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A Ty1 Integrase Nuclear Localization Signal Required for Retrotransposition

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Ty1 retrotransposition in *Saccharomyces cerevisiae* requires integrase (IN)-mediated insertion of Ty1 cDNA into the host genome. The transposition components are assembled in the cytoplasm and must cross the nuclear envelope to reach the genomic target, since, unlike animal cell nuclear membranes, the yeast cell nuclear membrane remains intact throughout the cell cycle. We have identified a bipartite nuclear localization signal (NLS) in IN required for Ty1 transposition (Ty1 IN) that directs IN to the nucleus. Mutations in the NLS that specifically abolish nuclear localization inactivate transpositional integration but do not affect reverse transcription, protein processing, or catalytic activity in vitro. No additional Ty1-encoded proteins are required for IN nuclear localization. Intragenic complementation experiments suggest that Ty1 IN functions as a multimer and contains two distinct domains, one required for integration and the other for nuclear localization. Nuclear targeting of the preintegration complex by an IN NLS may prove to be a general strategy used by retrotransposons and retroviruses that infect nondividing cells.

Ty1 elements belong to a family of retrotransposons that replicate through an RNA intermediate in the budding yeast *Saccharomyces cerevisiae* (for reviews, see references 3 and 19). Two overlapping open reading frames, *TYA1* and *TYB1*, are analogous to retroviral *gag* and *pol* genes, respectively. *TYA1* encodes nucleocapsid proteins that form the structural components of virus-like particles (VLPs). *TYB1* encodes the catalytic proteins protease (PR), integrase (IN), and reverse transcriptase (RT)/RNase (RH).

An essential step in Ty1 transposition is the cytoplasmic assembly of VLPs. Within VLPs, linear cDNA is synthesized by RT/RH. IN catalyzes the integration of this cDNA into the genome (5, 12, 39, 47). In vivo studies have shown that IN and the terminal nucleotides of Ty1 cDNA are required for transpositional integration but not for homologous recombination of Ty1 cDNA with resident elements (13, 47). Ty1 IN contains motifs common to retroviral INs, including the N-terminal HHCC and the core D,D35E catalytic domain (33).

For Ty1 integration to occur, the VLP or a subparticle preintegration complex (PIC) containing at least IN and Ty1 cDNA must return to and transit the nuclear membrane to access a genomic target. Little is known about how Ty1 elements return to the nucleus. Since the yeast nuclear membrane remains intact throughout the cell cycle (8) and since Ty1 VLPs, which are 60 nm in diameter (20, 38), exceed the 25-nm size limit for active transport of a particle across the nuclear pore complex (for a review, see reference 23), an intact nuclear envelope presents a potential barrier to the Ty1 PIC. This problem is analogous to that of retroviral infection of nondividing cells. Whereas most oncoretroviruses require mitotic nuclear membrane dissolution for infectivity (35, 46), lentiviruses, such as human immunodeficiency virus type 1 (HIV-1) and visna virus, can infect terminally differentiated cells (7, 24, 34, 42, 52). Translocation of the HIV-1 PIC across the nuclear membrane appears to require or be augmented by the matrix (MA) protein, which contains a nuclear localization signal (NLS), and the Vpr protein (6, 15, 36, 51).

Here, we demonstrate that Ty1 IN enters the yeast nucleus with no requirement for additional Ty1 element-encoded proteins. IN nuclear localization is mediated by a C-terminal NLS. The IN NLS appears to be bipartite but with small basic motifs separated by a large spacer region. Point mutations in the NLS basic motifs block transpositional integration but affect Ty1 cDNA homologous recombination less severely. Intragenic complementation analyses indicate that the Ty1 IN NLS region is functionally separate from the catalytic domain and that Ty1 IN probably functions as a multimer.

MATERIALS AND METHODS

Yeast strains and media. Yeast strains DG1251 (yGS37; *MAT*α *ura3-167 trp1-GB his3-*Δ*200 spt3-101*) and DG1286 (yGS38; *MAT*α *ura3-167 trp1-GB his3-*Δ*200 spt3-101 rad52-GB*) are isogenic derivatives of strain GRF167 (2, 47). Strain DG531 (*MAT*a *ade1-100 ura3-52 leu2-3,2-112 his4-519 spt3-101*), an isogenic *spt3* derivative of strain BWG1-7A, was used for indirect immunofluorescence (IIF) of pGTy1 due to its property of having dispersed VLPs (20, 55). DG1377 is a transformant of RDKY 1293 (*MAT*α *ura3-52 trp1 leu2-*Δ*1 his3-*Δ*200 pep4*::*HIS3 prb1-*Δ*1.6R GAL*) containing the Ty1 IN expression plasmid pGTy1-IN. All media were prepared as described by Sherman et al. (48).

Plasmid constructions. Construction of the pGTy1-IN expression plasmid has been described elsewhere (40). Green fluorescent protein (GFP)-LacZ-IN fusion plasmids contained genes coding for either full-length IN, N-terminal deletions of IN, and/or C-terminal truncations of IN that were generated by PCR (41) by using primers complementary to specific sequences of IN. The GFP-LacZ vector was a gift from P. Silver and contains a *GAL1*-promoted GFP-LacZ fusion. To fuse the IN gene in frame to *lacZ*, the vector was digested with *Sac*I, which recognizes a unique site in *lacZ*, and *Hin*dIII, which recognizes a unique site 3′ of the *lacZ* open reading frame. In the ligation reaction, this fragment was replaced with a *lacZ* fragment containing a 5′ *Sac*I site and a 3′ *Not*I site. The ligation reaction included restricted vector and IN fragments comprising a three-fragment unidirectional ligation. Forward IN primers contained a *Not*I restriction site followed by a frame-correcting nucleotide and IN sequences. Reverse primers contained a *Hin*dIII restriction site, an ochre stop codon, and IN sequences.

