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The Cys-His Motif of Ty3 NC Can Be Contributed by Gag3 or Gag3-Pol3 Polyproteins

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The major structural proteins capsid and nucleocapsid (NC) of the *Saccharomyces cerevisiae* retroviruslike element Ty3 are produced as domains within the Gag3 and Gag3-Pol3 precursor polyproteins. Ty3 NC contains one copy of the conserved motif $CX_2CX_4HX_4C$ found in most retroviral NC proteins. We show here that NC proteins derived by processing of these different precursor species differ at their carboxyl termini. To determine whether the Cys-His motifs of these nascent NC domains contribute differently to replication, Gag3 and Gag3-Pol3 fusion proteins containing wild-type or mutant Cys-His domains were expressed from separate constructs. Although the Cys-His box was shown to be essential for polyprotein processing of a wild-type Ty3 element, this domain could be contributed from Gag3 or as part of Gag3-Pol3. These data suggest that the functions of the retroviral NC Cys-His domain contributed from Gag and Gag-Pol are redundant.

Retroviruses express a high level of the major structural proteins and a lower level of catalytic proteins from the same RNA. Major structural proteins are produced from the first open reading frame (ORF). Differential expression of a polyprotein which includes the major structural protein domains as well as catalytic protein domains is accomplished by a low level of programmed translational readthrough from the first ORF into the downstream frame(s) (35). One consequence of this is that the major structural proteins encoded in the first ORF are contributed to the nucleoprotein complex in both Gag and Gag-Pol precursor polyprotein contexts. These proteins include capsid (CA) and nucleocapsid (NC) (21). It is likely that CA must be contributed from both Gag and Gag-Pol fusion proteins for inclusion of Gag and Gag-Pol in the particle. The NC domain has been shown to be essential for stable inclusion of RNA in the particle, and it has a variety of activities in vitro which suggest additional functions in the replication pathway. The NC domain is also produced as part of Gag and Gag-Pol polyproteins. Thus, it is possible that these different activities are associated with the NC domain in different contexts.

The *Saccharomyces cerevisiae* retrotransposon Ty3 encodes CA and NC proteins (31, 32, 39) with similarity to those found in retroviruses (17, 18, 28). In particular, Ty3 NC is similar in size to retroviral NC and contains one copy of the conserved motif $CX_2CX_4HX_4C$. These experiments were undertaken to investigate the role of NC in retrotransposition and to determine whether the Cys-His domains of NC within the Gag3 and Gag3-Pol3 precursor polyprotein contexts have separable essential functions.

Ty3 is composed of 340-bp long terminal repeats (LTRs) flanking an internal domain of 4.7 kbp. Transcription of the 5.2-kb genomic RNA begins and ends in the 5′ and 3′ LTRs, respectively (15). The Ty3 RNA is translated into proteins which form viruslike particles (VLPs) about 50 nm in diameter and 156S ± 5S in size. In addition to RNA and Ty3 proteins, the particle fraction displays reverse transcriptase (RT) activity and contains full-length Ty3 DNA (31). The presumed primer for Ty3 replication is $tRNA_i^{Met}$, which is complementary to the putative primer binding site which begins two nucleotides downstream of the U5-internal domain junction.

Ty3 contains two ORFs, *GAG3* and *POL3*, corresponding to the retroviral *gag* and *pol* genes. *POL3* overlaps *GAG3* by 38 nucleotides in the +1 frame. The *GAG3* gene encodes the precursor polyprotein $Pr38^{GAG3}$, which is processed to a 26-kDa CA species and a NC species of about 9 kDa. A minor 31-kDa species ($p31^{GAG3}$) which contains the CA but not the NC domain is produced. The *POL3* ORF is translated when a ribosomal frameshift results in translational readthrough from *GAG3* into *POL3*, resulting in production of the precursor polyprotein $Pr173^{GAG3\text{-}POL3}$ (24, 40). The estimated ratio of Gag3 structural polyprotein to Gag3-Pol3 catalytic polyprotein precursor in the particle is 13:1 (31, 40). The polyprotein $Pr173^{GAG3\text{-}POL3}$ is processed to $p39^{GAG3\text{-}POL3}$, a 16-kDa aspartyl protease (PR), a $p115^{POL3}$ species which includes integrase (IN) and RT domains, a 55-kDa RT, and 61- and 58-kDa IN species (31, 39). Additionally, the region between PR and RT could encode a protein as large as 10.5 kDa (39). The $p39^{GAG3\text{-}POL3}$ protein is processed to CA and NC. The different sizes of the Gag3- and Gag3-Pol3-derived structural polyproteins (38 and 39 kDa) (39), which presumably differ at the *GAG3-POL3* junction, suggested that NC derived from Gag3 might be physically distinguishable from NC processed from Gag3-Pol3.

Ty3 NC has similarity to its retroviral homolog. Retroviral NC proteins, with the exception of spumavirus NC protein (43), contain one or two copies of the conserved $CX_2CX_4HX_4C$ motif, or Cys-His box. Cys-His motifs composing putative metal fingers have been found in a variety of proteins that interact with nucleic acids but may be involved in protein-protein interactions as well (16). Isolated NC protein from several retroviruses has been shown to bind zinc (7), and the metal finger motif is essential for production of infectious virus (2, 4, 22, 30, 47–49). In retroviruses in which there are two copies of the Cys-His box, the amino-terminal (proximal) copy of the motif is the more highly conserved and virus infectivity is more sensitive to changes in this copy (12, 18, 29, 48). In Moloney murine leukemia virus and in Ty3, there is a single metal finger motif which resembles the proximal copy of the Cys-His box, C-F-X-C-X_3-G-H-X_3-E-C, where position C +1 is occupied by an aromatic residue, position C +7 is glycine,

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and the residue at position C +12 has a carbonyl group. These versions of the motif are also similarly spaced within the mature Gag- and Gag3-derived proteins. In Moloney murine leukemia virus, the first cysteine in the motif begins at residue 26 and the NC protein is 56 amino acids (aa) in length (57). The Ty3 Gag3 Cys-His motif begins at aa 34, and NC is 57 aa in length.

The roles of the NC moiety are not well defined. In vitro, NC has been shown to bind single-stranded nucleic acid relatively nonspecifically (38, 55) and to promote dimerization of genomic RNA (8, 49, 51, 53) and positioning of the primer tRNA for reverse transcription (51, 52). Less is known about activities of the precursor Gag or Gag-Pol polyproteins, which are probably initially involved in recognizing and binding genomic RNA. Pr65, the Gag precursor from murine leukemia virus containing the NC domain, was shown to bind polyethenoadenylic acid more tightly than the NC derivative, p10 (38). When immunoblots of human immunodeficiency virus type 1 (HIV-1) proteins were probed with nonspecific and HIV-1 RNAs, the Gag precursor protein bound HIV-1 RNA specifically under certain conditions (42). Retroviral cores defective in the Cys-His motif of NC are not stably associated with normal levels of genomic RNA (2, 4, 22, 30, 48, 49), further suggesting that this domain is involved in recognition or stabilization of the packaged RNA. Some mutations in NC, however, result in partial packaging phenotypes but completely block replication (47). These results imply that the conserved motif of NC performs critical functions in addition to stabilizing packaging of the RNA. Nevertheless, in vitro experiments have recently shown that the basic region flanking the metal fingers may be the region which is most critical for dimerization of the genomic RNA and annealing of the tRNA primer (19, 20). Thus, while the zinc finger is essential in vivo, its contribution in the context of the Gag and Gag-Pol polyproteins to retroviral replication is not well understood.

In Ty3, as in all retroviruses, NC is produced as part of both major structural and catalytic precursor proteins. These NC domains are in molecularly distinct contexts and are produced at different levels. If the carboxyl terminus of NC from Gag3 (NC^{GAG3}) were formed by translation termination and the carboxyl terminus of NC from Gag3-Pol3 ($NC^{GAG3\text{-}POL3}$) were formed by proteolytic processing at the position identified as the amino terminus of PR (39), then the mature NC species would differ. $NC^{GAG3\text{-}POL3}$ would be 19 aa longer than NC^{GAG3}. In Moloney murine leukemia virus, NC is also found in multiple forms, with p10′ being 4 aa longer than p10 (34). Similar to the organization of Ty3, the site of frameshifting of HIV-1 is within NC. Therefore, NC derived from the Gag-Pol precursor is also predicted to differ from NC derived from the Gag precursor (36, 58). The two species of NC might have discrete functions. For example, packaging or scaffolding functions demanding a high molar ratio of protein to RNA (38) might require NC supplied by Gag3. On the other hand, RT species bind primer tRNAs specifically (3, 50). Dimerization and positioning of the tRNA might therefore be carried out primarily by NC associated with RT in the Gag3-Pol3 context.

Because the function of NC in the life cycle of a retrotransposon had not been examined previously, we first determined the effects of mutating Ty3 NC on VLP formation and retrotransposition. To examine the roles of NC^{GAG3} and $NC^{GAG3\text{-}POL3}$ individually, yeast cells were transformed with two constructs, one of which expressed Gag3 and one of which expressed Gag3-Pol3. Gag3-derived proteins were produced from a construct containing a deletion of most of the *POL3* reading frame. Gag3-Pol3 was made from a construct in which one nucleotide within the overlap region between *GAG3* and *POL3* was deleted to create a single ORF. These constructs were mutated separately to determine whether NC^{GAG3} and $NC^{GAG3\text{-}POL3}$ are both essential for Ty3 replication. In this report, we address the following questions: (i) are there discrete NC^{GAG3} and $NC^{GAG3\text{-}POL3}$ species and (ii) if so, are the functions of the Cys-His boxes of these species separable? (iii) What are the effects of mutations in NC^{GAG3} and $NC^{GAG3\text{-}POL3}$ Cys-His motifs on Ty3 transposition and particle assembly?

MATERIALS AND METHODS

Recombinant constructs. All recombinant DNA techniques were performed essentially as described in reference 6. In many of the experiments described below, a system of helper and donor plasmids was used. Helper plasmids encoded unmarked elements competent to supply Ty3 proteins for retrotransposition of the marked donor. Donor plasmids encoded Ty3 elements marked with the *HIS3* gene but unable to transpose independently. Because the genetic marker was inserted into the *POL3* frame, these donors could also serve as a source of Gag3 protein. The high-copy-number plasmids in these experiments were derived from pEGTy3-1 (32), a plasmid from which Ty3 can be induced by growth on galactose-containing media. In this plasmid, the *GAL1-10* upstream activating sequence (37) is fused upstream of the Ty3 promoter. The vector backbone contains bacterial Amp^r and origin of replication sequences, as well as yeast 2 μm and *URA3* sequences. Yeast cells containing this plasmid can be selected for by growth on medium lacking uracil or selected against by growth on medium containing 5-fluoroorotic acid (5FOA) (10). The high-copy-number plasmid donor pEGTy3-H (14) is the same as pEGTy3-1 except that the Ty3 element is disrupted by insertion of a 0.9-kb *Bam*HI fragment containing the yeast *HIS3* gene at nucleotide 4882 in the IN-coding region of Ty3.

