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Mutations in the Ty3 Major Homology Region Affect Multiple Steps in Ty3 Retrotransposition

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The *Saccharomyces cerevisiae* retroviruslike element Ty3 encodes the major structural proteins capsid (CA) and nucleocapsid in the *GAG3* open reading frame. The Ty3 CA protein contains a sequence ($QGX_2EX_5FX_3LX_3H$, where H is a hydrophobic residue) which has not been observed in other retrotransposons but which is similar to the major homology region (MHR) described for retrovirus CA. In this study the effects of mutations in the Ty3 MHR on particle formation, processing, DNA synthesis, and transposition were examined. Each of the mutations tested resulted in severe defects in transposition, with disruption occurring prior to or at particle formation, subsequent to particle formation and prior to completion of DNA synthesis, and subsequent to DNA synthesis. Changing the Q in the motif to R had relatively little effect on particle formation but decreased transposition to about 13% of that of a wild-type element. Changing G to A or V almost completely eliminated the formation of intracellular particles, possibly by disruption of CA-CA interactions. Changes introduced at the position of E resulted in blocked processing, blocked DNA synthesis, or a block at some post-reverse transcription step, depending on the nature of the mutation introduced. These results showed that the integrity of the Ty3 MHR is required for multiple aspects of Ty3 replication involving CA. These functions are independent of extracellular budding and of infection, aspects of the retroviral life cycle which are not recapitulated in replication of the Ty3 retrotransposon.

The *Saccharomyces cerevisiae* retroviruslike element Ty3 encodes only two major structural proteins, capsid (CA), which has an apparent molecular mass of 26 kDa, and nucleocapsid (NC), a protein with an apparent molecular mass of 9 kDa (24). These proteins are encoded in the first open reading frame, which is designated *GAG3*. Catalytic proteins are encoded in the second open reading frame, *POL3* (24), and are produced as Gag3-Pol3 fusion proteins (29) by occasional frameshifting from *GAG3* into *POL3* (16). Although most of the proteins encoded by Ty3 represent clear homologs of retrovirus proteins on the basis of common sequence motifs, retrovirus CA proteins have fewer distinctive common sequence features than catalytic proteins. The designation of CA was initially based on the lack of a requirement for matrix-specific features, such as interaction with the envelope or envelope protein, position relative to NC in the polyprotein, and the presence of a partial sequence motif found in some retroviral CA domains (see below). Of the two major structural proteins, Ty3 CA may play the more fundamental role in particle formation. A Gag3 protein missing the NC-coding region is sufficient for particle formation and can complement a mutant in which only Gag3-Pol3 fusion protein is made (29). NC is 57 amino acids in length and includes one copy of the conserved metal finger motif found in retroviral NC proteins. Although it is essential for transposition, it apparently is not required at the same level as the CA protein (29, 36). The Ty3 element provides a system for elucidating the function of CA proteins in retroviruslike element particle morphogenesis.

The *gag* genes of simple retroviruses typically encode MA, CA, and NC (53). Additional proteins are also encoded but are more variable in representation among different viruses. The requirements for various domains of the Gag polyprotein for particle formation, budding, and infectivity have been probed by mutagenesis and functional studies with the type D virus Mason-Pfizer monkey virus (M-PMV); type C animal viruses, such as murine leukemia virus (MLV) and Rous sarcoma virus (RSV); and lentiviruses, such as human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus. The role of MA in localizing particle formation has been established (reviewed in reference 8). Signals for membrane localization of type C viruses occur in MA. In the case of the type D virus M-PMV, MA is implicated in targeting of intracellular assembly (41). The results of structural analysis of MA proteins from HIV-1 and simian immunodeficiency virus are consistent with a role in oligomerization of particle proteins (32, 39). An RSV mutant with the CA- and NC-coding regions deleted can produce low-density particles which are released from cells (52). Expression of simian immunodeficiency virus MA protein from a recombinant vector results in the release of particles from cells (23). In addition, mutations in MA, at least some of them in regions predicted to be involved in oligomerization, can disrupt particle formation (21, 35, 42, 54). In at least some cases, proteins persist, suggesting that it is assembly rather than stability which is affected. Together these results suggest that MA has a role in both localization and oligomerization aspects of the assembly pathway.