Point mutations in the putative NLS region of IN for GFP visualizations were

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constructed by PCR oligonucleotide mutagenesis in which the reverse PCR primer incorporated the codon for the desired amino acid substitution(s). Each reverse primer also contained an ochre stop codon and a *Hin*dIII restriction site. The primers and their sequences are as follows (with codon substitutions in boldface type and restriction sites underlined): K596G-GFP, CCGGGCCAAGCTTTTA(A)ATTTCAGTTTCATTATCTTCTAATGATCTTTT**ACC**ACTG*; K596,597G-GFP, CCGGGCCAAGCTTTTA(A)ATTTCAGTTTCATTATCTTCTAATGATCT**ACCACC**ACTG*; K596,597G/R598T-GFP, CCGGGCCAAGCTTTTA(A)ATTTCAGTTTCATTATCTTCTAATGAA**GTACCACC**ACTG*; E601,604,606Q/D602N-GFP, CGGCCCAAGCTTTTA(A)AT**CTG**AGT**C TG**ATT**ATTCTG**TAATGATCTTTTCTTACTG*. In these sequences, "(A)" indicates sequence complementary to Ty1 nucleotide 3861 and "G*" indicates sequence complementary to Ty1 nucleotide 3822. The forward primer contained a *Not*I restriction site followed by a frame-correcting nucleotide and in-frame IN gene sequences beginning at Ty1 nucleotide 3627 (IN amino acid residue 530) in the case of the basic motif mutations. The fragment with acidic-domain mutations included IN amino acids 1 to 607. PCR and ligation conditions were as previously described (40). For transposition assays, the point mutations coding for IN-K596G (a K-to-G mutation at residue 596) and IN-K596,597G were introduced into pGTy1-H3m*his3*AI (10) by oligonucleotide-directed mutagenesis (designated pGTy1-H3*his3-AI* in the text). An IN gene fragment was generated by PCR by using reverse primers that contained a codon for the amino acid substitution K→G and a *Ksp*I restriction site. The oligonucleotide primers used and their sequences are as follows (with restriction sites underlined and codon substitutions in boldface type): K596G-pGTy1, GGGCCGCCCGCGGAGGTT CTAAACTACGCATATTCTTAGTATTCCATGTGTCTCGTGATACCTTA ATTTCAGTTTCATTATCTTCTAATGATCTTT**ACC**ACTGTTG; K596, 597G-pGTy1, GGGCCGCCCGCGGAGGTTCTAAACTACGCATATTCTTA GTATTCCATGTGTCTCGTGATACCTTAATTTCAGTTTCATTATCTTCT AATGATCT**ACCACCA**CTGTTG. For each primer, the C in the fourth position of the restriction site represents sequence complementary to Ty1 position 3915, and the 3′ G represents sequence complementary to Ty1 position 3819. As a control for PCR amplification, a wild-type (WT) fragment was also generated by using a reverse primer of the same length as those listed above. The forward primer for PCR-amplified fragments contained a *Sal*I restriction site, which is unique in sequences coding for Ty1 IN and IN beginning at Ty1 nucleotide 2173. Vector and insert fragments were digested with *Sal*I and *Ksp*I and ligated as reported previously (40). Amino acid substitutions at positions 628, 629, and 630 were constructed by oligonucleotide mutagenesis of pGTy1-H3*his3-AI* (10). A unique *Ksp*I restriction site was introduced into this vector at Ty1 nucleotide 3915 by PCR with an oligonucleotide initiating at the *Pvu*II site at nucleotide 3944 and containing the mutation. This mutation did not alter the amino acid sequence of Ty1 IN. Complementary oligonucleotide pairs containing the sequences coding for mutations at amino acids 628, 628 and 629, and 628 to 630 were annealed and ligated into this vector at the *Ksp*I and *Pvu*II sites.

NLS point mutations were initially cloned into *URA3*-marked pGTy1-H3*his3-AI*. An in-frame linker insertion mutation in the catalytic domain of Ty1 IN *in-2600* (12, 39), has also been introduced into *URA*3-marked pGTy1-H3*his3-AI* (47). WT Ty1-H3*his3-AI* was cloned into vectors that carry either the *URA3* or *TRP1* marker. WT, NLS mutant, and *in-2600* pGTy1-H3*his3-AI* sequences all contain a unique *Bst*EII restriction site at nucleotide position 1792, which is within the Ty1 PR-coding region, and a unique *Nhe*I restriction site at nucleotide 639 within the inverted *HIS3* marker at the 3′ end of Ty RT. By using these restriction sites, fragments from either mutant were subcloned into vectors marked with either *TRP1* or *URA3*. Mutant clones were checked by diagnostic restriction site polymorphisms. The NLS *in*-K596,597G mutant contains a *Tsp*RI site not present in the WT IN gene, and the *in-2600* linker insertion includes an *Mlu*I site (12, 39). Recombinant plasmids were introduced into yeast by lithium acetate transformation (22).

GFP visualization. Yeast cells transformed with GFP-LacZ-IN constructs were isolated as single colonies. Cells were inoculated into 5 ml of SC−ura (synthetic complete medium lacking uracil) with 5% raffinose and grown overnight at 30°C. To induce protein expression, the cells were pelleted and resuspended in 5 ml of SC−ura with 2% galactose. Following induction for 2.5 h and a chase period of 1 h in SC−ura with 2% glucose at 30°C, the cells were fixed by adding 0.75 ml of 37% formaldehyde and then incubated for 1 h at 30°C. The cells were then washed twice in solution P (0.1 M potassium phosphate buffer [pH 6.5], 1.2 M sorbitol). Dithiothreitol was added to a final concentration of 25 mM, and following a 10-min incubation at room temperature, zymolyase was added to a final concentration of 0.3 μg/ml and the cells were incubated with gentle agitation at 30°C for 45 min. This incubation time resulted in approximately 80% spheroplasts. The spheroplasts were pelleted and resuspended in solution P. Twenty microliters of spheroplast suspension was applied to polylysine-coated wells of a chamber slide and allowed to attach for 15 min. After aspiration of excess spheroplasts, 0.5% Nonidet P-40 in solution P was added, and the wells were incubated at room temperature for 5 min. Attached spheroplasts were rinsed once with solution P and twice with solution AB2 (0.1 M Tris [pH 9.5], 0.1 M NaCl) and air dried. Antifade (*p*-phenylenediamine) containing 200 ng of DAPI (4′,6-diamidino-2-phenylindole) was used as the mounting medium, and coverslips were sealed with clear nail polish.

Cells were visualized with a Zeiss Axiophot fluorescence microscope with a 100× Plan-NEOFLUAR objective and fluorescein isothiocyanate (FITC) and DAPI filters.

Yeast IIF of ectopically expressed IN. Yeast IIF was carried out essentially as described by Pringle et al. (43). Primary antibody B2 (21) was added in phosphate-buffered saline (PBS) containing 1 mg of bovine serum albumin (BSA) at a 1:200 dilution. The secondary antibody, FITC-conjugated goat anti-rabbit immunoglobulin G (whole molecule; Sigma) in PBS-BSA, was added to the slides at a 1:400 dilution. Visualization of immunofluorescence involved the use of the same optical system described above for GFP.

Yeast IIF of pGTy1. Strain DG531, transformed with either WT or NLS mutant pGTy1-H3*his3-AI* plasmids, was grown overnight at 30°C in SC−ura with 2% raffinose and induced for VLP expression in SC−ura with 2% galactose at 20°C for 24 h. Cells were fixed with 1/10 volume of fresh 37% formaldehyde for 90 min at 30°C and then washed twice with solution SK (1 M sorbitol, 50 mM KPO_4 [pH 7.5]). Cell walls were digested in 1 ml of solution SK with 1.4 mM 2-mercaptoethanol and 34 ng of zymolyase at 30°C for 15 min. Spheroplasts were washed twice in solution SK and applied to a chamber slide prepared as described above. After 5 min, excess cells were aspirated and the wells of the chamber slide were washed twice with 15 μl of solution SK. The slides were fixed by immersion in methanol at −20°C for 6 min followed by acetone at −20°C for 30 s. The spheroplasts were then blocked by using 3% BSA in PBS for 20 min at room temperature. Primary antibody (8B11) was applied at a 1:4,000 dilution in PBS-BSA and incubated in a humidified chamber at room temperature for 2 h. The antibody was removed, and the cells were washed five times with 15 μl of PBS. The secondary antibody, FITC-conjugated goat anti-mouse immunoglobulin G (whole molecule; Sigma), was applied at a 1:2,000 dilution in PBS-BSA and the slides were incubated as described for the primary antibody. After aspiration of the secondary antibody and washing with PBS, mounting medium containing DAPI was applied as described above, and coverslips were sealed with clear nail polish.

VLP isolation and characterization. VLPs were isolated by established methods (12). Assays for one-ended IN catalytic activity and immunoblotting procedures were carried out as described previously (40). The oligonucleotide substrate used in this investigation was based on U5 long-terminal-repeat sequences.

Transposition assays. The effect of NLS mutations on Ty1 transposition was measured as the frequency of histidine prototrophy by using pGTy1-H3*his3*-AI (10). Six early-stationary-phase cultures of each strain grown in SC−ura with 2% raffinose were diluted 1:100 into SC−ura with 2% galactose and grown for either 3 (for DG1251) or 4 (for DG1286) days at 20°C. The cells were plated on SC−ura+glucose plates to determine the titer and on SC−ura−his+glucose plates to determine the number of histidine prototrophs.