Site-directed oligonucleotide mutagenesis was used to alter the Cys-His box of Ty3 NC. A PCR Mate 391 DNA synthesizer (Applied Biosystems) was used to make the mutagenic oligonucleotides. Plasmids transformed into *S. cerevisiae* were rescued by transformation into *Escherichia coli* HB101 (F^- *hsd-20* [r_B^- m_B^-] *recA13 leuB6 ara-14 proA2 lacY1 galK2 rpsL20* [Sm^r] *xyl-5 mtl-1 supE44* λ^-), and their identities were confirmed by sequence analysis and restriction digestion. An oligonucleotide with the sequence GTTCAGGCGCTGTCCCTCTTTC was used to change one nucleotide (underlined), converting the conserved histidine in the Cys-His box of the NC protein at aa 42 of NC to a glutamine (H42Q). This change was introduced into the wild-type (wt) helper element on pEGTy3-1 and into the *HIS3*-marked donor Ty3 element on pEGTy3-H, creating plasmids pKO365 and pKO418, respectively. An oligonucleotide with the sequence CAATAGAAGCTTAGCCG was used to change the first conserved cysteine at aa 34 of the wt element on pEGTy3-1 to serine (C34S), resulting in plasmid pKO375. To express Gag3, but not Gag3-Pol3, from a high-copy-number plasmid, pEGTy3-H and its mutant derivative pKO418 were cleaved with *Bst*EII and *Sal*I, and the *POL3* regions of the Ty3 genomes, spanning nucleotide 1773 downstream of the PR-coding region to nucleotide 3132 within the RT-coding region, were removed, resulting in plasmids pKO604 and pKO620, respectively. These elements retain the *HIS3* marker which disrupts the IN-coding region. The Gag3 protein expressed from pKO604 contains a wt Cys-His box, while the Gag3 expressed from pKO620 contains the H42Q mutation. To achieve a more severe mutant phenotype, the C34S mutation was introduced into pKO620, creating the

double mutant pKO667. These constructs were expressed and produced CA and NC but not RT or IN. A donor (pKO668) that is predicted to be defective for frameshifting was constructed by using oligonucleotide mutagenesis to change the serine codon immediately after the shift site from AGU, which is decoded by a minor isoacceptor, to UCU, which is decoded by a major isoacceptor. Pausing caused by low levels of the minor isoacceptor is required for frameshifting (24). Loss of frameshifting blocks production of any proteins derived from Gag3-Pol3. The high-copy-number *HIS3*-marked donor plasmid pJK314 is a derivative of pEGTy3-1 that encodes a 34-kDa form of CA. It was described previously (40).

The low-copy-number helper plasmids pJK311AC and pJK311AC-OL have been previously described (40). Plasmid pJK311AC is a derivative of pEGTy3-1 in which *CEN4* sequences replace 2 μm sequences, and *TRP1* replaces *URA3*. In addition, a *Bam*HI site is inserted at nucleotide position 297, upstream of the initiating ATG. Plasmid pJK311AC-OL is similar except that an adenine residue within the overlap region between the first and second ORFs has been deleted, thereby fusing the *GAG3* and *POL3* reading frames. The low-copy-number helper plasmids pJK311AC, which is wt, and pJK311AC-OL, which contains the *GAG3-POL3* fusion, were modified by introduction of the H42Q mutation, resulting in plasmids pKO635 and pKO619, respectively. pKO619 was further modified by introduction of the C34S mutation, creating plasmid pKO666, which expresses a doubly mutant NC.

Yeast strains and culture conditions. Yeast strains were cultured by standard methods (56). *S. cerevisiae* TMy18 (*MAT***a** *trp1-H3 ura3-52 his3-Δ200 ade2-101 lys2-1 leu1-12 can1-100* ΔTy3 *bar1*::*hisG GAL3*$^+$), a derivative of yVB110 which contains no endogenous copies of Ty3 (9, 32, 46), was used for transposition assays. *S. cerevisiae* 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) was used for Ty3 VLP preparations because it is defective in Ty1 transcription (11, 62). These strains were transformed by electroporation with the plasmids listed above. Cultures were grown in YPD (1% yeast extract, 2% peptone, 2% glucose) to an A_{600} of about 0.3 to 0.4, as determined in an LKB Ultrospect II spectrophotometer. The cells were pelleted and suspended in YPD at an A_{600} of about 10. A cell volume of 200 μl was mixed with 2 μg of plasmid DNA, placed in a 0.4-cm cuvette, and electroporated in a Gene Pulser (Bio-Rad) at 0.8 V and 25 μF. Cells were plated immediately on selective media. Transformed AGY-9 and TMy18 cells were grown in uracil-minus, tryptophan-minus synthetic medium as necessary to select for cells which retained the plasmid(s). Cultures of the parental strains were grown on synthetic complete medium. Transcription of the Ty3 elements was induced by growth in medium containing 2% galactose as a carbon source. Uninduced cultures were grown in medium containing 2% glucose. Two separate transformants were used for each transposition assay. One or two transformants in at least two separate experiments were used for all other assays.

VLP preparation. One-liter cultures were grown to an A_{600} of 0.9 to 1.1, as determined in an LKB Ultrospec II spectrophotometer, in synthetic medium containing galactose. About 30 ml of cells was removed for extraction of total nucleic acid by the method of Elder et al. (23). VLPs were prepared from extracts of the remaining cells as described previously (39). Extracts were layered onto 70%/30%/20% sucrose step gradients in 10 mM EDTA–10 mM *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid–KOH (HEPES; pH 7.8)–100 mM KCl and centrifuged at 83,000 × *g* for 3 h at 4°C. Four milliliters from the 70%/30% interface fractions was pooled and split into two equal parts. One half was centrifuged in a Ti50 rotor at 96,000 × *g* for 1 h at 4°C to concentrate the VLPs. Proteins were suspended in 15 mM KCl–10 mM HEPES (pH 7.8)–5 mM $MgCl_2$–10% glycerol. Protein concentrations were determined by Bradford assay (13). The other half was diluted 1:1 in 0.3 M sodium acetate and extracted with phenol-chloroform-isoamyl alcohol (25:24:1). The nucleic acid was precipitated with ethanol. The concentration of recovered nucleic acid was determined by reading the A_{260}.

RNA and DNA analysis. For RNA analysis, nucleic acid fractionated on gradients or total RNA was glyoxylated (44) and separated by electrophoresis in 1.1% agarose gels. The nucleic acid was transferred essentially as described for Northern (RNA) blotting (60) to Duralon-UV membranes (Stratagene) in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0]) on a PosiBlot pressure blotter (Stratagene) and cross-linked in a UV Stratalinker 1800 (Stratagene). DNA from the internal region of Ty3 was labeled with [α-^{32}P]dATP by the random primer method (25, 26). Filter-bound nucleic acid was hybridized to this internal probe and washed as described previously (15) and then exposed to Hyperfilm-MP (Amersham). Filter-bound nucleic acid was then stripped in 50% formamide–50 mM Tris (pH 8.0)–0.05 mM EDTA–0.0125% $NaPP_i$–0.0005% bovine serum albumin–0.0005% Ficoll–0.0005% polyvinylpyrrolidone–0.025% sodium dodecyl sulfate (SDS). Stripping occurred during two 80°C incubations of 45 min each. Membranes were reprobed either with the yeast *ACT1* gene labeled with [α-^{32}P]dATP or with an oligonucleotide from the *GAL1* gene end labeled with [γ-^{32}P]ATP and processed as described above for autoradiography.

For DNA analysis, equal volumes of the pooled, phenol-extracted fractions were treated with 0.2 μg of RNase A in a total volume of about 20 μl for 30 min at 37°C and then fractionated by electrophoresis on 0.8% agarose gels. DNA size markers were *Hin*dIII-digested lambda DNA fragments. After the gels were washed as described by Southern (59), the DNA was transferred to Duralon-UV membranes in 10× SSC and cross-linked in a UV Stratalinker 1800. Filter-bound nucleic acid was probed with a DNA fragment containing the internal region of Ty3 as well as with *Hin*dIII-digested lambda DNA fragments labeled with [α-^{32}P]dATP (25, 26), washed, and exposed to Hyperfilm-MP.

Whole cell extraction. Twenty-five-milliliter cultures of cells were pelleted and washed in 3 ml of whole cell extract buffer containing 25 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 0.1 mM EDTA, 50 mM KCl, and 10% glycerol. A mix of 250 μl of whole cell extract buffer made 0.25 mM in dithiothreitol (DTT) and 4.2 μg of aprotinin was prepared for each sample. Cells were suspended in 180 μl of whole cell extract buffer plus DTT plus aprotinin and vortexed with glass beads. Cells were vortexed at maximum speed for 15 s and then plunged into ice for 15 s. This was repeated five times. After the liquid was transferred to a new tube, 70 μl of whole cell extract buffer plus DTT plus aprotinin was added to the tube containing the glass beads. The tube was vortexed again, and the liquid in it was pooled with the first extraction liquid and centrifuged in a Eppendorf microcentrifuge (Brinkmann). The supernatant was transferred to a new tube. Protein concentration was determined by Bradford assay (13).

Immunoblot analysis. Proteins from VLPs or whole cell extracts were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred electrophoretically by the wet-transfer method (61) to Pall Biodyne A membranes (ICN) or Hybond-ECL membranes (Amersham). Small proteins from VLPs were fractionated by tricine-SDS-PAGE (54) and

transferred electrophoretically to Hybond-ECL membranes. Either the Genius system (Boehringer Mannheim) or the ECL system (Amersham) was used to detect proteins bound by either the anti-CA, anti-NC (31), or anti-IN (33) antibodies (Abs). In the case of the VLP proteins, these were polyclonal Abs raised against synthetic peptides from the Ty3 CA, NC, and IN sequences. Proteins from whole cell extracts were reacted with polyclonal Ab raised against the entire CA protein (46).

