecular weight standards were lambda DNA digested with *Hin*dIII, and the sizes are indicated on the left.

position 490, resulting in a protein truncated by 46 residues. The last 11 residues of this protein differ from the wild-type sequence. These mutations were also introduced into a helper Ty3, and transposition was monitored as described above. Neither CΔ27 nor FSΔ46 was able to complement transposition of the donor, indicating an inability to supply functional proteins in *trans* (Fig. 2). Thus, preservation of at least some portion of the carboxyl-terminal 27 residues of Ty3 IN is required for transposition.

Conservative substitutions of the Asp and Glu residues of the D,D(35)E motif which abolished transposition did not affect levels of Ty3 DNA. The amount of full-length reverse-transcribed DNA in VLPs isolated from cells expressing the D225E, E261D, and D225E/E261D mutants was determined by Southern analysis using a probe to the internal (non-LTR) portion of Ty3 DNA (Fig. 3). Nucleic acid from the VLPs of the mutants contained wild-type amounts of reverse-transcribed DNA (Fig. 3A). After normalization for CA content, equal amounts of VLPs were electrophoresed on an SDS–10% polyacrylamide gel. This was performed in triplicate with identical samples. One gel was stained with Coomassie blue (Fig. 4A), and the protein in the other two gels was transferred to nitrocellulose membranes and used for Western blot (immunoblot) analysis with antibodies to IN or RT (Fig. 4B or C, respectively). The wild-type mature IN protein actually occurs as two species (61 and 58 kDa). The size difference is due to differential amino-terminal processing (47). The amounts of mature VLP RT and IN proteins (Fig. 4) were approximately the same as those in wild-type VLPs.

Truncation of the carboxyl-terminal region of IN resulted in decreased amounts of reverse-transcribed DNA in vivo and decreased RT activity in vitro but did not affect the processing or amount of mature RT. The level of full-length reverse-transcribed DNA in VLPs isolated from cells expressing the truncated mutants was determined as described for the missense mutants (Fig. 3). Very little Ty3 DNA was detected in cells expressing each of the three IN truncation mutants. A trivial explanation for the effect of IN carboxyl-terminal truncations on DNA synthesis was that alteration of the structure