Complementation analysis. The following pGTy1-H3*his3-AI* plasmids were introduced into strain DG1286 by transformation: WT/*TRP1* (*TRP1*-based wild-type pGTy1-H3*his3-AI*), WT/*URA3* (*URA*-based wild-type pGTy1-H3*his3-AI*), *in-2600*/*TRP1* (*TRP1*-based mutant pGTy1-H3*his3-AI*/*in-2600*), *in-2600*/*URA3* (*URA3*-based mutant pGTy1-H3*his3-AI*/*in-2600*), *in-K596,597G*/*TRP1* (*TRP1*-based mutant pGTy1-H3*his3-AI*/*in-K596,597G*, and *in-K596,597G*/*URA3* (*URA3*-based mutant pGTy1-H3*his3-AI*/*in-K596,597G*). Plasmid segregation analyses were performed on each transformant. Qualitative transposition assays were performed as described previously (47) with strains containing the following pGTy1-H3*his3-AI* plasmids: WT/*TRP1* and WT/*URA3* (strain DG1798), *in-2600*/*TRP1* and *in-2600*/*URA3* (DG1814), *in-K596,597G*/*TRP1* and *in-K596,597G*/*URA3* (DG1802), WT/*TRP1* and *in-2600*/*URA3* (DG1815), WT/*URA3* and *in-2600*/*TRP1* (DG1804), WT/*TRP1* and *in-K596,597G*/*URA3* (DG1797), WT/*URA3* and *in-K596,597G*/*TRP1* (DG1803), *in-2600*/*TRP1* and *in-K596,597G*/*URA3* (DG1801), *in-K596,597G*/*TRP1* and *in-2600*/*URA3* (DG1813). Quantitative transposition assays were performed as described previously (10) with strains containing WT/*TRP1* and WT/*URA3* (strain DG1798), *in-2600*/*TRP1* and *in-2600*/*URA3* (DG1814), *in-K596,597G*/*TRP1* and *in-K596,597G*/*URA3* (DG1802), *in-2600*/*TRP1* and *in-K596,597G*/*URA3* (DG1801), *in-2600*/*TRP1* and WT/*URA3* (DG1804), and *in-K596,597G*/*TRP1* and WT/*URA3* (DG1803). In the complementation experiments, the transposition efficiency was defined as the number of His^+ Trp^+ Ura^+ cells divided by the number of Trp^+ Ura^+ cells present after galactose induction. The complementation efficiency was calculated by dividing the transposition efficiency obtained with a given strain, e.g., DG1801(*in-2600*/*TRP1*, *in-K596,597G*/*URA3*), by the transposition efficiency obtained with strain DG1798.

RESULTS

Ty1 IN localizes to the nucleus. The expression system used for the purification of catalytically active recombinant IN (40) provided an effective approach for studying cellular localization of IN. Briefly, yeast strain DG1377 contains the IN-coding region of Ty1 fused to the *GAL10* promoter of plasmid pRDK249 (28). When cells are grown in galactose, ectopically expressed Ty1 IN accumulates in the nucleus. Nuclear localization of Ty1 IN is demonstrated by the colocalization of

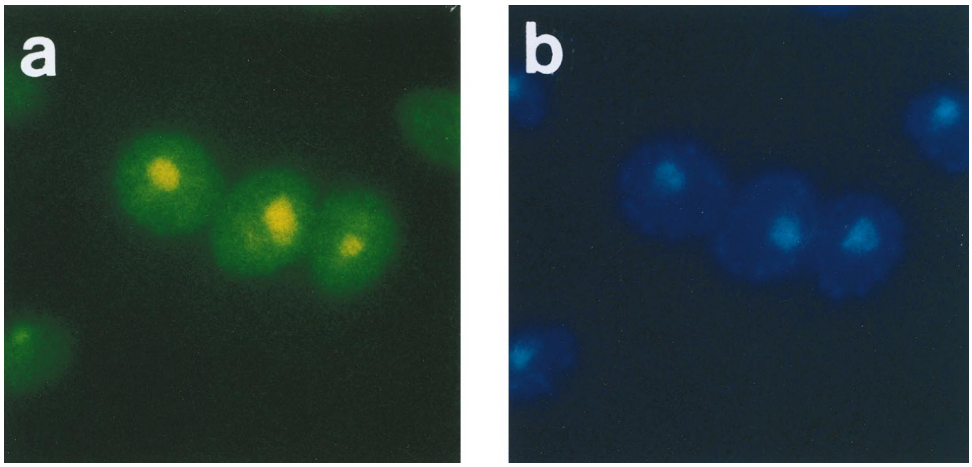

FIG. 1. Nuclear localization of Ty1 IN visualized by IIF (a) and DAPI counterstaining of nuclei (b).

antibody B2 (21) visualized by IIF (Fig. 1a) and nuclear staining by DAPI (Fig. 1b).

IN NLS maps to the C terminus. PCR-generated IN gene fragments were fused in frame to a *lacZ* coding region with an N-terminal GFP-coding sequence expressed from the *GAL1* promoter to determine which region of IN contains sequences required for nuclear localization (Fig. 2). GFP-LacZ alone was evenly distributed throughout the cytoplasm (Fig. 2a), whereas GFP–LacZ–full-length IN exhibited strong nuclear localization (Fig. 2b) identical to that observed by IIF of cells expressing IN ectopically (Fig. 1). Progressive N-terminal deletions of IN up to amino acid residue 401 did not inhibit nuclear partitioning of the remainder of the fragment (Fig. 2c to e). Conversely, C-terminal deletions resulted in cytoplasmic distribution of GFP-LacZ-IN fragments (Fig. 2f to h). A fragment containing IN residues 421 to 623 resulted in nuclear localization of the GFP-LacZ-IN fragment fusion (Fig. 2i). Deleting residues 421 to 520 from this fragment did not prevent GFP-LacZ-IN targeting of the nucleus (Fig. 2j). Within the region of residues 521 to 623, there is only one sequence of contiguous basic amino acids characteristic of classical NLSs, i.e., K596, K597, and R598 (designated basic region 1 [BR1]). An identical basic region comprising amino acid residues 628 to 630 (BR2) was also considered a candidate NLS. To test BR2 as an NLS without BR1 in the GFP-LacZ fusion system, we deleted residues 530 to 622. This fragment also localized to the nucleus (Fig. 2k).

To determine whether the KKR sequence comprising BR1 plays a role in nuclear localization, or if the fragment shown in Fig. 2j contains a cryptic NLS, we analyzed GFP-LacZ-IN fusions with increments of 6 or 7 amino acids from residues 595 to 614 added to a fragment containing residues 530 to 594 (Fig. 3a to d). The minimally sufficient fragment tested that showed nuclear targeting included both BR1 and seven additional amino acids (EDNETEI; acidic domain) (Fig. 3c). The karyophilic property of this fragment was not altered by adding amino acid residues 608 to 614 (Fig. 3d). Therefore, either BR1 plays no role in nuclear targeting or it is necessary but not sufficient. To test the necessity of BR1, we introduced amino acid substitutions into BR1 within the fragment containing residues 530 to 607 (Fig. 4). These substitutions were IN-K596G, IN-K596,597G, and IN-K596,597G/R598T. Figure 4a shows a WT fragment derived independently from that illustrated in Fig. 3c. The single amino acid change IN-K596G resulted in partial nuclear localization, whereas IN containing double (IN-K596,597G) and triple (IN-K596,597G/R598T) amino acid substitutions did not accumulate in the nucleus.