Time course assay. AGY-9 cells were transformed with pEGTy3-1, pKO365, and pKO375 and grown to an A_{600} of 0.2 to 0.4, as determined in an LKB Ultrospec II spectrophotometer, in the nonrepressing carbon source raffinose. Galactose was added to the cultures to a concentration of 2% to induce Ty3 transcription. Twenty-five milliliters of cells was removed at this time, and proteins were isolated from whole cell extracts. Two hours later, glucose was added to the cultures to a concentration of 2% to repress further Ty3 synthesis. Twenty-five milliliters of cells was removed at this time, and proteins were isolated from whole cell extracts. The cells were allowed to grow another 2 h in glucose, for a total of 4 h postinduction. Twenty-five milliliters of cells was removed, and proteins were isolated from whole cell extracts.

Transposition assay. Transposition of high-copy-number *HIS3*-marked Ty3 donor elements complemented in *cis* with Gag3 proteins and in *trans* with Gag3 and Gag3-Pol3 proteins expressed from low-copy-number helper elements was monitored as conversion of host cells that had lost the donor plasmid to His^+. The effects of mutations in NC from the *HIS3*-marked donor element (Gag3) and from the unmarked helper (Gag3 and Gag3-Pol3 or Gag3-Pol3 alone) were determined. Yeast strain TMy18 was transformed with the *HIS3*-marked donor plasmid pJK314 (CA only), pKO604 (Gag3, wt NC), pKO620 (Gag3, H42Q NC mutant), pKO667 (Gag3, H42Q and C34S NC double mutant), or pKO668 (wt Gag3 and unable to frameshift) and with the helper plasmid pJK311AC (wt), pJK311AC-OL (wt NC, *GAG3-POL3* fusion), pKO635 (H42Q NC mutant), pKO619 (H42Q NC mutant, *GAG3-POL3* fusion), or pKO666 (H42Q and C34S NC double mutant, *GAG3-POL3* fusion) to supply necessary proteins in *trans*. Transformants were selected on synthetic medium minus uracil and tryptophan. Two transformants of each type were streaked onto selective medium containing either 2% glucose, to repress, or 2% galactose, to induce, transcription of the Ty3 elements. After 3 days, five colonies from each transformant were patched onto YPD to allow for loss of the plasmids. Two to three days later, these patches were replica plated onto 5FOA-containing, histidine-minus medium to select for cells which had lost the *URA3*-marked donor plasmid but retained the *HIS3*-marked Ty3 element. The cells grown on galactose were compared with the cells grown on glucose to distinguish growth which was due to transposition of the marked Ty3 element from background recombination events.

RESULTS

Ty3 NC^{GAG3} is distinct from $NC^{GAG3\text{-}POL3}$. Initial analysis of Ty3 NC on an SDS–15% polyacrylamide gel showed one species with a mobility of about 9 kDa. Edman degradation of this species identified a single amino terminus of NC (39). However, there was evidence that Gag3 is produced de novo as a 38-kDa protein and is also processed from Gag3-Pol3 as a 39-kDa species. In cells expressing solely the Gag3-Pol3 fusion protein, the 39-kDa species, but not the 38-kDa species, is apparent (31, 40). This finding suggested that in addition to an NC species with its carboxyl terminus formed by translational termination, there was a species with its carboxyl terminus formed by proteolytic processing. These NC species would differ by 19 residues. Tricine-SDS-PAGE was used to differentiate heterologous forms of NC. The Ty3 proteins in wt VLPs were fractionated on gels and stained with Coomassie brilliant blue G (Sigma) (Fig. 1A) or transferred to membranes and probed with an anti-NC Ab. Two precursor proteins and three smaller proteins containing NC were identified by immunoblot analysis (Fig. 1B). As Gag3-derived proteins are present in the particle at a level 13 times the level of Gag3-Pol3-derived proteins, the major NC protein is deduced to represent the product of Gag3 processing. It has an apparent mobility of about 9 kDa and is referred to as NC^{GAG3}. The protein of lower abundance has an apparent mobility of 11 kDa. The level and mobility of this protein are consistent with those expected for $NC^{GAG3\text{-}POL3}$. The derivation of the protein of lowest abundance and apparent mobility of 7 kDa is not known. Experiments described below in which only Gag3 or Gag3-Pol3 products were expressed confirmed that the 9-kDa species is derived from $Pr38^{GAG3}$ and that the 11-kDa species is derived from $p39^{GAG3\text{-}POL3}$.

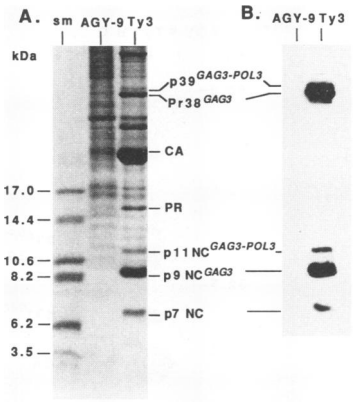

FIG. 1. Resolution of multiple NC species by tricine-SDS-PAGE analysis. Thirty micrograms of protein per sample concentrated from the 70%/30% interface of sucrose step gradients was fractionated by tricine-SDS-PAGE on 16.5% gels. The Ty3 proteins are indicated. (A) Proteins from AGY-9 cells (AGY-9) and AGY-9 cells transformed with pEGTy3-1 (Ty3) stained with Coomassie brilliant blue G. Size markers (sm) are indicated. (B) Immunoblot of the same samples as in panel A, transferred to a Hybond-ECL membrane and probed with the anti-NC Ab. The proteins were visualized with the ECL system and exposed to Hyperfilm-MP for 2 min.

Mutations in the NC Cys-His box can decrease processing and stability of Ty3 VLP proteins. Mutations in retroviral NC have been shown to disrupt stable association of RNA with the retroviral core. The protein and nucleic acid contents of Ty3 wt and NC mutant particles were examined to determine the effect of mutating the NC Cys-His motif on Ty3 particle morphogenesis. Proteins from AGY-9 and from AGY-9 transformed with a high-copy-number plasmid expressing either the wt (pEGTy3-1), the H42Q mutant (pKO365), or the C34S mutant (pKO375) NC were examined. These elements each encoded wt CA, PR, RT, and IN proteins. Ty3 VLPs were analyzed by probing immunoblots with anti-CA (Fig. 2A) and anti-IN (Fig. 2B) Abs. The mutant elements formed particles which appeared in the same fractions of the gradient as wt particles. However, there were much lower levels of particle proteins in preparations from cells expressing the mutant Ty3 elements. In addition, the particles containing mutant NC differed from wt particles in the proportion of processed

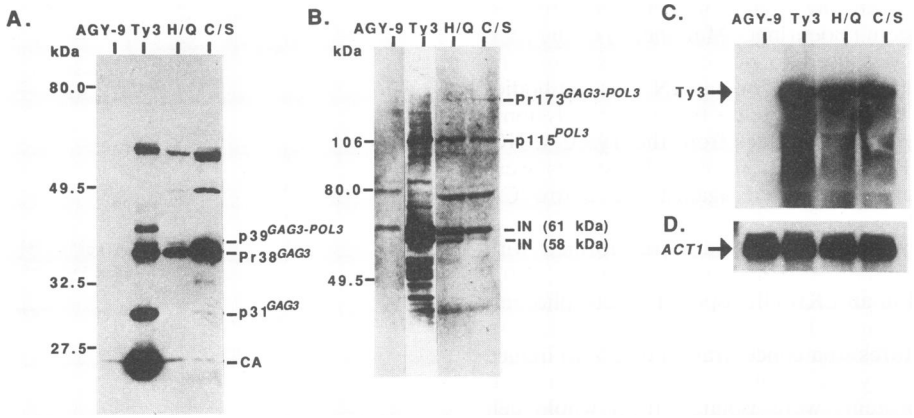

FIG. 2. Analysis of full-length NC mutants. Lanes contain extracts from nontransformed cells (AGY-9) and AGY-9 cells transformed with the following high-copy-number plasmids carrying Ty3 elements: wt on pEGTy3-1 (Ty3), H42Q NC mutant on pKO365 (H/Q), and C34S NC mutant on pKO375 (C/S). (A and B) Immunoblot analysis of protein processing in NC mutants. Thirty micrograms of protein concentrated from the 70%/30% interface of sucrose step gradients was fractionated on SDS–12% (A) or 10% (B) polyacrylamide gels, transferred to Biodyne A membranes, and probed with an anti-CA Ab by using the ECL system (Amersham) (A) or an anti-IN Ab by using the Genius system (Boehringer Mannheim) (B). (A) The immunoblot was exposed to Hyperfilm-MP for 3 min. (B) The wt lane was exposed to Hyperfilm-MP for 30 s; the other lanes were exposed for 5 min. The Ty3 proteins are labeled at the right of each autoradiogram, and the positions of migration of protein size markers are indicated at the left. (C and D) Analysis of genomic and nongenomic total nucleic acid. Ten micrograms of total RNA from cells expressing Ty3 plasmids was separated by electrophoresis on 1.1% agarose gels and processed as described in Materials and Methods for Northern analysis. Nucleic acids were transferred to Duralon-UV membranes as described in Materials and Methods. (C) The nucleic acid was probed with a DNA fragment of the internal region of Ty3 labeled with [α-^{32}P]dATP, washed, and exposed to Hyperfilm-MP for 25 h. (D) The filter was stripped and reprobed with the yeast *ACT1* gene labeled with [α-^{32}P]dATP, washed, and exposed to Hyperfilm-MP for 12 h.

proteins. In the wt distribution of Ty3 proteins, the mature 26-kDa CA predominated, with lesser amounts of p31^{GAG3}, Pr38^{GAG3}, and p39$^{GAG3\text{-}POL3}$ species. In the two NC mutants, little or no processed CA or p31^{GAG3} was present (Fig. 2A). Analysis of immunoblots of VLP proteins probed with the anti-IN Ab showed that the precursor Pr173$^{GAG3\text{-}POL3}$ was present at higher levels relative to processed forms than in wt cells (Fig. 2B). Thus, mutations in the conserved Cys-His motif affected both the total level of Ty3 proteins and the proportion of processed proteins.

Although equal amounts of total protein were loaded in all lanes, the absolute amount of Ty3 proteins present in the mutant strains was much less than the amount present in the wt strain. Formally, reduced levels of proteins could result from instability in the mRNA or protein. Northern blot analysis of total RNA from cells containing either the wt element on pEGTy3-1 or the mutant elements on pKO365 and pKO375 showed that Ty3 RNA was present at equal levels in the three types of transformants (Fig. 2C). Thus, the decrease in the amount of Ty3 protein present was not due to a change in the steady-state level of Ty3 mRNA.