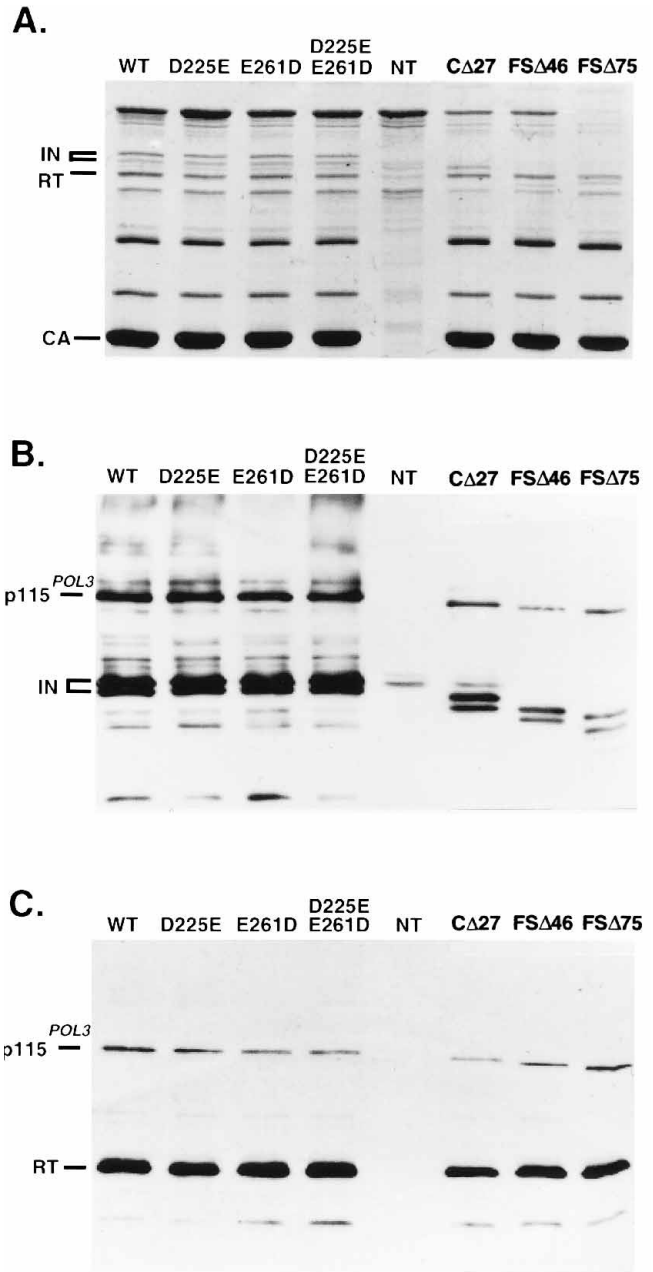

FIG. 4. Immunoblot analysis of VLP proteins from cells expressing Ty3 IN mutants. (A) VLPs were isolated from cells expressing wild-type Ty3 (WT) or one of the IN mutant Ty3 elements (D225E, E261D, D225E/E261D, CΔ27, FSΔ46, or FSΔ75) or not transformed (NT). Equal amounts of VLP protein (~10 μg) from each sample were separated on a denaturing SDS–10% polyacrylamide gel and then stained with Coomassie blue. The IN doublet (61 and 58 kDa), RT (55 kDa), and the major structural protein, CA, are indicated to the left. Samples identical to those in panel A were transferred to nitrocellulose (Hybond ECL; Amersham) and probed with a polyclonal rabbit anti-IN immunoglobulin G antibody (B) or a polyclonal rabbit anti-RT immunoglobulin G antibody (C). The IN doublet, RT, and p115^{POL3} proteins are labeled on the left.

of IN within the Gag3-Pol3 polyprotein resulted in structural aberrancies which caused rapid turnover of the VLPs and/or inhibited correct polyprotein processing. Rapid turnover, rather than accumulation, of the Gag3 and Gag3-Pol3 polyprotein precursors was observed previously for a protease-deficient Ty3 mutant (47). To determine whether the defective IN

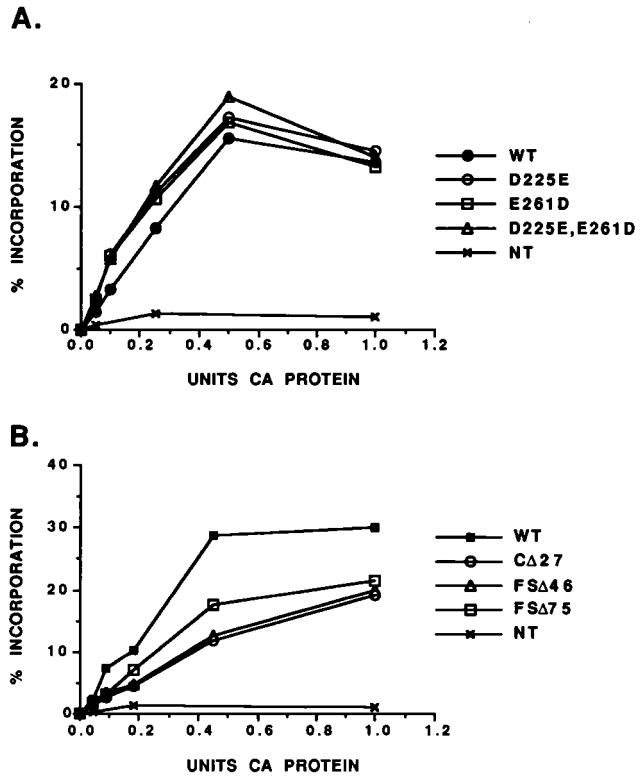

FIG. 5. In vitro RT activity of Ty3 IN mutants. Equal amounts of VLP protein were used for all of the samples: wild-type Ty3 (WT) and each of the IN mutants. RT activity was calculated as the percentage incorporation of [α-^{32}P]dGTP at 26°C in 2.0 h, using a poly(C) template and an oligo(dG) primer. The percentage incorporation was plotted as a function of CA units (corresponding to 1 to 5 μg of protein). (A) D,D(35)E mutants; (B) carboxyl-terminal truncation mutants. NT, protein from AGY-9 cells that were not transformed with a Ty3 plasmid.

was associated with low levels of Ty3 particles, VLP proteins expressed from wild-type Ty3 and from each of the Ty3 IN mutants were analyzed. Equal amounts of total protein from VLP preparations were examined by separation on an SDS–10% polyacrylamide gel followed by Coomassie staining. Each mutant produced approximately wild-type amounts of VLPs, as judged from the amount of the major structural protein, CA (data not shown). Thus, rapid particle turnover was not the cause of the lower amount of DNA.