The GFP analysis illustrated in Fig. 3c and Fig. 4c to d indicates that nuclear localization of GFP-LacZ requires BR1 and adjacent C-terminal residues. Although the minimal number or the exact character of the C-terminal residues has not been determined, four of the seven residues are acidic (EDNETEI; acidic residues underlined). To determine whether any of these residues plays a role in nuclear localization or if only random sequences are required C-terminal to BR1, we introduced point mutations into this region by which all three glutamic acid residues were replaced with glutamine and by which asparagine was substituted for the aspartic acid residue. These four substitutions also prevented nuclear accumulation of the GFP-LacZ-IN fragment (Fig. 4e).

To test whether the two basic motifs, BR1 and BR2, are interdependent, full-length IN containing point mutations in the basic motifs was expressed in the *spt3* mutant strain DG1251, in which genomic Ty1 elements are not transcribed (53). The expression vector contains neither *GFP* nor *lacZ* sequences and has been used previously for expressing recombinant Ty1 IN (40). IIF analysis showed that WT IN localized to the nucleus in strain DG1251 (Fig. 5a), indicating that IN localization does not require the expression of genomic Ty1 element proteins. Point mutations in either BR1 (Fig. 5b) or BR2 (Fig. 5c) resulted in the cytoplasmic distribution of IN. In this context, WT sequences in either BR1 or BR2 cannot compensate for mutations in the other motif. By using GFP analysis, nuclear localization was observed with BR1 alone when BR2 was deleted. Conversely, BR2 alone was also sufficient to localize a GFP-LacZ-IN fragment to the nucleus. However, in IIF analyses of ectopically expressed IN or the complete pGTy1 element, missense mutations in BR1 or BR2 in the complete IN protein prevented nuclear localization. These differences in the NLS activity of BR1 and BR2 may reflect the method of visualization or the effects of a deletion versus a missense mutation. Since the two basic regions are identical, a mutation that renders one basic region neutral may be more severe than a deletion.

Ty1 IN nuclear localization during retrotransposition. Ty1 IN was shown to translocate to the nucleus during Ty1 transposition by an analysis of cells expressing the entire Ty1 element (2) for IN localization by IIF (Fig. 5d). For this experiment, pGTy1-H3*his3-AI* (10) was expressed in strain DG531, in which VLPs are evenly dispersed throughout the cytoplasm

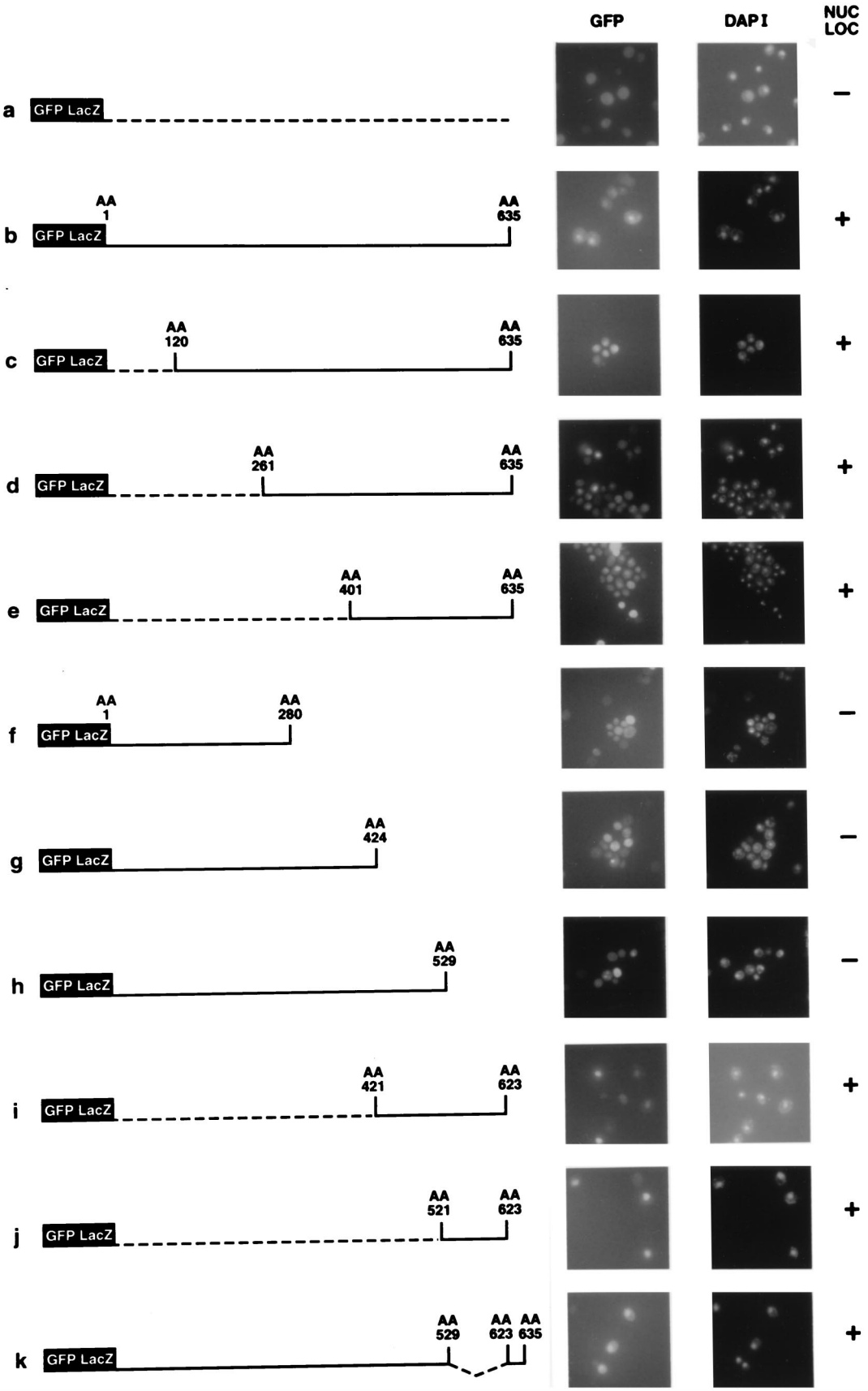

FIG. 2. Mapping of IN NLS by nuclear localization (NUC LOC) of GFP-LacZ-IN fragment fusions. (a) GFP-LacZ only; (b) GFP–LacZ–full-length IN; (c to e) N-terminal deletions; (f to h) C-terminal deletions; (i to j) N- and C-terminal deletions; (k) internal deletion from amino acid 530 to 622. Dashed lines represent deleted sections of IN. Numbers represent amino acid (AA) residues of IN at junctions or termini.