The likely effects of mutations in the NC Cys-His box on the stability and processing of proteins are difficult to assess in VLPs accumulated over a 42-h period of expression in galactose. The levels of Ty3 species in extracts of cells induced for only 2 h followed by growth in the repressing carbon source glucose for another 2 h were therefore assessed (Fig. 3). In extracts from cells expressing wt Ty3, mature CA appeared after 2 h in galactose and predominated after an additional 2 h. Although mutant Gag3 precursor appeared in 2 h, no mature CA appeared after an additional 2 h. As in the earlier experiments, levels of mutant proteins were very low relative to levels of wt proteins. The low levels of total mutant proteins suggested that these proteins are very unstable. However, the relative levels of Gag3 and CA also differed between wt and mutant elements. Although it is formally possible that the

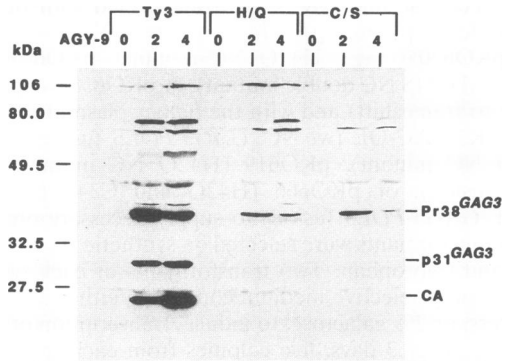

FIG. 3. Precursor processing and stability in wt and mutant Ty3 VLPs. AGY-9 cells transformed with high-copy-number plasmids carrying Ty3 elements (wt on pEGTy3-1 [Ty3], H42Q NC mutant on pKO365 [H/Q], and C34S NC on pKO375 [C/S]) were grown on the nonrepressing carbon source raffinose. At zero time (lanes 0), galactose was added to a concentration of 2% to induce Ty3 transcription. One portion of the cells was removed, and proteins were extracted. Two hours later (lanes 2), glucose was added to a concentration of 2% to repress further Ty3 synthesis. One portion of the cells was removed, and proteins were extracted. The cells were grown for 2 h in glucose, or 4 h postinduction (lanes 4). One portion of the cells was removed, and proteins were extracted. Whole cell extracts were prepared from 25 ml of these cells at the specified time points, as well as from nontransformed AGY-9 cells grown continuously in galactose (AGY-9), as described in Materials and Methods. Fifty micrograms of protein from whole cell extracts taken at time points 2 (lanes 2) and 4 (lanes 4) and 20 μg of protein from whole cell extracts taken at time point 0 (lanes 0) and from nontransformed cells (lane AGY-9) was fractionated on SDS–12% polyacrylamide gels, transferred to Hybond-ECL membranes, and probed with an anti-CA Ab, using the ECL system. The immunoblot was exposed to Hyperfilm-MP for 5 min. The Ty3 proteins are labeled at the right, and the positions of migration of protein size markers are indicated at the left.

processed CA protein is much less stable than the precursor, there is no obvious reason that this should be the case. The presence of precursor but absence of mature CA in cells expressing mutant NC during the period that the mature CA is accumulating in cells expressing wt NC argues that precursor is available but that processing is decreased by the mutation in the NC Cys-His box.

Whether mutating the Cys-His box of the Ty3 NC affected association of Ty3 RNA with the VLP was also tested. Nucleic acids were extracted from VLPs, separated on agarose gels, and transferred to Duralon-UV membranes. Total and VLP nucleic acids were hybridized with a radiolabeled, Ty3-specific probe to compare total and VLP nucleic acids from mutant elements with nucleic acids from cells expressing the wt element. To monitor an mRNA induced similarly to Ty3 mRNA, a radiolabeled *GAL1*-specific oligonucleotide was also used to probe total and VLP nucleic acids. Nucleic acids were examined from cells expressing full-length, unmarked elements encoding wt (pEGTy3-1), H42Q mutant (pKO365), or C34S mutant (pKO375) NC. Both Ty3 and *GAL1* RNAs were present at similar levels in all total RNA samples (Fig. 4A and B). The level of VLPs in fractions from cells expressing NC mutant elements was reduced; consequently, the level of the Ty3 RNA was also reduced in samples from these cells. In addition, Ty3 VLP fractions contain nongenomic RNAs as well as genomic Ty3 RNA. It is, therefore, difficult to determine quantitatively to what extent association of Ty3 genomic RNA is affected by the mutations in the Cys-His box.

Full-length, replicated Ty3 DNA was not detected by Southern blot analysis in samples from cells expressing mutant NC (Fig. 4C). The absence of Ty3 DNA in cells expressing the mutant NC domain would be consistent with reduced levels of genomic RNA. It would also be consistent with reduced levels of processed RT, since unprocessed RT has been shown to be inactive (39). The decrease in processed RT levels reflects both lower levels of total Ty3 protein and a reduced proportion of processed protein. The effect of reduced Ty3 genomic RNA on replication cannot be evaluated.

The effect of a missense mutation in the NC Cys-His box on transposition depends on the context of its expression. A helper/donor assay was used to determine the effects of mutations in the NC Cys-His motif on Ty3 transposition. In the experiments described above, only one source of Ty3 protein and RNA was present in cells. In the transposition assays, two Ty3-expressing plasmids were present; one expressed an element with a deleted internal domain providing CA or Gag3 and truncated Gag3-Pol3 proteins and a transposition marker (donor), and one expressed an element providing Gag3 and Gag3-Pol3 proteins (helper). Three of the high-copy-number plasmids carrying donor Ty3 elements were pKO604, pKO620, and pJK314 (Fig. 5). The pKO604 donor Ty3 contains a deletion extending 3′ of the PR-coding sequence to the last one-fifth of the RT-coding sequence and an insertion of the *HIS3* gene into the coding sequence of IN. Thus, although it produced $p39^{GAG3\text{-}POL3}$, it did not produce a precursor which contained both $NC^{GAG3\text{-}POL3}$ and RT. Plasmid pKO620 is identical to pKO604 except that it encodes the H42Q mutation in NC. Plasmid pJK314 is different in that the only Ty3 protein expressed is CA. The element on pJK314 is deleted from nucleotide 1131 at the 5′ end of the NC-coding region to nucleotide 4882 at the 3′ end of the IN-coding region. Thus, it makes a 34-kDa protein including all of CA and the first 7 aa of NC. The 34-kDa protein was previously shown to form particles when expressed alone from pJK314 (40). The two low-copy-number plasmids carrying helper Ty3 elements used in the first assay were pJK311AC (wt NC) and pKO635 (H42Q

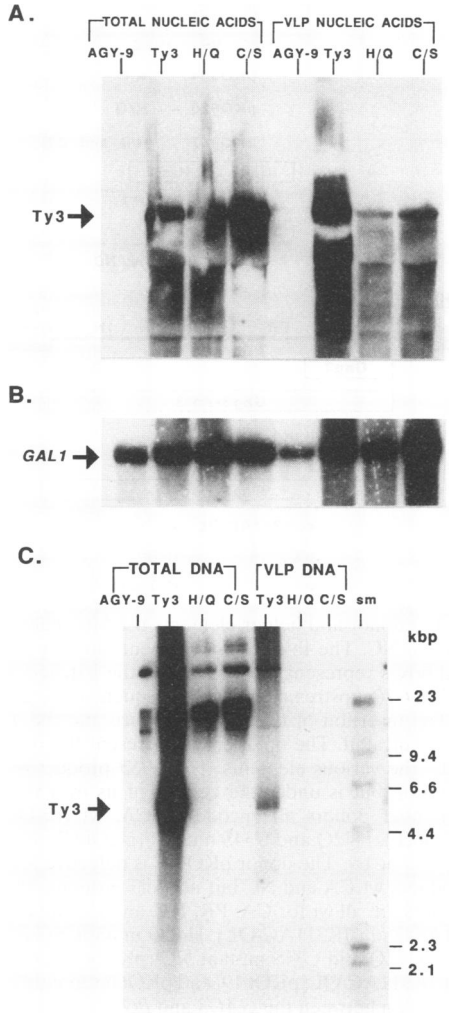

FIG. 4. Ty3 RNA and DNA in cells expressing Ty3 NC mutant and wt elements. Ten micrograms of total RNA from cells expressing Ty3 plasmids and equal volumes representing from 6 to 21 μg of nucleic acids from the phenol-extracted, pooled VLP fractions of the 70%/30% interface of sucrose step gradients were separated by electrophoresis on 1.1% agarose gels and processed as described in Materials and Methods for Northern analysis (A and B) or on 0.8% agarose gels and processed as described in Materials and Methods for Southern analysis (C). Nucleic acids were transferred to Duralon-UV membranes as described in Materials and Methods. The lanes contain nucleic acids from nontransformed AGY-9 cells (AGY-9), and AGY-9 cells transformed with pEGTy3-1 (Ty3), pKO365 (H/Q), or pKO375 (C/S). Total nucleic acids and VLP nucleic acids are marked. (A) The nucleic acid was probed with a DNA fragment of the internal region of Ty3 labeled with [α]-^{32}P]dATP, washed, and exposed to Hyperfilm-MP for 3 h. (B) Nucleic acid on the same filter was stripped, reprobed with a [γ-^{32}P]ATP-end-labeled oligonucleotide complementary to *GAL1* gene transcripts, washed, and exposed to Hyperfilm-MP for 6 days. (C) The same amounts of nucleic acids were treated with 0.2 μg of RNase A for 30 min at 37°C, separated on agarose gels, and transferred to Duralon-UV membranes. The DNA samples were probed with a DNA fragment of the internal region of Ty3 labeled with [α-^{32}P]dATP and exposed to Hyperfilm-MP for 12 days. The full-length Ty3 DNA is indicated; higher bands represent plasmids.