Although VLPs were not destabilized by the IN truncation mutants, local structural changes in IN could have caused global structural changes in the precursor polyprotein which inhibited processing and resulted in a decreased amount of mature RT (47). Previous studies have shown that RT activity is severely reduced in mutants which are affected in processing activity. VLP samples containing equal amounts of CA protein were examined by immunoblot analysis to determine the relative amounts of the RT and IN proteins. The IN species from each of the truncation mutants (CΔ27, FSΔ46, and FSΔ75) were smaller than wild-type IN proteins and occurred as doublets of approximately the sizes predicted for truncated versions of the 61- and 58-kDa IN species on the basis of the positions of the stop codons terminating IN synthesis (Fig. 4B). The truncated IN proteins appeared to be present in slightly lower amounts than wild-type IN, suggesting that the mutant species were less stable. On the basis of the immunoblot assay with the anti-RT antibody, there appeared to be equal amounts of RT synthesized for all of the truncation mutants, and the amount and size of this species were comparable to those in wild-type VLPs (Fig. 4C).

To determine whether RT specific activity is diminished in VLPs containing mutated IN, RT activity was measured in vitro. VLPs from cells expressing each of the mutant Ty3 elements and representing equal amounts of CA were serially diluted and used in a standard exogenous RT assay. RT activity was measured as the incorporation of [α-^{32}P]dGTP, using a poly(C) template and oligo(dG) primer. Mutations in the conserved residues of IN did not affect RT activity (Fig. 5A). However, each of the IN carboxyl-terminal mutations caused a decrease in RT activity of mutant VLPs relative to the wild-type level. The activity of the mutant VLPs was still significantly higher than background (Fig. 5B). Thus, while the size and amount of the 55-kDa RT species were not affected by the carboxyl-terminal truncations of IN, the product of reverse transcription was decreased both in vivo and in vitro.

The 115-kDa VLP protein is an RT-IN fusion protein. Previously, a 115-kDa protein was observed in Ty3 VLP preparations and detected with an anti-IN antibody (35). The size of this protein was affected by the frameshift mutation in Ty3-2 which caused a carboxyl-terminal truncation of each mature IN protein (35). It therefore was postulated that the 115-kDa species represented a processing intermediate. Its size was consistent with that predicted for a fusion of the 55-kDa RT species and the 61-kDa IN species. In the immunoblot analysis of VLP protein shown in Fig. 4B and C, it was demonstrated that the a 115-kDa protein was detected by both anti-RT antibody and anti-IN antibody. In addition, two new mutations that caused truncation of the mature IN species at the C terminus resulted in truncation of the 115-kDa species by the same amount. Like the full-length 115-kDa species, the truncated forms of this protein were detected by both antibodies. The mature IN species of the truncation mutants were present in lower amounts than wild-type IN, but there did not appear to be a resulting accumulation of the precursor 115-kDa protein (at least not higher than wild-type levels), suggesting that both the RT-IN and IN truncation species were inherently unstable.

Ty3 preintegrative DNA is 2 bp longer at each end than the integrated element. The position of the putative Ty3 PBS is 2 bp offset from the junction of the U5 region and the internal domain. Thus, replication by a retroviruslike mechanism would result in addition of 2 extra nt to at least one 5′ end of the DNA, which would ultimately result in addition of extra nucleotides to the 3′ complementary end as well. To test whether this occurs in Ty3 replication, the structure of the termini of Ty3 DNA from wild-type VLPs was examined, using a method similar to that used to identify retroviral DNA ends (8, 30, 67). The nucleic acid was extracted from sucrose gradient fractions containing Ty3 VLPs and treated with RNase A and *Hin*fI. This restriction enzyme is predicted to release unique-sized fragments from the U3 and U5 ends of full-length linear Ty3 DNA. The products of digestion were separated on denaturing polyacrylamide gels. Sequence ladders were run in lanes adjacent to the VLP DNA samples to serve as molecular weight standards. The sequence ladder used for determination of fragment sizes from the U3 minus strand (3′ end of the Ty3 minus strand) and U3 plus strand (5′ end of the Ty3 plus strand) was primed with an oligonucleotide annealed to the plus strand of the downstream Ty3 LTR. This oligonucleotide represented the 5′-most 20 nt of the minus strand of the predicted product of *Hin*fI digestion at the site closest to the 5′ end of the LTR. The sequence ladder used for determination of the sizes of fragments from the U5 plus strand (3′ end of the