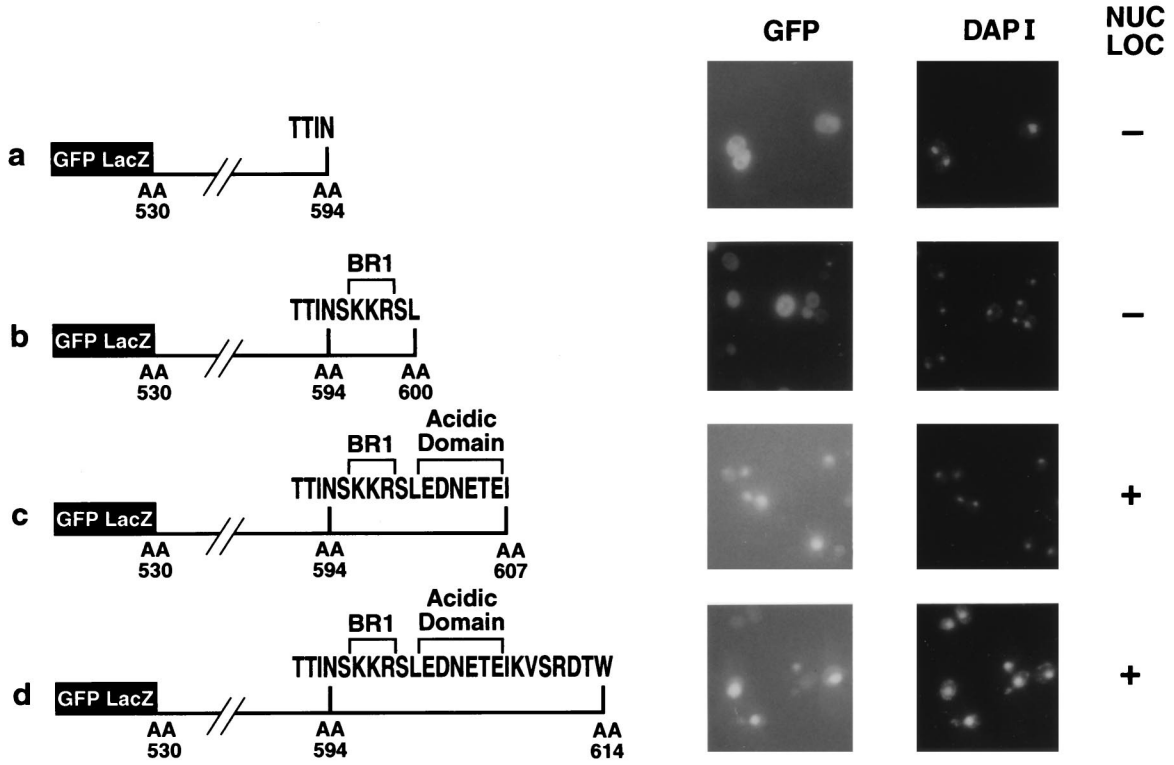

FIG. 3. Incremental inclusion of amino acids (AA) 594 to 614 to determine residues required for IN nuclear localization (NUC LOC). (a) GFP-LacZ-IN fragment fusion from IN AA residues 530 to 594. AA 591 to 614 are designated by single-letter abbreviations. (b) GFP-LacZ-IN fragment fusion from AA 530 to 600. (c) GFP-LacZ-IN fragment fusion from AA 530 to 607. (d) GFP-LacZ-IN fragment fusion from AA 530 to 614. BR1 is indicated.

(20, 55). This property reduces background immunofluorescence resulting from antibody cross-reaction with VLP-associated IN. Monoclonal antibody 8B11 (12) was used as the primary antibody. Unlike ectopically expressed IN, in which virtually every cell of the population exhibited nuclear accumulation of IN, pGTy1-H3*his3-AI* expression resulted in a less uniform staining pattern of IN. Many cells showed minimal levels of fluorescence, and of those that demonstrated significant staining, about half showed IN nuclear localization. The majority of cells expressing Ty1 with the BR1 double mutation IN-K596,597G showed only cytoplasmic distribution of IN (Fig. 5e). The number of cells displaying IN nuclear localization was shown to be statistically significant in cells expressing WT pGTy1-H3*his3-AI* compared with cells expressing the BR1 mutant plasmid by scoring random fields of cells of each type as either IN localized to nuclei or IN evenly distributed throughout the cytoplasm. Almost 41% (123 of 303) of the cells expressing WT pGTy1-H3*his3-AI* showed IN nuclear accumulation, whereas 4.3% (13 of 303) of the cells expressing the NLS mutation showed IN associated with the nucleus. Chi-square analysis indicated that this result is highly significant ($P < 0.001$). A defect in nuclear localization was also observed with a BR2 mutation (data not shown).

NLS point mutations reduce Ty1-H3*his3-AI* transposition. Representative point mutations in BR1 and BR2 were introduced into pGTy1-H3*his3-AI* to assay transposition. This element is marked with the *his3-AI* indicator gene, in which the *HIS3* gene is disrupted by an artificial intron (AI) in the antisense orientation (10). Upon galactose induction of a WT element, His^+ cells result from a retrotransposition process requiring precise splicing of the AI and reverse transcription of the element. If transpositional integration is defective, Ty1 cDNA may undergo *RAD52*-dependent homologous recombination with genomic Ty1 elements to generate His^+ prototrophs (47). WT and NLS mutant elements were expressed in strains DG1251 (*spt3 RAD52*) (Table 1) and DG1286 (*spt3 rad52*) (Table 2) to distinguish cDNA recombination from retrotransposition (47). A Ty1 element containing a five-codon linker insertion that disrupts the catalytic D,D35E region, *in-2600* (12, 39, 47), was included for comparison. Because the vector carrying the *in-2600* mutant element is slightly different from that containing the NLS mutant, the cognate WT pGTy1-H3*his3-AI* plasmid of each mutation was also assayed for histidine prototrophy. In the *RAD52* strain DG1251 (Table 1), the single point mutation IN-K596G resulted in no reduction in the frequency of histidine prototrophs, but multiple mutations in either BR1 or BR2 resulted in a 5- to 10-fold reduction in the frequency of histidine prototrophs compared with that of the WT. The *in-2600* mutation resulted in a 2.5-fold reduction in His^+ frequency. Thus, the NLS multiple mutations and the catalytically inactive *in-2600* mutation resulted in similar, though not greatly reduced, His^+ frequencies. When transposition alone was assayed in the *spt3 rad52* strain DG1286 (Table 2), multiple mutations in BR1 and BR2 as well as the *in-2600* mutation resulted in marked decreases in transposition levels, ranging from 62- to 670-fold. Similar results were also obtained in a separate experiment using clones from a PCR fragment that was derived independently. These observations further support the requirement for and the interdependence of BR1 and BR2 in Ty1 transposition. The moderate effect of the NLS mutations on combined cDNA recombination and transposition levels (Table 1), compared to the drastic reduction in transposition alone (Table 2), indicates that DNA synthesis during the process of reverse transcription is not the