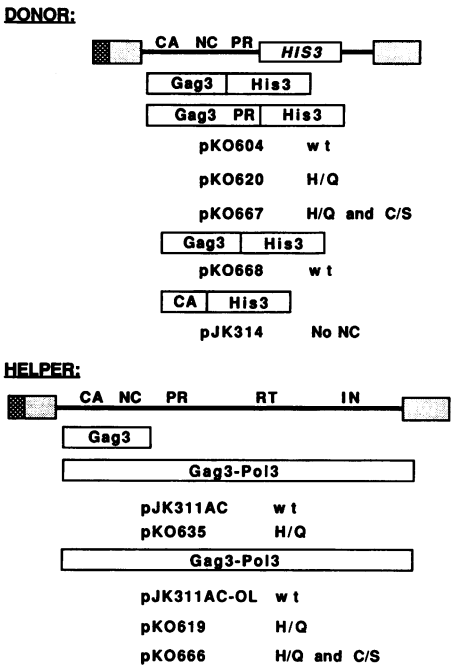

FIG. 5. Ty3 donor and helper elements used to analyze the effects of mutations in NC. The thick lines represent internal Ty3 sequence, the stippled boxes represent the LTRs, and the hatched boxes represent the *GAL1-10* upstream activating sequence fused to the Ty3 promoter. The insertion of the *HIS3* sequence and the Ty3 proteins encoded are indicated. The open boxes represent the protein precursors made by the various elements. The *HIS3* product is not synthesized as a fusion but is under the control of its own promoter. The high-copy-number donors all encode wt CA, wt (pKO604), H42Q mutant (pKO620), H42Q and C34S mutant (pKO667) or no (pJK314) NC, and no RT or IN. The donor pKO668 is defective for frameshifting and produces wt CA and NC but no Pol3 proteins. The low-copy-number helpers are all wt for CA, PR, RT, and IN and contain either wt (pJK311AC and pJK311AC-OL), H42Q mutant NC (pKO635 and pKO619), or H42Q and C34S mutant NC (pKO666). In addition, the helpers on pJK311AC-OL, pKO619, and pKO666 contain a deletion in the overlap region between the *GAG3* and *POL3* genes, so that only a Gag3-Pol3 polyprotein is formed.

NC). We first examined the effect of wt or mutant NC domains contributed from the *GAG3* reading frame of high-copy-number donor elements in combination with wt or mutant NC domains contributed from low-copy-number helper elements on the ability of the marked donor element to move.

The transposition phenotypes of cells expressing wt helper together with wt or mutant donors are shown in Fig. 6A. All combinations which included wt helper resulted in transposition. The three donor constructs were each tested with the H42Q mutant helper that produced Gag3 and Gag3-Pol3 (Fig. 6B). This assay showed that when mutant helper was expressed together with a donor that did not contribute NC (pJK314), transposition did not occur. When mutant helper was expressed with the wt NC domain (pKO604), transposition did occur. Surprisingly, when H42Q NC domains were expressed from both helper and donor, transposition also occurred. This result was at odds with the phenotypes of the two separate mutant Ty3 elements, expressed from the single plasmids pKO365 (H42Q) and pKO375 (C34S), in which particle maturation and replication were severely disrupted. It suggested that this mutation of a conserved histidine in the Cys-His motif was leaky and that the severity of the mutant phenotype depended on the ratio of Gag3 to Gag3-Pol3, or that the expression from separate donor and helper elements alleviated the deficiency in particle formation.

NC produced in *trans* to RT can suppress the effects of a Gag3-Pol3 Cys-His box mutation on transposition. The wt helper system described above allowed us to examine the function of mixed populations of helper and donor Gag3 and Gag3-Pol3 but did not allow separate analysis of NC made in *cis* and *trans* to RT because any mutation introduced into the helper was contained in both Gag3 and Gag3-Pol3 proteins. Separate analysis was necessary to test whether the subpopulation of NC made in *cis* to RT has a specialized role in replication. Therefore, a helper Ty3 in which the *GAG3* and *POL3* reading frames were fused near the frameshift site, encoded on plasmid pJK311AC-OL, was used. The Ty3 element on this plasmid was mutated by introducing the H42Q mutation into the NC-coding domain to give the helper element on pKO619. The wt and fusion helper elements were expressed in combination with the donor elements described above. It was predicted that if the NC Cys-His box synthesized in *cis* with RT had a unique function, then only Ty3 combinations with wt NC in the *GAG3-POL3* fusion (OL) helper would show mobility.

All three donor elements transposed when expressed together with the wt OL helper on pJK311AC-OL. The element on plasmid pKO604, which supplied wt CA and NC at high levels, transposed at a very high rate when expressed together with the wt OL helper (Fig. 6C). The Ty3 donors on pJK314 and pKO620, which encode CA and CA plus mutant NC, respectively, also transposed, although at a lower level than the donor expressing wt Gag3. This result argued, as described previously (40), that NC contributed from the Gag3-Pol3 precursor was sufficient for transposition. As shown by Kirchner et al., transposition of marked donors producing CA (pJK314) and complemented with the wt OL helper is significantly above the level of transposition of marked donors not encoding protein (40). The fact that the level of transposition of marked donors producing mutant NC is similar to that of the marked donor producing CA alone (Fig. 6C) suggests that the mutant Gag3 must be incorporated into the VLP. Mutations in the conserved positions of the Cys-His box of NC did not interfere with the function of that domain contributed from the wt helper (Fig. 6C). Partially mutant structural proteins can interfere with the function of wt proteins and confer a dominant negative phenotype. The fact that interference was not observed, even when mutant NC was expressed from the high-copy-number donor, suggested that the NC domain was not grossly misfolded. A much higher frequency of transposition was observed in experiments with the OL Ty3 helper elements than with the nonfused helper elements. This result argued that the proteins encoded by the *POL3* gene are normally limiting. When they are produced at greater than wt levels, transposition increases. With the expression of *GAG3-POL3* fusion helpers, the *GAG3* gene products became limiting so that differences in transposition between mutant and wt NC-contributing donors were evident.

Whether the Cys-His box of $NC^{GAG3\text{-}POL3}$ provided in *cis* with RT performs a function which cannot be performed by the Cys-His box of NC^{GAG3} was examined by expressing the H42Q OL helper on pKO619 with the three donors (Fig. 6D). The donor element which contributed the CA domain but no NC (pJK314) did not transpose when combined with the H42Q OL Ty3. Since this donor transposed efficiently with the wt OL helper, the H42Q mutation in Gag3-Pol3 appeared to inactivate an essential function of NC. An alternative formal possi-

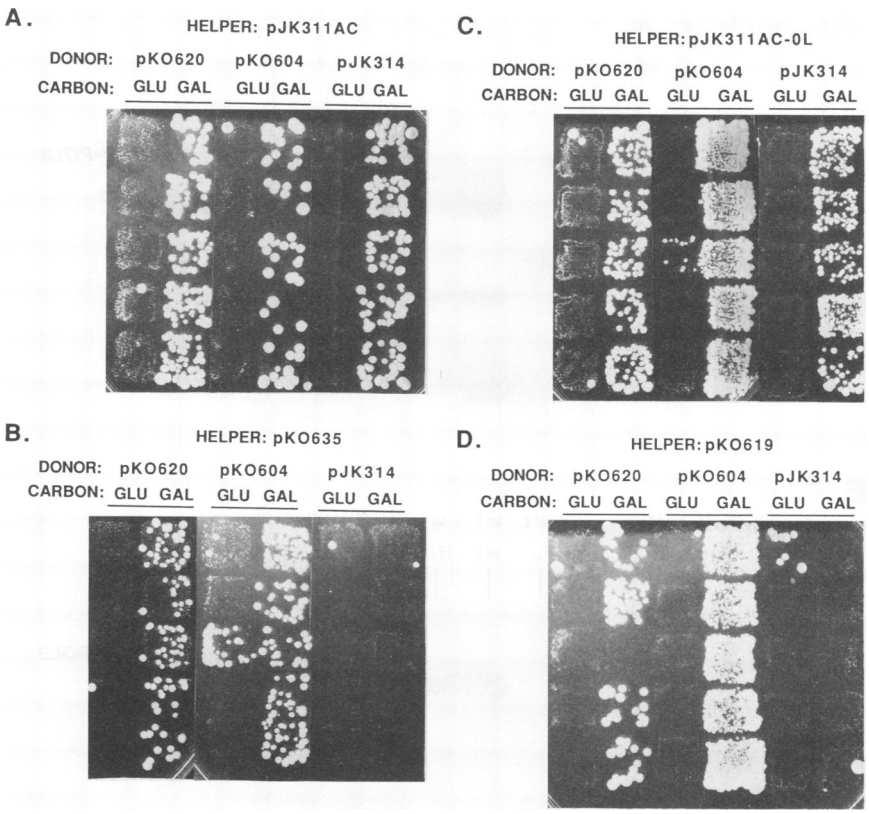

FIG. 6. Effects of mutations in the NC^{GAG3} and $NC^{GAG3\text{-}POL3}$ Cys-His boxes on transposition. TMy18 cells were transformed with the low-copy-number helper plasmid pJK311AC (wt NC) (A), pKO635 (H42Q NC) (B), pJK311AC-OL (wt NC, *GAG3-POL3* fusion) (C), or pKO619 (H42Q NC, *GAG3-POL3* fusion) (D) and high-copy-number donor plasmid pJK314 (no NC), pKO604 (wt NC), or pKO620 (H42Q NC). Transformants were streaked onto selective synthetic medium containing either glucose (GLU), to repress transposition, or galactose (GAL), to induce transposition, and grown for 3 days. Five isolated colonies of each transformant were then patched onto YPD nonselective medium and grown for 2 days to allow for loss of the donor plasmid. Cells were then replica plated onto medium containing 5FOA and lacking histidine to select for the acquisition of a genomic, marked donor Ty3. Growth on medium lacking histidine and containing 5FOA is shown.

bility is that the 34-kDa protein expressed from pJK314 allows function of the wt NC but interferes with function of the mutant NC. The donor element on pKO620 which contributed H42Q NC transposed at a low level when coupled with the H42Q OL helper. Thus, the mutant phenotype of the helper could be suppressed in the presence of donor Gag3 but not CA expression. Transposition of the wt donor (pKO604) was the same whether the Gag3-Pol3 supplied from the OL helper was mutant or wt. This result was consistent with the interpretation that the NC Cys-His box produced in *cis* with RT has no unique function in replication.

NC produced in *trans* to RT can suppress the effects of a Gag3-Pol3 Cys-His box mutation on particle formation. Extracts from cells containing induced Ty3 elements on the low-copy-number OL helper plasmid pJK311AC-OL (wt NC) or pKO619 (H42Q mutant NC) and the high-copy-number donor plasmid pJK314 (CA), pKO604 (CA and wt NC), or pKO620 (CA and H42Q mutant NC) were fractionated by velocity sedimentation on sucrose step gradients. Proteins were pelleted from the 70%/30% interface fraction, separated by SDS-PAGE, transferred to Hybond-ECL membranes, and probed with the anti-CA Ab (Fig. 7A). The wt NC donor on pKO604 transposed very efficiently with both wt and mutant NC OL helpers, and the Gag3 proteins were processed efficiently. The H42Q mutant donor on pKO620 also transposed with wt and mutant OL helpers, although at a lower rate than the wt NC donor. When the H42Q donor was present with the H42Q helper on pKO619, a large part of the *GAG3*-encoded protein was present as the mature CA. The CA expressed from the element on pJK314 permitted neither normal processing nor transposition when present with the H42Q helper on pKO619. The additional H42Q mutant NC present in the strain containing plasmids pKO620 and pKO619, compared with the strain containing pJK314 (which does not encode NC) and pKO619, was sufficient to allow a low level of transposition. Thus, particle processing correlated with the transposition activity determined by the helper/donor assay.