The details of the role of CA in particle formation, budding, and infectivity are not known. HIV-1 mutants in which MA has been completely or almost completely deleted nonetheless assemble intracellularly, indicating that some functions capable of mediating oligomeric interactions reside within non-MA portions of Gag (15, 30, 45, 51). Consistent with this, mutations in the CA-coding domain have been shown to affect particle assembly and virion infectivity. Expression of *gag* or proviruses with deletions, insertions, and point mutations in the CA-coding domain can block particle formation or cause particles

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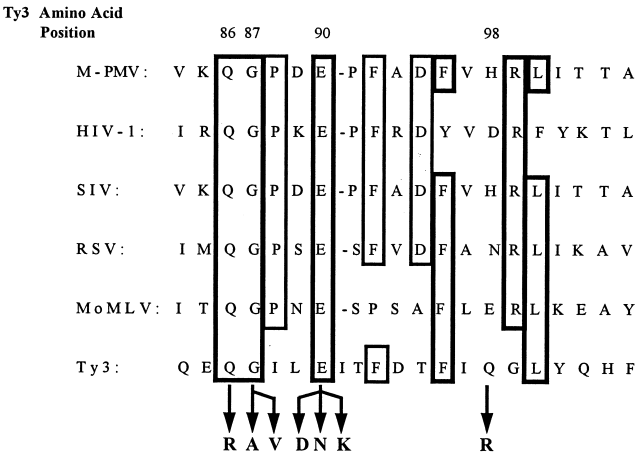


FIG. 1. Alignment of retrovirus and Ty3 MHR domains. M-PMV, HIV-1, simian immunodeficiency virus (SIV), RSV, Moloney MLV (MoMLV) (47), and Ty3 MHR sequences are shown. Positions of conserved residues are boxed. The most highly conserved positions are in boldface boxes. Positions of Ty3 amino acids are numbered from the first Met in the *GAG3* open reading frame. Changes introduced into the Ty3 CA MHR by site-directed mutagenesis are indicated.

released to be heterogeneous, low in density, or noninfectious (9, 11, 31, 40, 46, 47, 50, 52). Linker insertion mutations in the CA domain of an MLV Gag–β-galactosidase fusion disrupted the inclusion of this protein into virion-density particles, but not its release from the cell in vesicles, when expressed in *trans* with wild-type virus (26). At least in some cases, reduced particle formation does not appear to be attributable solely to destabilization of the component proteins. These findings in vivo are consistent with evidence for the abilities of HIV-1 CA to oligomerize in vitro (12) and of mutations in HIV-1 CA to disrupt self-reactivity of HIV-1 Gag fusion proteins in the two-hybrid system (20). However, these in vivo experiments do not show particle formation by viruses expressing a domain consisting exclusively of CA.

The lack of a high degree of protein sequence conservation among different retrovirus CA proteins has made it difficult to infer which domains are likely to have a fundamental role in particle formation and to implicate particular motifs or predicted tertiary structures in this function. However, a sequence designated the major homology region (MHR) has been identified in representatives of type B, C, and D retroviruses and lentiviruses (47, 53). This region consists of about 20 amino acids, 6 of which are highly conserved. An alignment of this centrally located region in the CA proteins from 10 different retroviruses showed the sequence (H)XQGX$_2$E(S)X$_3$FX$_2$RLX$_2$(SH), where (H) indicates a hydrophobic residue and (S) indicates P, S, or T. The MHR motif occurs in the vicinity of the N- and B-tropic determinants of MLV (10, 37). The MHR is not universal among retroviruses, since it does not occur in spumaretroviruses (33). A sequence related to the MHR motif also occurs in the Ty3 CA protein in the form QGX$_2$EX(S)X$_3$FX$_3$LX$_3$(H) (Fig. 1). It has not been identified in other retrotransposons, including Ty1, another element of *S. cerevisiae* (6).

The contribution of the MHR domain to viral particle formation and infectivity has been studied for M-PMV (47), HIV-1 (31, 46), and RSV (9) by mutational analysis. Depending on the system under investigation and the nature of the mutant, mutations in the MHR can cause defects in particle formation, budding, and infectivity. The fact that several positions of the MHR can be changed without blocking budding of virions argues that at least some aspects of particle assembly and budding are not dependent on the intact motif. These studies showed incorporation of Gag-Pol into particles in the case of HIV-1 (31) and RSV (9). However, a recent paper (46) reported reduced inclusion of Gag-Pol fusion polyproteins in particles formed by Gag expressed in *trans*, caused by particular mutations in the Gag-Pol protein MHR domain. This defect was not observed when the mutation was present in both Gag and Gag-Pol proteins produced in *trans*, perhaps because of concurrent kinetic changes in assembly. This could also explain the failure of previous studies to document this aspect of the MHR phenotype. Despite the ability of mutations in CA to disrupt particle formation, budding, and infectivity, the ability of viruses with quite extensive deletions in CA to bud suggests that CA is not essential for particle formation and release but that it contributes to proper packing of the core and to infectivity. Together, the studies of MA and CA in retroviruses suggest that domains contributing to subunit oligomerization are redundant.