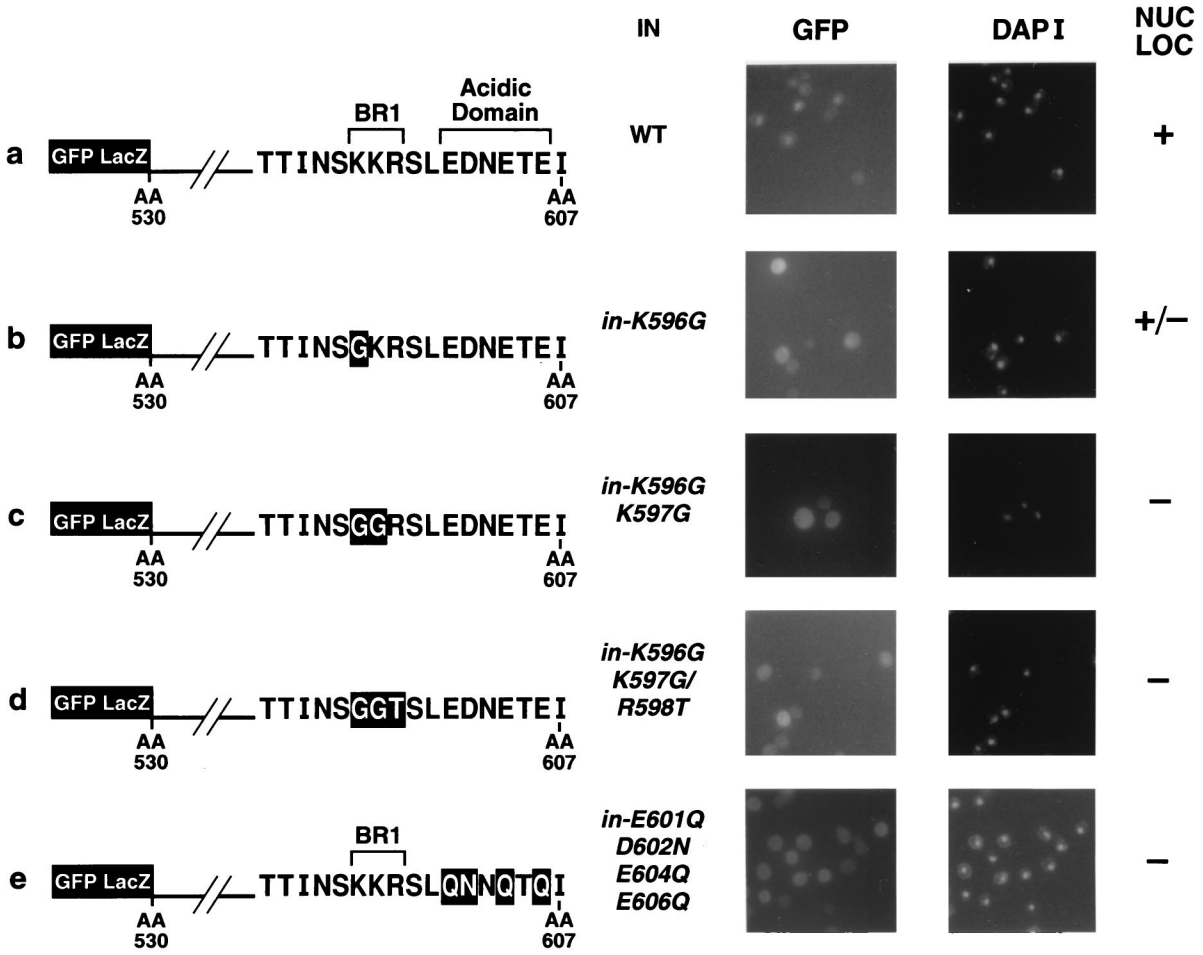

FIG. 4. Effect of point mutations on nuclear localization (NUC LOC) of GFP-LacZ-IN fragment fusions. (a) WT sequences of IN fragment from amino acid (AA) residues 530 to 607; (b) IN-K596G single point mutation; (c) IN-K596,597G double point mutation; (d) IN-K596,597G/R598T triple point mutation; (e) point mutations in the acidic domain IN-E601,604,606Q/D602N. The site of BR1 is indicated.

limiting factor responsible for reduced transposition in the NLS mutants. If this were the case, the frequencies of cDNA recombination and transposition should be similar, as has been shown for strains with mutations in Ty1 PR and RT/RH (47).

NLS mutant VLPs are enzymatically active. VLPs were purified from galactose-induced cells expressing WT and NLS mutant pGTy1 plasmids to determine whether NLS mutations affect other steps in retrotransposition. VLPs were assayed for proteolytic processing and RT and IN catalytic activity. NLS mutant VLPs displayed RT activity similar to that of WT VLPs (data not shown). Furthermore, the IN-K596,597G mutation in BR1 did not reduce Ty1 PR processing, since comparable levels of processing intermediates and mature IN were observed with mutant and WT VLPs (Fig. 6a). The NLS mutant IN was as catalytically active as WT IN in vitro (Fig. 6b). Similar results were obtained with VLPs containing the IN-K628,629G/R630T mutation in BR2 (Fig. 6c and d). From these experiments, we infer that DNA binding is unaffected. A frameshift linker insertion at amino acid residue 587, which resulted in translation termination upstream of the IN NLS, has also been shown to retain in vitro IN catalytic activity (5). A reduction of catalytic activity is therefore not responsible for the reduction in transposition levels of the NLS mutants.

Intragenic complementation between an IN NLS and a D,D35E mutation. Using an intragenic complementation test in which the *in-2600* catalytically inactive mutation and the IN-K596,597G NLS mutation were coexpressed in the same cell, we determined whether the catalytic core and NLS region of Ty1 IN are separate domains and whether Ty1 IN functions as a multimeric protein. For this experiment, the mutations were subcloned into *TRP1*- and *URA3*-based pGTy1-H3*his3-AI* plasmids, and various combinations of plasmids were introduced into strain DG1286. The transformants were analyzed for Ty1 transposition as monitored by their ability to form His^+ colonies in a qualitative patch test (47). Initial experiments suggested that efficient intragenic complementation occurred between *in-2600* and IN-K596,597G and that both IN mutations were recessive. Similar results were obtained regardless of whether the IN mutations or WT sequences were present on the *TRP1*- or *URA3*-based pGTy1-H3*his3-AI* plasmids. Whether recombination between mutant plasmids generated a WT Ty1 element was tested by recovering *TRP1*- and *URA3*-based pGTy1 plasmids from 14 independent His^+ colonies from strain DG1801(*in-2600*/*TRP1*, *in-K596,597G*/*URA3*) and analyzing the plasmids by restriction digestion to detect restriction site polymorphisms in the IN mutations. All 28 plasmids contained the *Mlu*I and *Tsp*RI restriction sites that identify *in-2600* or IN-K596,597G mutations, respectively.

Intragenic complementation was quantitated by determining the level of Ty1 transposition (10) in strains DG1798(WT/