The VLPs from cells expressing Gag3-Pol3 OL helpers and *HIS3*-marked donors were also examined by tricine-SDS-PAGE (Fig. 7B) in which different NC species could be resolved. In the combination of elements expressed from pJK311AC-OL and pJK314, $NC^{GAG3\text{-}POL3}$, but not NC^{GAG3}, is encoded. The blot showed the 39-kDa Gag3 precursor and the 11-kDa NC species but no 38-kDa and no 9-kDa NC species, thus arguing that the 11- and 9-kDa proteins are derived from Gag3-Pol3 and Gag3, respectively. A summary of the proposed derivation of different Ty3 species is shown in Fig. 8.

In addition to disrupting proteolytic processing in Ty3, introduction of mutations into the Cys-His box of NC could have affected RNA association with the particle. Therefore, the transformants used in the transposition assay were also examined for Ty3 RNA. RNAs from cells in which OL helpers

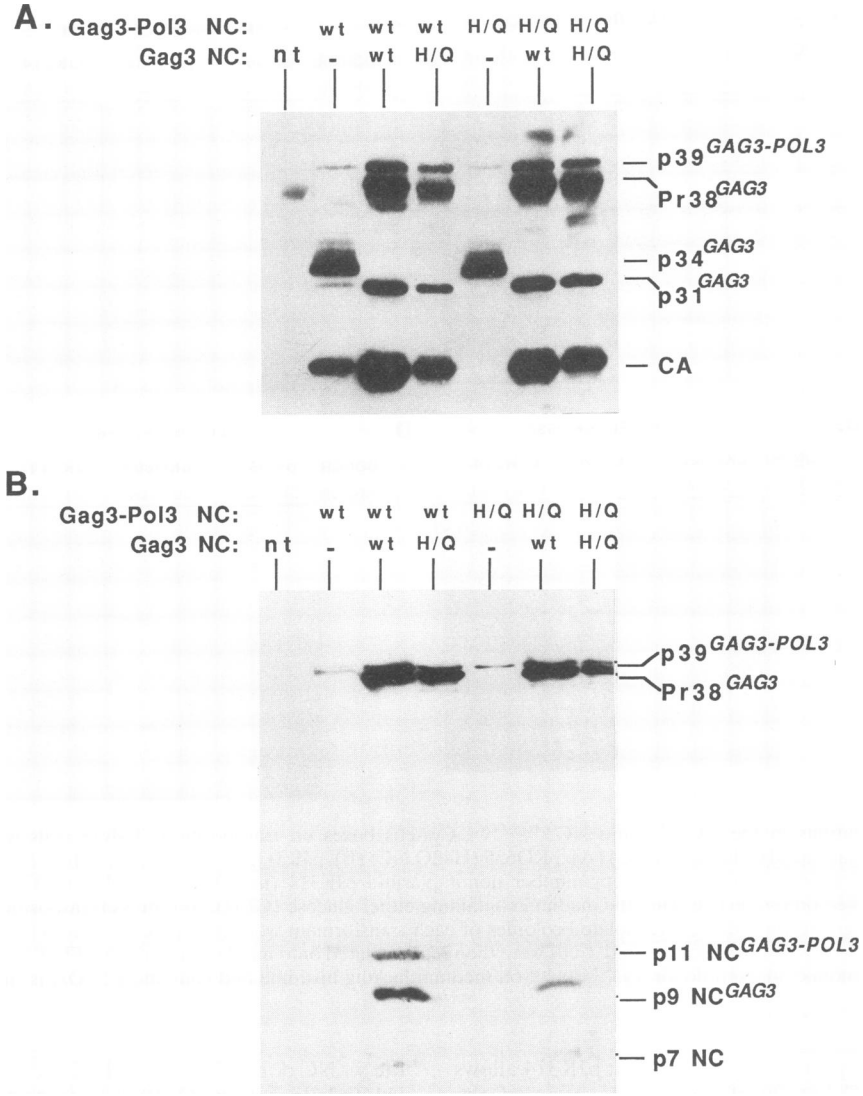

FIG. 7. Effects of mutations in the NC^{GAG3} and $NC^{GAG3\text{-}POL3}$ Cys-His boxes on VLP morphogenesis. Thirty micrograms of protein concentrated from the 70%/30% interface of sucrose step gradients was fractionated by SDS-PAGE on SDS–12% polyacrylamide gels (A) or by tricine-SDS-PAGE on tricine-SDS–16.5% polyacrylamide gels (B), transferred to Hybond-ECL membranes, probed with the anti-CA Ab, and exposed to Hyperfilm-MP for 2 min (A) or probed with the anti-NC Ab and exposed to Hyperfilm-MP for 30 min (B). Lanes contain extracts from nontransformed TMy18 cells (nt) or TMy18 cells transformed with a combination of low-copy-number OL helper plasmid pJK311AC-OL (wt) or pKO619 (H/Q) and *HIS3*-marked high-copy-number donor plasmid pJK314 (−), pKO604 (wt), or pKO620 (H/Q). The combinations of helpers and donors are indicated above the lanes, and the Ty3 proteins are labeled at the right of each autoradiogram. Note that strain TMy18 does not have endogenous Ty3 elements and that the smudge in lane nt in panel A is an artifact.

containing wt NC (pJK311AC-OL) or H42Q mutant NC (pKO619) were coexpressed with the *HIS3*-marked donor elements containing no NC (pJK314), wt NC (pKO604), or H42Q mutant NC (pKO620) were examined on Northern blots (Fig. 9). Here, probing with radiolabeled Ty3 showed a doublet of bands representing the helper and donor RNAs in lanes containing total RNA as well as lanes containing RNA from the VLP fractions (Fig. 9A). The Ty3 donor expressed on pJK314 does not hybridize to the Ty3 probe used and so was not visualized. Reprobing the blot with a radiolabeled fragment of the *HIS3* gene showed that the lower band within the doublet represented the *HIS3*-marked donor RNA (data not shown). A faster-migrating species hybridized to the Ty3-specific probe and weakly to the *HIS3*-specific probe. It is presumed to represent a RNA species that has its 5′ end in the upstream LTR and its 3′ end in the upstream portion of the *HIS3* gene. The level of Ty3 RNA in the particle fraction was reduced severalfold when the high-copy-number donor *GAG3* was mutant but was not as strongly affected by mutations in *GAG3* of the Gag3-Pol3 fusion helper element. The representation of the helper RNA was preferentially reduced when NC^{GAG3} was mutant but was not noticeably reduced when $NC^{GAG3\text{-}POL3}$ was mutant. The levels of both Ty3-related RNAs in the particle fraction were drastically reduced when both donor and helper NC domains were mutant. Because the level of replication (Fig. 9C) correlated loosely with the levels of RNA, these experiments did not offer additional insight into replication-specific roles of the Cys-His motif. The decreased

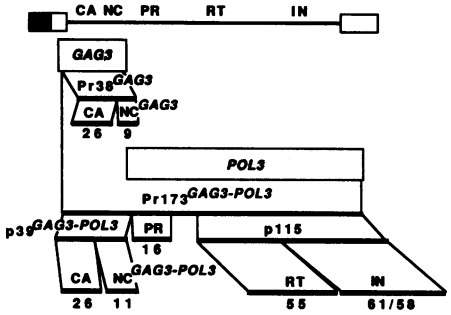

FIG. 8. Summary of the relationship of mature Ty3 proteins to coding regions. The open boxes represent the LTRs, and the striped box represents the *GAL1-10* upstream activating sequence fused to the 5′ LTR. The stippled boxes represent the ORFs, and the thick horizontal lines represent the Ty3 proteins. Protein names are indicated above the lines; sizes in kilodaltons are indicated below the lines. Cleavages are indicated by the thin vertical and slanted lines. Proteins of unknown origin, $p31^{GAG3}$, the 7-kDa NC species, and the putative protein between PR and RT, are not shown. This diagram is not drawn to scale. No implications with respect to the order of processing events are intended.

efficiency of transposition associated with mutant NC correlated well with the multiple defects in VLPs formed.

The NC Cys-His domain is not required in *cis* for RT function. The foregoing results were consistent with a model whereby NC could be contributed in *cis* or *trans* to RT. However, our interpretation of these experiments was limited by the leaky nature of the H42Q missense mutation which resulted in a weak, but positive, transposition phenotype of the mutant donor together with the mutant OL helper. Because of this result, we could not conclude that transposition of the wt donor in combination with the mutant Gag3-Pol3 OL helper was solely due to the wt Gag3 protein and not due to residual Gag3-Pol3 function. To achieve a more severe mutant phenotype, a second mutation, the C34S mutation, was introduced into the coding region for the NC Cys-His box of the donor in pKO620 to create pKO667, a double-mutant donor. This mutation was also introduced into the NC coding domain of the helper in pKO619 to create pKO666 carrying a Gag3-Pol3 OL helper encoding a doubly mutant NC protein.

Transposition occurred when the wt Gag3-Pol3 OL helper was present with each of the three donors (Fig. 10A) or when the H42Q mutant Gag3-Pol3 OL helper was present with the wt and H42Q single-mutant donor but not when the H42Q mutant Gag3-Pol3 OL helper was present with the H42Q and C34S double-mutant donor (Fig. 10B). Thus, the double mutation did not display a leaky phenotype in this context. Transposition occurred when the double-mutant OL helper was present with the wt donor or single-mutant donor but not when it was present with the double-mutant donor (Fig. 10C). Thus, the double mutation in either the donor- or the helper-derived NC did not eliminate transposition in combination with a wt source of NC. These results argued that neither the donor nor the helper NC Cys-His domain has a unique function.

To further probe the respective functions of Gag3 and Gag3-Pol3, the effects of double mutations in both proteins on particle formation were investigated (Fig. 11). In all cases in which transposition occurred, particle formation was also normal. However, in the two combinations in which transposition did not occur, the double-mutant donor (pKO667) present with either the single-mutant (pKO619) or double-mutant (pKO666) helper, little or no mature CA was visible. The double mutation in either the donor or the helper did not eliminate particle formation, but the elements on the donor appeared to be more sensitive to this double mutation, because there was little particle formation when the double-mutant donor was combined with the single-mutant helper, but particle maturation occurred when the double-mutant helper was present with the single-mutant donor.