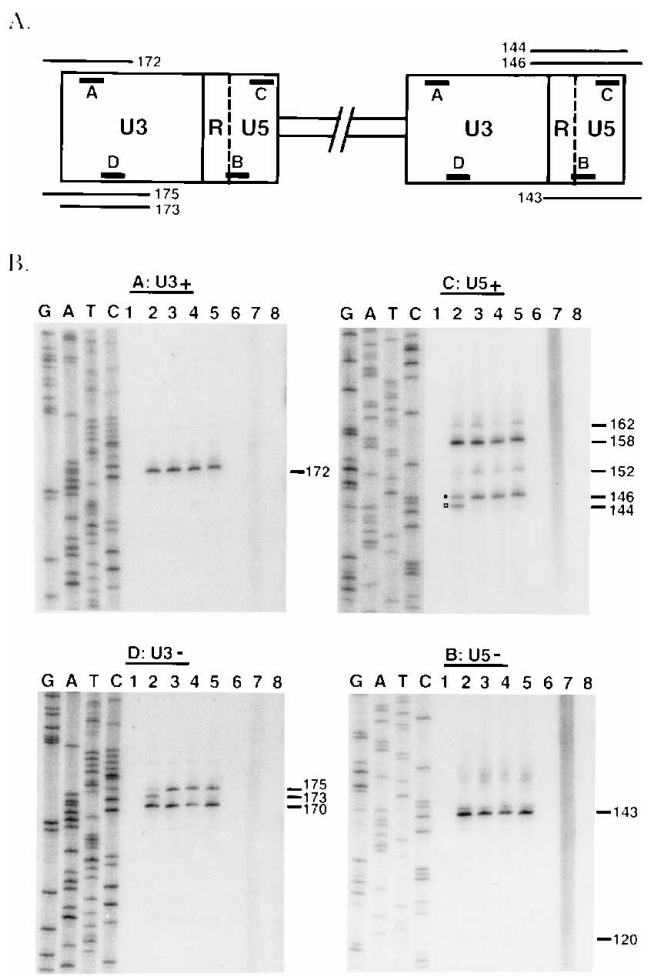

FIG. 6. Characterization of the terminal structure of Ty3 DNA and detection of the putative minus-strand strong-stop DNA. (A) Schematic diagram of Ty3 with the LTR regions U3, R, and U5 labeled in each LTR. The thick lines represent the relative positions of annealing of each of the four oligonucleotide probes, A, B, C, and D. The vertical dashed line represents the heterogeneity of transcription termination in Ty3. The sizes of *Hin*fI fragments predicted to be released from the ends when Ty3 linear DNA is digested with this enzyme are indicated. There are two *Hin*fI sites in the Ty3 LTR. (B) Southern analysis of *Hin*fI-digested VLP DNA probed with oligonucleotide A, B, C, or D as described in Materials and Methods. Sequence ladders representing the 3′ termini of Ty3 DNA were run in adjacent lanes. A different ladder was used for the U3 and U5 ends of the DNA. The DNA was probed with one of four oligonucleotides (A, B, C, or D) as indicated at the top. The blots were exposed to a PhosphorImager screen for 5 days and then scanned in a PhosphorImager (Molecular Dynamics) and imaged with ImageQuant software (Molecular Dynamics). The strand and LTR region detected by each probe are indicated. Fragment sizes are indicated in base pairs to the right of each panel. In each panel, the *Hin*fI-digested samples are the following: lane 2, wild-type DNA; lane 3, D225E; lane 4, E261D; lane 5, D225E/E261D; lane 6, DNA from nontransformed cells; and lane 7, pEGTy3-1 plasmid DNA. The DNA samples digested with RNase A but not *Hin*fI in each panel are the following: lane 1, wild-type DNA; and lane 8, carrier DNA. Black dots indicate fragments matching with two bases beyond the end of the LTR; open squares indicate fragments matching the end of the LTR. In the C: U5+ panel, the sequence ladder shown is a darker exposure of that portion of the blot in order to facilitate size comparisons.