The present study was undertaken to investigate the role of the MHR motif in retroviruslike particle function. In the retroviruslike element context, the capability of replicating the genomic RNA can be monitored in the absence of a requirement for extracellular infection. One potential limitation of such a mutagenesis study is that particular mutations will disrupt the overall structure of the protein and that the observed effects on the function of a particular domain will be indirect. Multiple changes were therefore introduced at two positions in the MHR, and the ability of mutant species to interact was tested in the two-hybrid system as well as in the native context. Mutations were introduced at the first three positions of the Ty3 MHR which align precisely with highly conserved positions in the retroviral MHR (Q-86, G-87, and E-90). The effects of these mutations on Ty3 CA-CA interaction, particle formation, processing, DNA synthesis, and transposition were determined. The results showed that particle formation is sensitive to mutations in this domain, and while some of these may be direct effects on CA-CA interactions, others are on the formation of structures required for stabilization of the particle, processing, and synthesis and integration of the DNA.

MATERIALS AND METHODS

Yeast strains and culture conditions. *S. cerevisiae* yTM443 (*MAT***a** *trp1-H3 ura3-52 his3-Δ200 ade2-101 lys2-1 leu1-12 can1-100* ΔTy3 *bar1::hisG GAL3*$^+$) (previously TMy18 [36]), a derivative of yVB110 which contains no endogenous copies of Ty3, was used for transposition assays (3, 25, 34). *S. cerevisiae* AGY-9 (*MAT***a** *ura3-52 his4-539 lys2-801 trp1-Δ63 leu1-Δ1 spt3*) (a gift from A. Gabriel and J. Boeke, The Johns Hopkins University), which is defective in Ty1 transcription (4, 55), was used for the viruslike particle (VLP) preparations. *S. cerevisiae* GGY1::171 (*gal4 gal80 ura3 his3 leu2*) carries an integrated *GAL1-lacZ* fusion gene and was used for the two-hybrid interaction assays (19, 22).

Yeast strains were transformed by electroporation as described previously (36). Cells were plated immediately on synthetic media lacking uracil and/or tryptophan to select for cells which retained the plasmid(s) (see below). Cultures of the parental strains were grown on synthetic complete medium. Transcription of the Ty3 elements was induced by growth on media containing 2% galactose as a carbon source. Uninduced cultures were grown on media containing 2% glucose.

Recombinant constructs. Recombinant DNA techniques were performed essentially as described in reference 1. Plasmids transformed into yeast strains 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^-). Plasmid identities were confirmed by sequence analysis and restriction digestion.

Ty3 elements on plasmids were expressed under control of the *GAL1-10* upstream activating sequence as described previously (25). There were two types of elements, those which encoded proteins and RNA required for transposition (helpers) and those which encoded the Ty3 genomic RNA and could be reverse transcribed into DNA but were disrupted by insertion of the *HIS3* gene (donors). The low-copy-number helper plasmid pTM843 contains a galactose-inducible Ty3 element and the bacterial *bla* and *ori* sequences, as well as yeast ARS/CEN

and *TRP1* sequences. Low-copy-number helper plasmids in combination with high-copy-number donor plasmids were used to monitor transposition of wild-type (wt) and mutant elements. Seven mutations which caused changes in the conserved amino acids of CA were individually introduced into pVB571, a plasmid which contains the 3.8-kbp region between the *Hin*dIII site upstream of Ty3-1 (2) and the internal *Sal*I site (nucleotide 3212 of Ty3-1) by site-directed oligonucleotide mutagenesis. A PCR Mate 391 DNA Synthesizer (Applied Biosystems) was used to make the mutagenic oligonucleotides. The mutations were then transferred to the complete element by exchanging the 3,009-bp *Bam*HI-*Sal*I fragment of pVB571 for the *Bam*HI-*Sal*I fragment of pTM843. The amino acid and nucleotide changes, numbered from the start of translation and from the upstream end of the element, respectively, were as follows: amino acid Q-86 to R (Q86R), nucleotide A-672 to G in pTM839; amino acid G-87 to A (G87A), nucleotide G-675 to C in pMH945; amino acid G-87 to V (G87V), nucleotide G-675 to T in pTM838; amino acid E-90 to D (E90D), nucleotide A-685 to C in pTM841; amino acid E-90 to N (E90N), nucleotides G-683 to A, A-685 to T, and A-688 to T in pTM837; amino acid E-90 to K (E90K), nucleotides G-683 to A and A-685 to G in pTM836; and amino acid Q-98 to R (Q98R), nucleotide A-708 to G in pTM840.