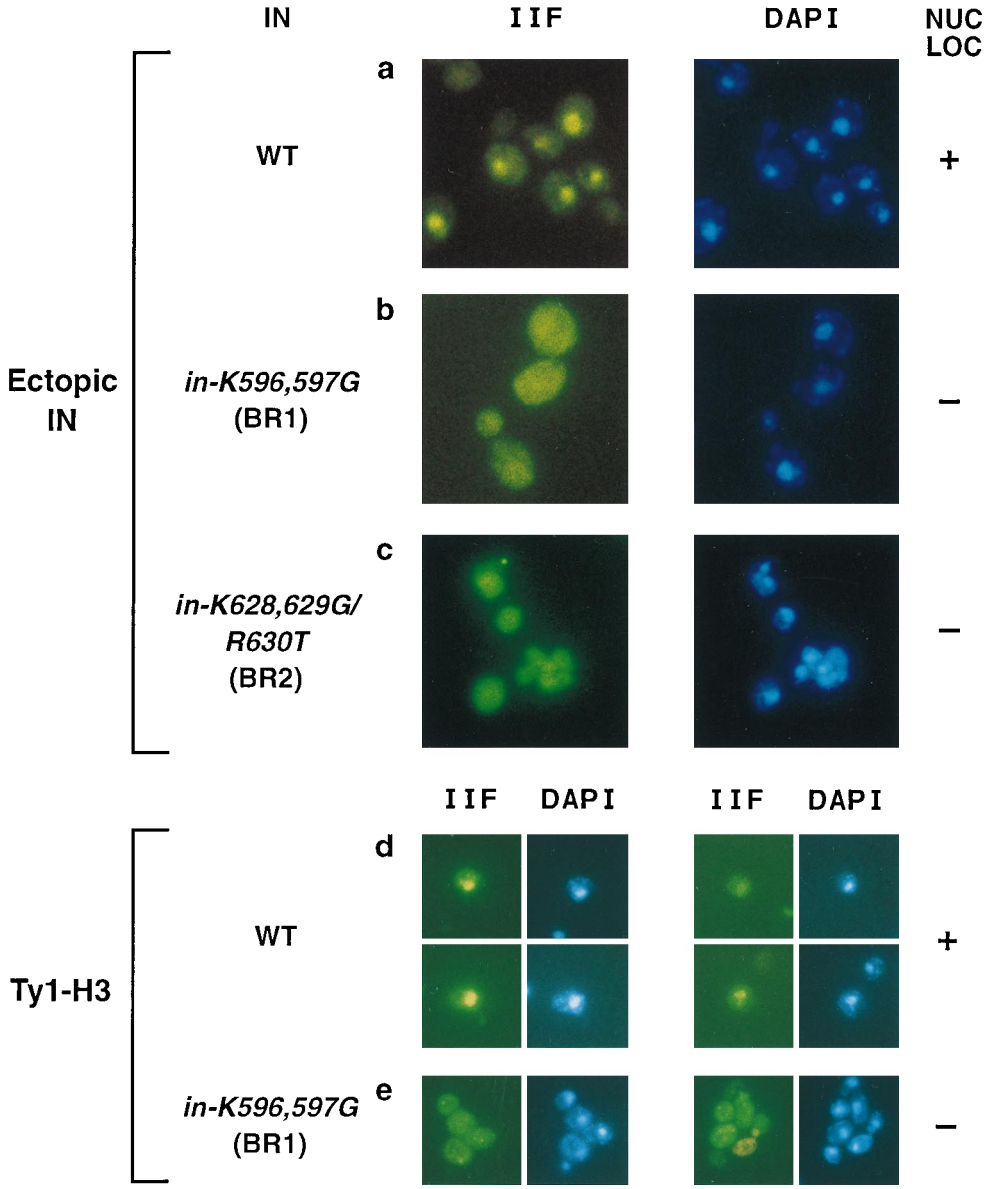

FIG. 5. IIF visualization of Ty1 IN. (a to c) Ty1 IN expressed ectopically; (d and e) Ty1 IN expressed from pGTy1-H3*his3-AI*, which expresses the entire Ty1 element. NLS point mutations and basic regions are indicated. Cells were counterstained with DAPI.

TRP1, WT/*URA-3*), DG1801(*in-2600*/*TRP1*, *in-K596,597G*/*URA3*), DG1814(*in-2600*/*TRP1*, *in-2600*/*URA3*), DG1802(*in-K596,597G*/*TRP1*, *in-K596,597G*/*URA3*), DG1803(*in-K596, 597G*/*TRP1*, WT/*URA3*), and DG1804(*in-2600*/*TRP1*, WT/*URA3*) (Table 3). As predicted, the IN mutant strains DG1814 and DG1802 were defective for transposition, showing more than a 100-fold reduction in His^+ formation relative to that of the WT. Coexpression of the WT and IN mutant pGTy1 plasmids in strains DG1803 and DG1804 restored transposition to the level observed with WT strain DG1798, indicating that the IN mutations are recessive. Coexpression of Ty1 elements containing *in-2600* and IN-K596,597G resulted in a transposition efficiency of 3.2% and a complementation efficiency of 40%, which was more than a 50-fold increase over those values for elements containing either IN mutation individually.

DISCUSSION

Two conclusions can be drawn from our data. First, the C terminus of Ty1 IN contains a region that functions as an NLS. Substitution of crucial amino acids in this region severely diminishes nuclear localization when IN is fused to GFP-LacZ, when IN is expressed ectopically in a biochemically active form, and, more relevantly, when IN is expressed as part of a functional Ty1 element. The IN NLS is absolutely required for Ty1 retrotransposition, since mutations that abolish nuclear targeting also reduce transposition to the equivalent of that of catalytically inactive IN. In addition, since VLP production, protein processing, reverse transcription, and in vitro IN activity remain at WT levels when nuclear localization is defective, the NLS region carries out a specific function during the process of retrotransposition.

TABLE 1. Transposition and cDNA recombination of IN mutants

Plasmid	Region	Efficiency[a]	
		Transposition and cDNA recombination (%)	Relative to that of WT
pGTy1-H3*his3-AI*	WT	8.0 ± 1.8	1.00
pGTy1-H3*his3-AI* derivative			
in-K596G	BR1	10.6 ± 4.0	1.32
in-K596,597G	BR1	1.4 ± 0.5	0.17
in-K628G	BR2	4.1 ± 1.8	0.51
in-K628,629G	BR2	0.8 ± 0.2	0.10
in-K628G,629G/R630T	BR2	1.4 ± 0.6	0.18
pGTy1-H3*his3-AI*[b]	WT	3.8 ± 1.6	1.00
pGTy1-H3*his3-AI/in-2600*	D,D35E	1.4 ± 0.8	0.36

[a] The combined efficiency of transposition and cDNA recombination is expressed as a percentage, determined by dividing the number of His^+ Ura^+ cells by the number of Ura^+ cells obtained in strain DG1251 (*RAD52 spt3*).

[b] Because the vector carrying the *in-2600* mutant element is slightly different from that containing the NLS mutant, the cognate WT pGTy1-H3*his3-AI* plasmid of each mutation was also assayed for histidine prototrophy (47).

Second, we provide genetic evidence that the Ty1 NLS is a functionally separate region by showing intragenic complementation between the NLS (IN-K596,597G) and a D,D35E catalytic core (*in-2600*) mutation. The C-terminal region of Ty1 IN contains approximately 300 amino acids that have no apparent homology or functional similarity to the shorter C-terminal regions of retroviral INs. No function has been definitively assigned to this region of Ty1 IN. Although this region may have additional functions, the NLS located at the extreme C terminus of the protein plays a critical role in retrotransposition. The intragenic complementation between the catalytic and NLS domains suggests that Ty1 IN probably functions as a multimeric protein in vivo. Although it is not yet known how many IN molecules are minimally sufficient for retrotransposition of the Ty1 cDNA element, the multimerization of several retroviral integrases and Mu transposase has been well characterized (1, 29, 50, 54). Our results suggest that a subset of NLS^- and IN^- proteins multimerize and form a PIC with a given cDNA molecule. The NLS function of catalytically inactive IN molecules translocates the PIC to the nucleus, while the catalytic activity of NLS mutant molecules performs the integration reaction. Combined in vitro and in vivo analyses of Ty1 IN multimeric interactions and definition of the Ty1 PIC are now possible. Furthermore, studies on Ty1 IN functional domains will enhance the biochemical comple-

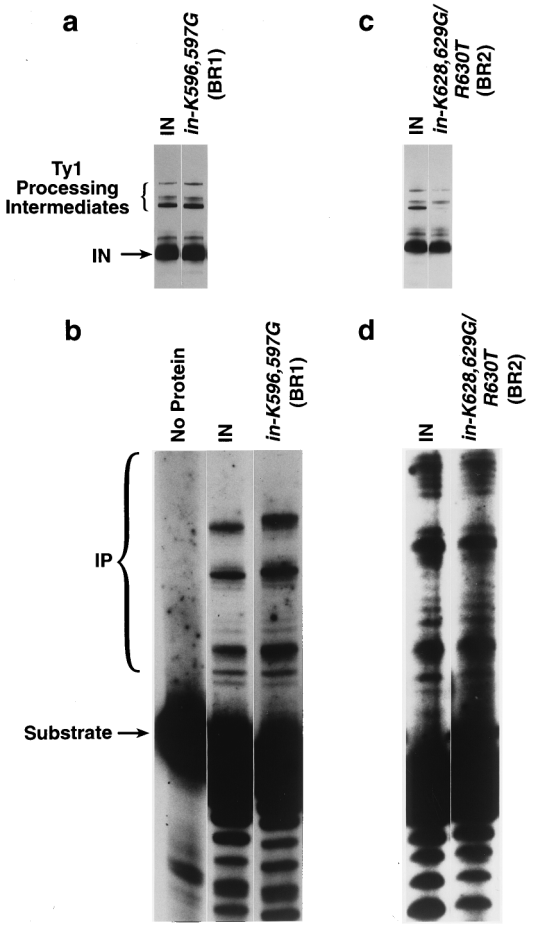

FIG. 6. In vitro activities of VLPs containing either WT or NLS mutant IN. Proteolytic processing (a and c) and in vitro catalytic activity (b and d) of WT and IN-K596,597G VLPs (a and b) and of WT and IN-K628,629G/R630T VLPs (c and d) are shown. IP, integration products.