Ty3 plus strand) and U5 minus strand (5′ end of the Ty3 minus strand) was primed with an oligonucleotide annealed to the minus strand of the upstream Ty3 LTR. This oligonucleotide represented the 5′-most 20 nt of the plus strand of the predicted product of *Hin*fI digestion at the site closest to the 3′ end of the LTR. The ladder fragments were identical in composition to the 3′-end sequences of Ty3. However, it was not possible to generate sequence ladders representative of the 5′-end fragments without making an assumption about the product of reverse transcription. The predicted sizes of the fragments released from the ends of the plus and minus strands of the linear Ty3 DNA were between 140 and 180 bp (Fig. 6A); therefore, all four ends could be examined by using the same gel conditions. Identical DNA samples were separated on four denaturing polyacrylamide gels, transferred to nylon membranes, and probed with one of four oligonucleotides (probe A, B, C, or D). Each oligonucleotide anneals to one of the four ends of the Ty3 DNA as diagrammed in Fig. 6A. *Hin*fI-digested DNA was examined from wild-type Ty3 VLPs (Fig. 6B, lanes 2) and from each of the IN mutants which produced Ty3 DNA (D225E, E261D, and D225E/E261D; Fig. 6B, lanes 3, 4, and 5, respectively, in each panel). Controls for nonspecifically hybridizing DNA species included *Hin*fI-digested DNA from AGY-9 cells which were not transformed with a plasmid (Fig. 6B, lanes 5), *Hin*fI-digested pEGTy3-1 DNA (Fig. 6B, lanes 6), and *Hin*fI-digested carrier DNA used to precipitate the digested samples (Fig. 6B, lanes 7). Undigested wild-type Ty3 VLP DNA treated with RNase A (Fig. 6B, lanes 1) was used as a control for the detection of any Ty3 DNA fragments that were not a result of *Hin*fI digestion. Probes for the 5′ ends of the plus and minus strands, probe A and probe B, respectively, each detected a single prominent species that was present in all four VLP samples, both wild-type and mutant, but not in the undigested control or in the nontransformed and plasmid DNA controls. Thus, there was a single major species for each 5′ end of Ty3 linear DNA. Each 5′-end DNA species migrated with a fragment in the sequence ladder equivalent to the size of the *Hin*fI fragment predicted to be generated if the preintegrative DNA has 2 additional nt at each end compared to the integrated element (172 bases for the U3 plus-strand fragment; 143 bases for the U5 minus-strand fragment). Probes for the 3′ ends of the plus and minus strands, probe C and probe D, respectively, each detected a species (146 and 175 bases, respectively; which migrated with the mobility of the sequence ladder fragment representing the LTR fragment plus 2 nt. Probes C and D also each detected a species that was 2 nt smaller (144 and 173 bases, respectively) and which migrated with the mobility of the sequence ladder fragment representing the end of the LTR. These species were not detected in any of the control samples. This experiment was also performed with wild-type Ty3 DNA that was digested with *Hha*I. DNA terminal fragments of the sizes predicted on the basis of the positions of the *Hha*I sites were detected (data not shown). This analysis showed that preintegrative Ty3 DNA is 2 nt longer than the integrated form at the 3′ ends of the plus and minus strands. The 5′-end fragments migrated with a mobility consistent with a 2-nt extension, as would be expected for the template for the 3′ ends. Therefore, the full-length product of Ty3 reverse transcription must be 2 bp longer at each end than the integrated DNA.

Conservative changes in the Asp and Glu residues of the D,D(35)E motif prevent processing of Ty3 DNA termini and integration. Mutations in the conserved Asp and Glu residues of the D,D(35)E motif in Ty3 IN abolished transposition (Fig. 2). In an in vitro assay for Ty3 integration in which VLPs are the source of both the DNA substrate and IN, Ty3 VLPs with the D225E, E261D, or D225E/E261D mutation in IN failed to catalyze integration (46). Since these mutants all synthesized approximately full-length Ty3 DNA, this in vitro result suggested that the IN protein was inactive and/or that the preintegrative DNA had some defect which prevented its use as a substrate. The most likely defect in the structure of the DNA

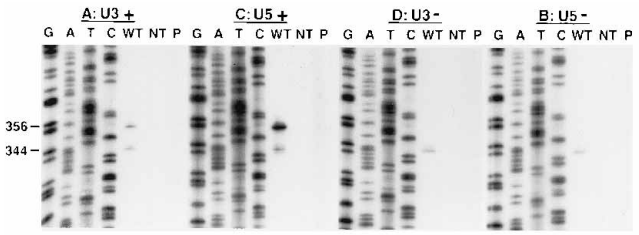

FIG. 7. Detection of Ty3 plus-strand strong-stop DNA. VLP DNA from cells expressing wild-type Ty3 (WT) or from cells that were not transformed (NT) and plasmid pEGTy3-1 DNA (P) were as described in Materials and Methods. Sequence ladders representing the predicted plus-strand DNA were run in adjacent lanes. The sizes of the DNA species detected are indicated in base pairs at the left. The probe used and the strand and LTR region that it detected are indicated at the top of each panel. The blots were exposed for 10 days to X-ray film (Hyperfilm; Amersham).