The high-copy-number donor plasmid pKO253, which was described previously (29), was used in these experiments to monitor transposition genetically. This plasmid was derived from pEGTy3-1 by replacement of Ty3 nucleotides 431 to 4979 with the yeast *HIS3* gene. Because of the deletion of most of the internal domain, this donor does not encode the Gag3 protein and can be replicated only when appropriate proteins are supplied in *trans.*

Two-hybrid assay. The two-hybrid assay, which uses fusions to the Gal4p DNA-binding and -activating domains (19) to monitor interactions between proteins, was used to monitor intermolecular contacts of capsid monomers. Strain GGY1::171 was transformed with a *LEU2*-marked pGAD2F-derived plasmid encoding the Gal4p activation domain fused to amino acids 7 to 207 in Ty3 CA and with a *HIS3*-marked pMA424-derived plasmid encoding the Gal4p DNA-binding domain fused to the same region encoding Ty3 CA. Mutant CA domains were cloned into the fusion vectors in several steps, as follows. pVB192 (3), which contains a half Ty3 element (*Hin*dIII to *Sal*I) in pIBI20, was mutated by oligonucleotide site-directed mutagenesis to introduce an *Eco*RI site at position 431 and *Bam*HI sites at positions 417 and 1034. This construct was designated pTM821. A separate construct had a *Bam*HI site at position 1103. The pTM821 DNA was then cleaved with *Bam*HI, and the isolated fragment was cloned into the pGAD2F vector at the *Bam*HI site. The pTM821 *Eco*RI-*Bam*HI fragment (nucleotides 431 to 1034) was cloned into the *Eco*RI-*Bam*HI sites of pMA424. The oligonucleotides used to introduce the MHR mutations into the half Ty3 element in pVB571 were also used to mutagenize the Ty3 sequences in pTM821. The *Bam*HI fragment of each of these constructs was then isolated and cloned into the pMA424 and pGAD2F vectors. For the wt and mutants, plasmids were screened by restriction digestion to identify isolates with fragments in the correct orientation. The final pMA424 constructs were designated as follows: Q86R, pKO657; G87V, pKO664; E90D, pKO662; E90N, pKO660; E90K, pKO661; and Q98R, pKO665. The final pGAD2F constructs were designated as follows: Q86R, pKO658; G87V, pKO653; E90D, pKO659; E90N, pKO655; E90K, pKO663; and Q98R, pKO654. One to four transformants were patched onto plates containing synthetic medium lacking histidine and leucine but with 10 times the level of amino acids in synthetic complete medium (43). After 3 days, the patches were replica plated onto synthetic medium containing the nonrepressing carbon source sucrose, 0.04 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (Sigma Chemical Co.) per ml, and amino acids except histidine and leucine. The interaction of fusion proteins restores Gal4p activity and mediates induction of the β-galactosidase gene, which results in the growth of blue colonies in X-Gal-containing medium. Colonies were evaluated for color development indicative of CA domain interactions 2 to 10 days after plating.

Analysis of Ty3 proteins and DNA in whole-cell extracts. Ty3 particles were monitored in whole-cell extracts to compare levels of Ty3 proteins and DNA in cells expressing Ty3 elements encoding mutant and wt MHR domains. AGY-9 cells transformed with the appropriate plasmid containing a wt or mutant Ty3 element were grown to an A_{600} of 0.2, as determined in an LKB Ultrospec II spectrophotometer, with the nonrepressing carbon source raffinose. Galactose was added to the cultures to a concentration of 2% to induce Ty3 transcription. After growth to an A_{600} of 0.9 to 1.1, portions of the culture were processed for protein (36) and nucleic acid (14) analyses as described previously.

Preparation of VLPs from cells. One-liter cultures in synthetic medium containing galactose were grown to an A_{600} of 0.9 to 1.1. Total nucleic acid was extracted by the method of Elder et al. (14) from cells collected from 30 ml of culture. VLPs were prepared from extracts of the remaining cells, as described previously (13, 24, 36). The nucleic acid concentration within the particles was determined by measuring the A_{260}. Protein concentrations were determined by the Bradford assay (5).