TABLE 2. Transposition of IN mutants

Plasmid	Region	Efficiency[a]	
		Transposition (%)	Relative to that of WT
pGTy1-H3*his3-AI*	WT	13.4 ± 3.2	1.00
pGTy1-H3*his3-AI* derivative			
in-K596G	BR1	9.3 ± 3.0	0.69
in-K596,597G	BR1	0.05 ± 0.02	0.0037
in-K628G	BR2	4.4 ± 1.2	0.33
in-K628,629G	BR2	0.22 ± 0.07	0.016
in-K628G,629G/R630T	BR2	0.02 ± 0.01	0.0015
pGTy1-H3*his3-AI*[b]	WT	2.5 ± 1.0	1.00
pGTy1-H3*his3-AI/in-2600*	D,D35E	0.01 ± 0.004	0.004

[a] Transposition efficiency is expressed as a percentage, determined by dividing the number of His^+ Ura^+ cells by the number of Ura^+ cells obtained in strain DG1286 (*rad52 spt3*).

[b] Because the vector carrying the *in-2600* mutant element is slightly different from that containing the NLS mutant, the cognate WT pGTy1-H3*his3-AI* plasmid of each mutation was also assayed for histidine prototrophy (47).

TABLE 3. Complementation of IN mutants

Strain	pGTy1-H3*his3-AI*[a]		Efficiency[b]	
	TRP1-based	*URA3*-based	Transposition (%)	Complementation
DG1798	WT	WT	7.8 ± 2.0	1.0
DG1801	*in-2600*	*in-K596,597G*	3.2 ± 0.6	0.4
DG1814	*in-2600*	*in-2600*	0.03 ± 0.001	3.8×10^{-3}
DG1802	*in-K596,597G*	*in-K596,597G*	0.06 ± 0.0002	7.7×10^{-3}
DG1803	*in-K596,597G*	WT	8.8 ± 1.0	1.1
DG1804	*in-2600*	WT	6.0 ± 1.7	0.8

[a] The strains carried the plasmids indicated. Derivatives of plasmid pGTy1-H3*his3-AI* are shown in italics.

[b] Transposition efficiency is expressed as a percentage, determined by dividing the number of His^+ Trp^+ Ura^+ cells by the number of Trp^+ Ura^+ cells. Complementation efficiency is the transposition of a given strain divided by that obtained in strain DG1798.

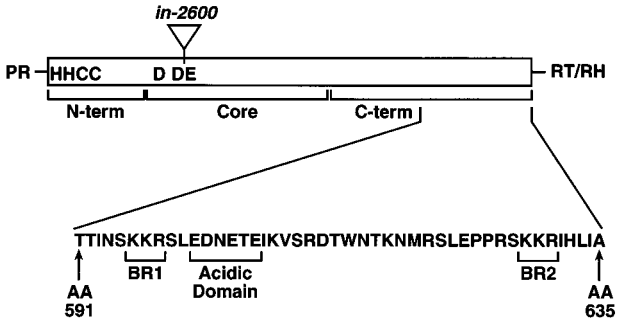

FIG. 7. Structure of Ty1 IN. Positions of the N terminus with the HHCC motif (N-term), the core domain with the D,D35E motif (Core), and the C terminus with amino acids comprising the NLS denoted as BR1, acidic domain, and BR2 (C-term) are illustrated. Single-letter amino acid abbreviations indicate the sequence between residues 591 and 635.

mentation studies performed with retroviral INs (14, 29, 50, 56).

The Ty1 IN NLS is an unusual bipartite sequence in that the two short basic motifs are identical, each composed of two lysines followed by an arginine (Fig. 7) (see also the accompanying paper [31]). Each of these small basic regions is atypical for a basic cluster NLS, but the 29-amino-acid spacer between the two basic regions is greater than usually reported for other bipartite NLSs (11). The length of this spacer is somewhat flexible, since a 12-bp in-frame linker insertion introduced into this region did not reduce transposition (39). Similar spacer length flexibility has also been reported for nucleoplasmin (45). The interdependence of the two basic motifs in the transposition assay and in IIF localization favors the hypothesis that this domain represents a nucleoplasmin-like bipartite NLS with an unusually long spacer.

The sequence context surrounding an NLS may contribute to the efficiency or temporal regulation of protein translocation (26, 49). We have shown that a region containing several acidic amino acids between IN residues 600 and 607 (Fig. 7) is necessary for GFP-LacZ-IN nuclear localization. Acidic domains have been shown to be an important feature of the NLSs of transcription factors (4), c-myc (37), and simian virus 40 large T antigen (27) since they may contain a casein kinase recognition site. Casein kinase-mediated phosphorylation of a serine near the NLS of simian virus 40 large T antigen enhances its nuclear import (27). Although the GFP-LacZ-IN fragment shown in Fig. 2k, in which both BR1 and the acidic domain are deleted, shows nuclear localization, a candidate casein kinase recognition site which approximates the same spacing relative to BR2 as that in wild-type IN was fortuitously created by this deletion. The significance of the Ty1 NLS sequence context is further supported by the fact that Ty1 IN contains another SKKR motif at residues 418 to 421, but the IN fragment containing this region as the only NLS candidate did not show nuclear localization.

The Ty1 IN NLS suggests a straightforward nuclear targeting pathway for the Ty1 PIC. Since Ty1 IN contains both an NLS and a catalytic function, the Ty1 PIC may consist minimally of a cDNA element and an IN multimer. HIV-1 integration in nondividing cells also requires nuclear targeting of a PIC. However, the HIV-1 PIC contains the viral proteins MA, Vpr, and IN, all of which have nuclear targeting potential (6, 18, 25, 52). MA contains a region with basic residues which might function as an NLS (6, 16), but IN is required to recruit MA into the virion core (17, 18). The inability of MA mutant viruses to infect terminally differentiated macrophages can be rescued by Vpr (25), although this function may not be the primary role of Vpr during HIV-1 infection (9). Like Ty1 IN, ectopically expressed HIV-1 IN localizes to the nucleus (18, 30) and has been recently shown to contain a C-terminal bipartite NLS that is recognized by the importin/karyopherin transport pathway (18). HIV-1 mediates nuclear transport of the PIC in monocyte-derived macrophages inoculated with high doses of virus, in some nondividing epithelial and fibroblast lines, and in neurons. Redundant viral factors resulting in the nuclear transport of the HIV-1 PIC may be necessary for infection of different types of nondividing cells. However, data also suggest that the HIV-1 IN NLS has additional functions, since NLS mutations block replication even in proliferating cells, and may interfere with IN multimerization. The remarkable similarity between the karyophilic potentials of HIV-1 and Ty1 INs suggests that the NLS function was strongly selected for during retroelement evolution. Other retroviral INs which have been reported to localize to the nucleus include avian sarcoma virus IN (32) and murine leukemia virus IN (44). The degree to which nuclear localization of these INs influences viral infectivity has not been determined.

In summary, this analysis has elucidated an important and novel aspect of Ty1 retrotransposition. An essential NLS in Ty1 IN suggests a working hypothesis to explain how the Ty1 PIC translocates to the nucleus. The VLP may undergo degradation analogous to viral uncoating to release the PIC. The PIC, containing IN, Ty1 DNA, and perhaps other Ty1 and cellular proteins, is then recognized by nuclear transport factors by the IN NLS. If IN-mediated integration is blocked, however, the PIC may release Ty1 cDNA that can undergo homologous recombination with genomic elements. Further biochemical and genetic dissection of the Ty1 PIC and nuclear transport will elucidate its composition, release from the VLP, and interaction with the nuclear translocation apparatus.

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