would be a lack of processing at the 3′ termini as a result of the inactivation of IN. The termini of DNA from the mutant VLPs were examined and compared with the termini of DNA from wild-type VLPs (Fig. 6). The same DNA species were observed for wild-type Ty3 and each of the mutants at the 5′ ends of the DNA (detected by probes A and B), but the pattern of DNA fragments from wild-type VLPs differed from that of the DNA from mutant VLPs at the 3′ ends (detected by probes C and D). As discussed previously, wild-type Ty3 DNA had a mixture of two species for each 3′ end. The larger species corresponded to the LTR fragment plus 2 nt and the other species, 2 nt smaller, corresponded to the LTR fragment without the 2 extra nt. In DNA from each of the three IN mutants, however, only the larger of the two species was detected. Even after a long exposure, the smaller species was not observed (data not shown). These results demonstrated that the activity of Ty3 IN is required for removal of the 3′-terminal dinucleotides.

In addition to the products of restriction digestion described above, other products of digestion that were unaffected by the mutations which changed D225 and E261 were observed. A prominent species detected by probe C was 12 nt larger than the fragment generated from the LTR. This 158-nt species could represent the *Hin*fI fragment from the 3′ end of the plus-strand strong-stop DNA if minus-strand DNA and the first 12 nt of the tRNA primer were used as a template. The origins of the two fainter species detected by probe C (152 and 162 nt) and the 170-base species detected by probe D are unknown. DNA species of unknown origin were also observed in the DNA samples digested with *Hha*I. However, species analogous to the 152-, 162-, and 170-nt species were not detected in the *Hha*I-digested DNA with the same probes. Therefore, these species may be aberrant products of restriction digestion specific to *Hin*fI. Because the D225E and E261D mutations abolished IN processing of the ends of Ty3 preintegrative DNA, it is unlikely that the unidentified DNA species are the result of IN endonuclease activity.

Ty3 plus-strand strong-stop DNA is present as a mixture of two species. To identify the plus-strand strong-stop DNA, DNA from wild-type VLPs was used in a Southern analysis similar to that described above. DNA from yeast cells that were not transformed, and the source plasmid from which Ty3 was expressed, were used as controls for nonspecifically hybridizing DNA species. In this experiment, however, the DNA was heated, quickly cooled on ice, and treated with RNase A for 30 min at 37°C. The sequence ladder used to size the plus-strand DNA was primed with a plus-strand oligonucleotide which had its 5′ end located at the start site of plus-strand synthesis, as determined from the analysis of DNA termini presented in Fig. 6. This site is two bases upstream of the U3 sequence in the downstream LTR. The DNA samples were separated on denaturing 8% polyacrylamide gels, transferred to nylon membranes, and hybridized with probe A, B, C, or D (Fig. 7). Probes A and C are both complementary to the plus strand of the LTR (Fig. 6). Each probe specifically detected two prominent strong-stop species, 356 and 344 bases. The Ty3 LTR is 340 bp in length. The more intense band represented a fragment of 356 bases, which is the size predicted for full-length Ty3 strong-stop DNA if it were primed 2 nt upstream of the LTR in the PPT and terminated 12 bases into the initiator $tRNA^{Met}$ attached to the 5′ end of the minus-strand DNA template. The less intense band represented a fragment of 344 bases, which is 4 bp longer than the LTR. Probes B and D, complementary to the minus strand, each detected the smaller of the two DNA species but not the larger, even though it was more abundant. All four probes (A, B, C, and D) also detected a minor species of 345 nt (faintly visible in Fig. 7). The species that were detected by both plus- and minus-strand probes may exist as double-stranded DNA molecules. Thus, Ty3 VLP DNA species include a form consistent with generation of plus-strand strong-stop DNA from an internal PPT primer.

DISCUSSION

Ty3 IN protein is larger than the typical retroviral IN protein, and Ty3 integrates with unusual specificity into the target genome. Nevertheless, Ty3 IN has the conserved residue at 12 of the 18 residues which are identical among HIV-1, Rous sarcoma virus (RSV), bovine leukemia virus, MoMLV, and human T-cell leukemia virus I IN proteins. It has a similar residue at four of the remaining six positions. Mutations in five of these six residues, which are not conserved in Ty3 IN, in HIV-1 IN did not block viral replication (this study and references 13 and 28). This finding argues that Ty3 IN is likely to perform the central functions mediated by retroviral IN. Retroviral IN has been demonstrated to catalyze both the removal of 2 nt from each of the 3′ ends of the replicated extrachromosomal retroviral DNA and the transfer of the 3′ ends to chromosomal target DNA. The sequences of LTR retrotransposons from a variety of organisms, however, do not predict the existence of these extra nucleotides (31), which suggests that these elements do not process the ends of the DNA by removing extra nucleotides. Although it has been shown with Ty1 VLP IN and recombinant IN that Ty1 IN is competent for strand transfer of different blunt-ended substrates, the ends of the replicated element have not been examined directly. The present study reports the demonstration of 2 extra bp at each end of Ty3 preintegrative DNA and further demonstrates that mutations introduced into the catalytic triad residues of IN block 3′-end processing. A subset of the Ty3 mutants examined resulted in loss of RT activity, implicating the IN domain in reverse transcription. Two plus-strand strong-stop species were observed. One was the size predicted for a species templated by primer. Because the Ty3 putative PBS is not 12 nt in length, this observation extends the surprising finding in the Ty1 system that a major plus-strand strong-stop DNA may not be an intermediate in transposition.