$NC^{GAG3\text{-}POL3}$ does not have a unique function in transposition. The experiments described above showed that NC does not have to be produced in *cis* to RT in order to function. Nevertheless, because the wt and mutant donors other than pJK314 (which does not produce NC) encode Ty3 protein through the carboxyl end of PR, it is possible that a truncated Gag3-Pol3 product is translated and processed into the 11-kDa $NC^{GAG3\text{-}POL3}$ and that this species has some unique function. We constructed a fifth donor in which frameshifting is predicted to be eliminated so that translation is terminated at the end of the *GAG3* frame (pKO668, fs^-). Frameshifting has been shown to depend on the codon for a minor serine isoacceptor postulated to cause ribosome pausing at the shift site (24). This mutation changed the codon to a serine codon for a more abundant isoacceptor not associated with frameshifting, without changing the coding sequence of NC. This donor was tested for transposition in combination with the wt and double-mutant OL helpers (Fig. 12A). Transposition occurred in both combinations. This finding demonstrated that the $NC^{GAG3\text{-}POL3}$ Cys-His motif, when produced in *trans* to RT, did not have a unique function in transposition. Comparison of the proteins from cells expressing either the wt donor (pKO604) or the donor which is unable to frameshift (pKO668) and the wt OL helper (pJK311AC-OL) or the double-mutant OL helper (pKO666) showed no differences in protein processing (Fig. 12B). This result showed that the $NC^{GAG3\text{-}POL3}$ Cys-His motif produced in *cis* to RT was not required for particle assembly or protein processing.

DISCUSSION

The Ty3 *GAG3* gene encodes the structural portion of Gag3 and Gag3-Pol3 polyproteins which are processed into CA and NC species. We have shown here that mature NC can be resolved into two major species which are derived from Gag3 and Gag3-Pol3 and differ at their carboxyl termini. Ty3 NC, like most retroviral NC species, contains the conserved motif $CX_2CX_4HX_4C$. In contrast to findings in retroviral systems, conservative mutations in the Cys-His motif disrupted processing of particle proteins. In the experiments reported here, we expressed Gag3 and Gag3-Pol3 independently and showed that either can act as the sole contributor of the NC Cys-His motif. Thus, these NC Cys-His domains delivered to the particle in different polyprotein contexts are functionally redundant.

NC is conventionally considered a major structural protein by virtue of high-level expression from the first ORF and its proposed role as an RNA scaffold. However, several findings suggest that the contributions of the Ty3 NC Cys-His box to the particle are of a nature which does not involve scaffolding interactions of this domain with RNA or require it stoichiometrically with other structural proteins. First, transposition occurred efficiently even when NC was supplied exclusively from Gag3-Pol3, which is typically present at 1/13 the level of Gag3. Second, mutations in the NC Cys-His domain of Gag3 did not interfere significantly with the function of wt NC or CA domains present in the particle, as might have been anticipated for a mutation in a structural protein. Third, although the CA

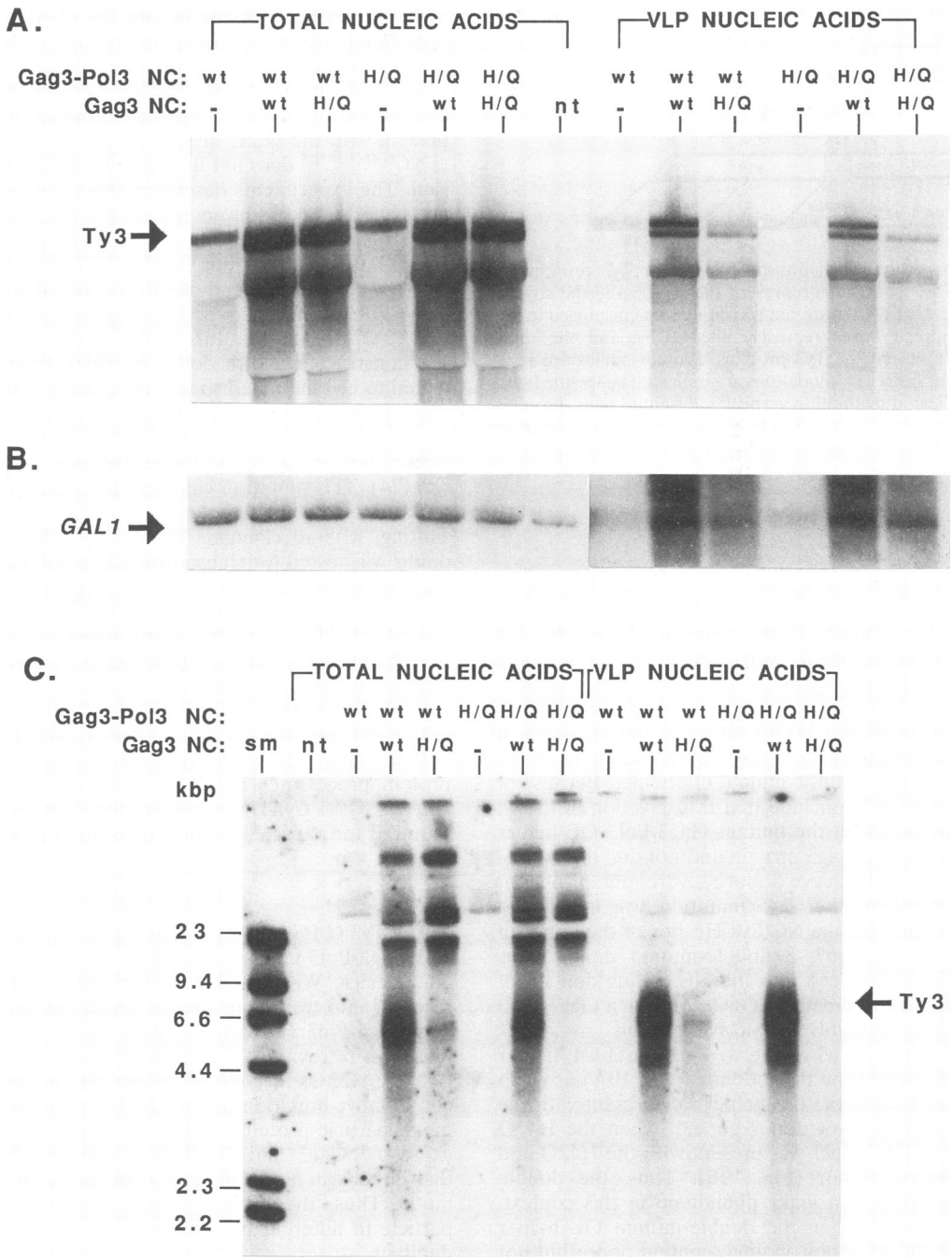

FIG. 9. Effects of mutations in NC^{GAG3} and NC$^{GAG3\text{-}POL3}$ on VLP RNA and DNA. Ten micrograms of total nucleic acid and equal volumes representing from 2 to 7 μg of nucleic acid from the phenol-extracted, pooled fractions of the 70%/30% interface of sucrose step gradients were separated by electrophoresis on 1.1% agarose gels and processed as described in Materials and Methods for Northern analysis (A and B) or on 0.8% agarose gels and processed as described in Materials and Methods for Southern analysis (C). Samples contained nontransformed TMy18 cells (nt) or TMy18 cells transformed with a combination of low-copy-number OL helper plasmid pJK311AC-OL (wt) or pKO619 (H/Q) and *HIS3*-marked high-copy-number donor plasmids pJK314 (−), pKO604 (wt), or pKO620 (H/Q). The combinations of helpers and donors are indicated above the lanes. (A) The RNA was probed with a DNA fragment of the internal region of Ty3 labeled with [α-^{32}P]dATP, washed, and exposed to Hyperfilm-MP for 3 h. (B) RNA on the same filter was stripped, reprobed with an oligonucleotide from the *GAL1* gene end labeled with [γ-^{32}P]ATP, washed, and exposed to Hyperfilm-MP. Exposure times were 5 h for the total RNAs and 8 days for the VLP RNAs. (C) The same amounts of nucleic acids were treated with 0.2 μg of RNase A for 30 min at 37°C prior to separation on agarose gels and transfer to Duralon-UV membranes. The DNA samples were probed with a DNA fragment of the internal region of Ty3 labeled with [α-^{32}P]dATP, washed, and then exposed to Hyperfilm-MP for 4 days. The full-length Ty3 DNA is indicated; higher bands represent plasmids.

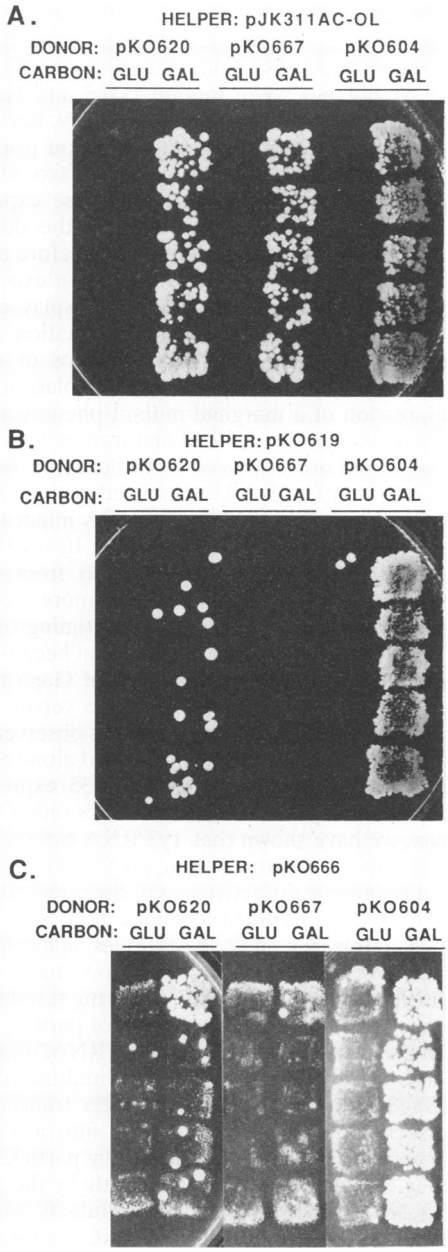

FIG. 10. Effects of double mutations in the Cys-His boxes of NC^{GAG3} and $NC^{GAG3\text{-}POL3}$ on Ty3 transposition. TMy18 cells were transformed with low-copy-number OL helper plasmid pJK311AC-OL (wt NC) (A), pKO619 (H42Q NC) (B), or pKO666 (H42Q and C34S NC) (C) and high-copy-number donor plasmid pKO604 (wt NC), pKO620 (H42Q NC), or pKO667 (H42Q and C34S NC). Assays were performed and are shown as described in the legend to Fig. 6.