Six mutations were introduced into the IN-coding domain of Ty3, and the effects on particle formation, IN and RT maturation, reverse transcription, 3′-end processing, and transposition were tested. The mutants, each of which failed to transpose, were blocked either at the stage of reverse transcription or in the integration of the replicated DNA. Mutations in the coding region for the carboxyl-terminal portion of Ty3 IN

truncated IN by 27, 46, or 75 residues. These mutations all severely disrupted reverse transcription, whether measured in an in vitro assay or by monitoring appearance of the 5.4-kbp Ty3 VLP DNA in vivo. The mobilities of IN and RT species were consistent with correct amino-terminal processing, suggesting that the proteins were not grossly aberrant. The amount of the 55-kDa RT did not appear to be significantly affected by these mutations. Previous studies identified a 115-kDa species which reacted with antibodies against Ty3 IN and was found only in cells expressing Ty3 (33, 35). In the current study, this 115-kDa species was again observed in immunoblotted preparations which were reacted with antibodies to Ty3 IN and also in preparations reacted with antibodies to Ty3 RT. Because Ty3 RT is encoded immediately upstream of IN and because a fusion of the 55- and 61-kDa domains would produce a protein of approximately the observed mass, the simplest explanation of the identity of the 115-kDa species is that this protein represents a fusion of RT and IN domains. Thus, three explanations for the diminution of RT activity in the IN truncation mutants present themselves: first, IN activity is directly required for reverse transcription; second, the activity of the 55-kDa RT protein is reduced because it is indirectly dependent on IN, perhaps for correct folding; and third, the activity of a heterodimeric RT composed of an RT subunit and a truncated RT-IN fusion subunit is attenuated, either because of inappropriate folding or because an essential domain is absent. The first model seems unlikely on the basis of the observation that mutations in the putative catalytic triad of IN which eliminated 3′-end processing and transposition did not affect RT activity in vitro or in vivo. The second and third possibilities are difficult to distinguish on the basis of the experiments performed in this study. There are precedents in retroviral systems and in Ty3 for mutations in one protein affecting the processing or activity of another protein as suggested in the second model. Linker and deletion mutagenesis of the IN-coding region of MoMLV, which has a monomeric RT, did not reveal any particular sensitivity of the RT domain to mutations introduced into IN (19, 68). In the case of HIV, the heterodimeric RT is composed of one polymerase-RNase H subunit and one polymerase subunit (76). These species are formed by differential carboxyl-terminal processing (55). Crystallographic studies have shown that domains of the different subunits which have the same primary structure fold quite differently in the distinct subunit contexts (49). Thus, the structure of the polymerase domain is affected by association in *trans* or *cis* with the RNase H domain. In this context, point mutations in the IN-coding domain have been found to have pleiotropic effects on virus replication, including effects on virion morphology and effects on RT activity (13, 28, 72). Thus, mutations in the Ty3 IN domain might affect RT activity without being part of the same heterodimeric molecule. There are, however, also precedents in retroviral systems for heterodimeric RT proteins as required in the third model. In the case of RSV, the RT is composed of alpha and beta subunits representing a polymerase-RNase H subunit and a polymerase-RNase H-IN subunit, respectively (32, 54, 65, 70). Although deletions of portions of the IN domain disrupt replication of RSV, the point at which replication is blocked in these mutants has not been determined (36). In this context, improper folding of the IN domain could directly disrupt the activity of the associated RT domain. The findings of both the requisite fusion species and the sensitivity of Ty3 RT activity to mutations in the IN domain are therefore consistent with, but do not prove, the hypothesis that Ty3 has an alpha-beta-type heterodimeric RT.

Conservative mutations introduced into positions of the D,D(35)E triad in Ty3 IN caused loss of 3′-end processing in vivo (this study) and in vitro (46). Changes introduced into the catalytic triad of RSV, MoMLV, and HIV IN have been shown to block 3′ dinucleotide removal, strand transfer, and disintegration in vitro, suggesting that these residues are required for nucleophilic attack on the phosphodiester bond of the target molecule. In addition, mutations of catalytic triad residues introduced into infectious clones of HIV-1 have been shown to block viral replication at some step after the nuclear import of replicated DNA and lead to the accumulation of circular DNA (13, 28, 68, 72, 80). Given that the extra nucleotides are present and processed in Ty3 and retroviruses but not in other LTR retrotransposons such as Ty1, what might be the function of such nucleotides? Ellison and Brown (25) have shown in vitro that integration complexes formed on substrates containing the 2 extra 3′ nt are more resistant to challenge by competitor DNAs than are species not containing these nucleotides. Thus, these extra base pairs could serve to stabilize the integration complex prior to target site contact. With respect to this model, it is interesting that Ty1, which does not have these extra nucleotides, both readily uses exogenous DNA and is relatively relaxed in its sequence requirements for the ends of donor molecules (7).

Other features of Ty3 transposition also resemble those of retroviral replication. In addition to the terminal fragments of full-length Ty3 DNA released by *Hin*fI digestion or *Hha*I digestion (data not shown), low-molecular-weight species present in RNase A-treated VLP DNA not digested with restriction enzymes were also identified. The most prominent of these hybridized to probes complementary to both the U3 and U5 regions and at 356 nt corresponded in length to the size of the LTR (340 nt) plus 16 nt. A 344-nt species was also observed. For retroviruses (reviewed in reference 74), Ty1 (59, 62), and gypsy(mdg) elements (1), plus-strand strong-stop species which are extended onto the tRNA primer of the minus-strand DNA are observed. In the case of Ty1, reverse transcription continues up to the first modified base of the tRNA primer or 12 nt past the end of the LTR (as cited in reference 52). For some retroviruses, this particular species is not the only plus-strand strong-stop species detected, but it is considered to be the one which transfers to the 3′ end of the minus-strand DNA. Experiments with viruses containing mutant PBS sequences showed that information could apparently be transferred from the tRNA into the PBS (64). In the case of Ty1 and Ty3, however, evidence suggests that the species using the primer tRNA as a template are not the active intermediates in replication. For both Ty1 and Ty3, in which the PBS sequences have only 10 and 8 nt of complementarity to the tRNA primer, respectively, information copied from the tRNA up to the first modified base is obviously not incorporated into the genomic sequence. In addition, mutations introduced into at least some internal positions of the Ty1 PBS are not corrected (52), also arguing that information is not transferred from the initiator $tRNA^{Met}$ template to the PBS. The 344-nt plus-strand, minor species observed for Ty3 which reacts with U3- and U5-specific probes would be consistent with a species which was primed at the PPT 2 nt upstream of the U3-internal domain junction and extended on a template from which the tRNA primer had been removed. Thus, it is possible that the 344-nt minor form represents the true replication intermediate while the 356-nt form accumulates as a dead-end species. A 344-nt form was also visualized by using the U3- and U5-specific probes for the minus strand. These findings are consistent with the existence of a double-stranded LTR species. Such a species could arise if a plus-strand, strong-stop species (possibly displaced by plus-strand synthesis originating at an upstream site) served as the

receptor for transfer of a minus-strand strong-stop species which was subsequently extended on the plus-strand template. This species would consist of a double-stranded LTR with 2 extra bp on each end and could, in theory, be equivalent to 344 bp. If Ty3 has a retroviruslike mode of replication, then a minus-strand strong-stop species of 120 nt is also predicted and has been observed in some experiments. In experiments to be described elsewhere, a species which is larger than 120 nt has also been observed (44).

In conclusion, these experiments have demonstrated for the first time that processing of 2 extra nt from the 3′ ends of extrachromosomal, retroelement DNA is not a fundamental distinction between retroviruses and retrotransposons. The carboxyl-terminal domain of retroviral IN has DNA binding activity. Whether this region in Ty3 IN interacts with host transcription factors and/or DNA is not yet known. The current study showed that this domain is essential for Ty3 RT activity. The existence of readily observable, processed and unprocessed extrachromosomal, genomic-length Ty3 DNA species in yeast cells expressing Ty3 should allow discrimination in vivo between domains of IN required for LTR processing and for integration into host DNA and may lead to a better understanding of the functional significance of the extra nucleotides.

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