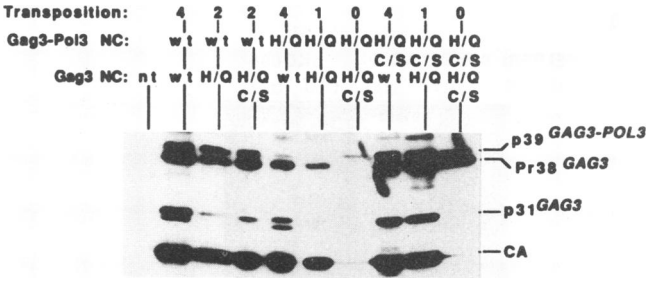

FIG. 11. Effects of double mutations in the Cys-His boxes of NC^{GAG3} and $NC^{GAG3\text{-}POL3}$ on VLP morphogenesis. Fifteen micrograms of protein concentrated from the 70%/30% interface of sucrose step gradients was fractionated on SDS–12% polyacrylamide gels, transferred to Hybond-ECL membranes, probed with the anti-CA Ab, and exposed to Hyperfilm-MP for 30 s. Lanes contain extracts from nontransformed TMy18 cells (nt) or TMy18 cells transformed with a combination of low-copy-number OL helper plasmid pJK311AC-OL (wt), pKO619 (H/Q), or pKO666 (H/Q C/S) and *HIS3*-marked high-copy-number donor plasmid pKO604 (wt), pKO620 (H/Q), or pKO667 (H/Q C/S). The combinations of helpers and donors are indicated above the lanes, and Ty3 proteins are indicated at the right. Levels of transposition are indicated above the combinations of helpers and donors, ranging from completely negative (0) to strongly positive (4).

domain showed CA-CA interaction in the Fields and Song two-hybrid system (27, 41, 45), NC showed neither CA-NC nor NC-NC interactions (data not shown). It has been hypothesized that the metal finger functions to condense the basic regions which flank the metal finger into a conformation which is appropriate for RNA binding (19, 20); it is possible that the mutations that we introduced into NC did not interfere with this interaction once genomic RNA was incorporated via the activity of wt NC. It is possible that deletions of the avian retrovirus NC metal finger domain did not block incorporation of genomic RNA, but rather blocked formation of a stable association of the RNA with the virus core (5). Our system did not allow us to distinguish between possible functions of the Ty3 NC Cys-His motif for incorporation as opposed to stabilization of genomic RNA.

The results of introducing single missense mutations into the Cys-His motif of Ty3 NC were unexpected in several respects. Biochemical analysis of the particulate fraction of cells in which Ty3 was expressed from a single high-copy-number element containing the H42Q or C34S missense mutation in the NC-coding domain of *GAG3* showed that particle formation occurred, although at a reduced level. In addition, levels of mature proteins were reduced relative to levels of precursors, suggesting that processing was reduced or that mature proteins were unstable compared with precursors. Because mature proteins were not detected over the time period that processing could be observed in wt particles, we favor the interpretation that processing is disrupted in the Cys-His mutant particles. This decreased processing of the Gag3 polyprotein was unanticipated, given previous reports of studies with retroviruses. In Rous sarcoma virus, mutation of the first conserved cysteine of the proximal Cys-His box to a serine, mutation of the conserved histidine to a proline (22), or deletion of one or both copies of the Cys-His box (48) did not affect the maturation of the Gag or Gag-Pol proteins. In the case of HIV-1, mutation of a cysteine in the metal finger of NC corresponded in one case with a slight decrease in levels of processed Gag protein (30), but not in other cases, including changing the first two cysteines of either the second motif or both motifs (1). The simplest resolution of the discrepancy is that the mutations in Ty3 proteins themselves directly disrupted the processing site and that this was not observed in retroviruses because specific mutations affect those proteins differently. However, Ty3 proteins containing mutant NC domains were processed, when expressed together with wt proteins, suggesting that the change in primary structure introduced by the mutations did not directly interfere with recognition of the processing site or with PR activity. A more tenable explanation of the result, therefore, is that processing was defective because the Gag3 sub-

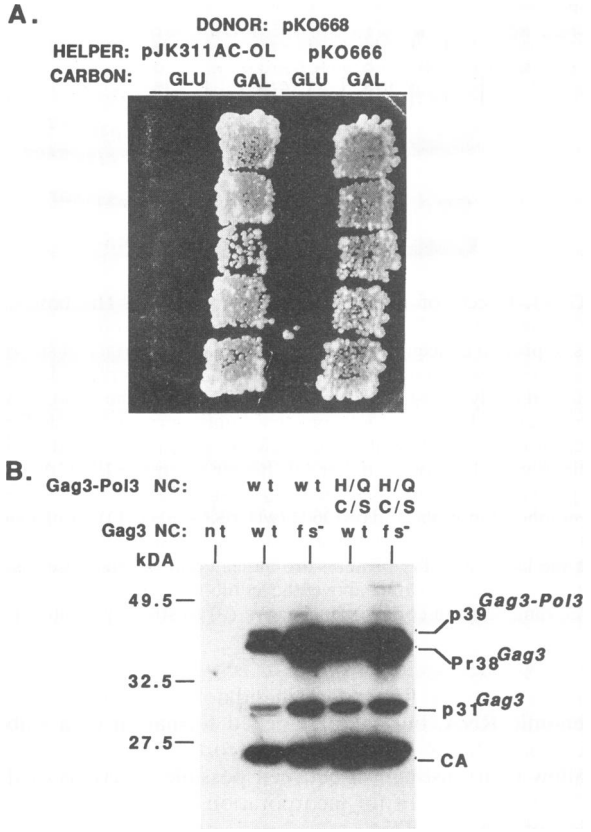

FIG. 12. Analysis of transposition and VLP morphogenesis in the absence of wt $NC^{GAG3\text{-}POL3}$. (A) Transposition assays of the frameshift-negative element on the high-copy-number *HIS3*-marked donor plasmid pKO668 (fs^-) and low-copy-number OL helper plasmid pJK311AC-OL (wt) or pKO666 (H42Q and C34S). Assays were performed and are shown as described in the legend to Fig. 6. (B) Immunoblot analysis of proteins concentrated from the 70%/30% interface of sucrose step gradients. Fifteen micrograms of protein was fractionated on SDS–12% polyacrylamide gels, transferred to Hybond-ECL membranes, probed with the anti-CA Ab, and exposed to Hyperfilm-MP for 30 s. Lanes contain extracts from nontransformed TMy18 cells (nt) or TMy18 cells transformed with a combination of low-copy-number OL helper plasmid pJK311AC-OL (wt) or pKO666 (H42Q and C34S) and *HIS3*-marked high-copy-number donor plasmid pKO604 (wt) or pKO668 (fs^-). The combinations of helpers and donors are indicated above the lanes, and the Ty3 proteins are indicated at the right.

strate and/or Gag3-Pol3-derived PR failed to fold properly. This could have been corrected by interactions with even a nonstoichiometric level of wt Cys-His box or, as we find more likely, by interaction with RNA incorporated or stabilized in the particle by wt NC. Why Ty3 processing might be more sensitive than retrovirus maturation to defects in association of genomic RNA with the particle is not clear. An interesting possibility raised by these results is that it is condensation around the genomic RNA which triggers proteolytic maturation of the Ty3 particle.

Surprisingly, the effect of the single H42Q missense mutation in the NC domain depended on the context of its expression. The phenotype of the Ty3 H42Q mutation was more dramatic when introduced to a single element, affecting Gag3 and Gag3-Pol3 in *cis*, than when introduced to Gag3 and Gag3-Pol3 expressed in *trans*. The former situation resulted in aberrant particles and no transposition; the latter situation yielded low but reproducible levels of transposition. Reversion of the helper or donor Ty3 could explain the discrepancy between the phenotypes when mutant Gag3 and Gag3-Pol3 are expressed in *cis* or *trans*. This seems unlikely, however, for the following reasons: (i) there was no selection prior to the transposition measurement which would have favored survival of revertants over mutant elements; (ii) these experiments were repeated with two transformants; (iii) the donor and helper plasmids were rescued and sequenced before and after the transposition experiments; and (iv) in some experiments, wt and single- and double-mutant constructs displayed distinct phenotypes. The most straightforward explanation of these results, therefore, is either that relaxed ratios of Gag3 to Gag3-Pol3, which may be a feature of the two-plasmid system, result in suppression of a marginal mutant phenotype or that there are kinetic barriers in particle maturation when mutant Gag3 and Gag3-Pol3 are produced from the same RNA. For example, if Gag3 and Gag3-Pol3 associated cotranslationally, assembly might occur more rapidly and RNA might be incorporated during a relatively narrow window. If, on the other hand, Gag3 and Gag3-Pol3 were translated from different RNAs, the assembly process might occur more slowly and exhibit lower stringency with respect to the timing of various steps. We tend to favor the second explanation because we did not observe gross alterations in the ratios of Gag3 to Gag3-Pol3 proteins in particles encoded by single versus multiple RNAs. Also, defective particle formation was observed for the high-copy-number plasmid pKO365 expressed alone as well as the low-copy-number mutant helper pKO635 expressed together with the donor pJK314, which encodes only CA.

In summary, we have shown that Ty3 RNA can be incorporated and replicated in a step requiring the Gag3 or Gag3-Pol3 Cys-His box. Despite the attractiveness of the notion that Gag3 or Gag3-Pol3 NC may perform a unique replication related function, such a function, if it exists, does not require the Cys-His motif. We further hypothesize, on the basis of a number of ancillary observations, the following testable model for Ty3 particle assembly: Multimerization of particle proteins is initiated in close proximity to the coding RNA, and particle assembly is a several-step process—primary multimerization of Gag3 and Gag3-Pol3 during or shortly after translation followed by association of mixed multimers into a VLP. The proper incorporation of Gag3 as reflected by particle stability and processing can be effected independently by the presence of the Gag3 NC Cys-His motif or by contacts with RNA incorporated via Gag3-Pol3. Although NC is essential to particle integrity, one-to-one interaction of mature CA and NC inside the particle either does not occur or is not critical. Finally, if Ty3 translation and protein multimerization take place in close proximity as proposed here, the translated RNA pool may be accessible to the packaging machinery, contrary to the separate genomic and translated pools proposed for retrovirus core morphogenesis. If this view is correct, our data are consistent with a model whereby Gag3-Pol3 association in *cis* with genomic RNA is favored, but association in *trans* is allowed.

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