DNA analysis. Ty3 DNA was fractionated by electrophoresis on 0.8% agarose gels and analyzed by the method of Southern (44) as described previously (7). *Hin*dIII-digested λ DNA fragments were used for DNA size markers. Filter-bound nucleic acid was hybridized to fragments from the internal region of Ty3 as well as to *Hin*dIII-digested lambda DNA fragments, which were labeled with [α-^{32}P]dATP by the random primer method (17, 18). The filters were washed as described previously (7) and exposed to Hyperfilm-MP.

Immunoblot analysis. Proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically by the wet-transfer method (49) to Hybond-ECL membranes (Amersham). The ECL system (Amersham) was used to detect proteins which reacted with either the anti-CA or anti-IN antibodies. These were polyclonal antibodies raised against the entire proteins (34).

Transposition assay. *TRP1*-marked, low-copy-number helper plasmids carrying wt or mutant Ty3 elements were used to supply all proteins necessary for transposition of a *HIS3*-marked element with almost the entire coding region of Ty3 deleted on a *URA3*-marked high-copy-number donor plasmid (pKO253). The yeast strain yTM443 was transformed with one helper plasmid and with pKO253. Transformants were selected on synthetic medium lacking uracil and tryptophan. The transposition frequency was determined by a modified P_0 analysis. Transformants were plated on synthetic medium lacking uracil and tryptophan and containing either glucose or galactose as the carbon source. After 3 days of growth on glucose-containing medium or 5 to 7 days of growth on galactose-containing medium (to achieve comparable sizes), colonies were replica plated to yeast extract-peptone-dextrose to allow loss of the *URA3*-marked donor plasmid. After 1 day, colonies were replica plated to medium lacking histidine and containing 5-fluoro-orotic acid to allow detection of cells which had lost the *URA3*-marked plasmid but retained the *HIS3*-marked Ty3 element. A total of 2,860 colonies grown on the repressing carbon source, glucose, were tested, and 331 of these colonies gave rise to at least one His$^+$, 5-fluoro-orotic acid-resistant papillation. Between 400 and 700 colonies were analyzed for each set of two transformants expressing a mutant Ty3 element. For each transformant type, the number of colonies representing the background was calculated and subtracted from the number of positive colonies, and the fraction of colonies having zero events was determined. By using the formula $P_0 = e^{-m}$, where P_0 is the probability of no events in a colony, the mean transposition frequency per colony (m) was determined. A plot of colony number per plate against percentage of positive colonies showed that at above 200 colonies per plate, there is a negative correlation between colony number and transposition frequency (data not shown); therefore, only data from plates with between 100 and 200 total colonies were used in this analysis.

RESULTS

Mutations in the MHR affect interactions between the CA domains of the VLP. CA expressed alone is sufficient for particle formation (29). It may therefore be inferred that a function of the CA domain within Gag3 and Gag3-Pol3 is to provide the scaffold upon which the proteins and nucleic acids within the VLP are arrayed. Within the nascent particle, the CA domain subunits would be expected to have strong interactions among themselves. The Gal4p two-hybrid fusion system has been used to demonstrate protein interactions (19). In one version of this system, the Gal4p DNA-binding domain (amino acids 1 to 147) is fused to the amino terminus of one protein and the Gal4p activation domain (amino acids 768 to 881) is fused to the amino terminus of a second protein. Interaction between the two hybrid proteins reconstitutes Gal4p activity and results in expression of the *lacZ* gene under Gal4p control in strain GGY1::171. In order to test for possible interaction between CA domains and to explore the possible contribution of the Ty3 MHR motif (Fig. 1) to this interaction, the region encoding Gag3 from nucleotide 431 to 1103 (on an *Eco*RI-*Bam*HI fragment) was fused in frame with *GAL4* sequences in pMA424. This included the region from codon 7 through codon 230, counting from the first Met codon in *GAG3*. The 21-amino-acid MHR extends from position 84 to position 104 in Gag3. The amino terminus of NC begins at amino acid 234. This pMA424 derivative was transformed into yeast strain GGY1::171. Transformants were grown and evaluated for Gal4p activity as described in Materials and Methods. Colonies containing solely the Gal4 DNA-binding domain-Gag3 fusion were blue, indicating that the Gal4p activation domain was not necessary for activity (data not shown). Inspection of the fused Ty3 Gag3 sequence showed that the carboxyl-terminal region predicted from the sequence is acidic in overall charge and is a potential activator. Therefore, a second set of fusion constructs was produced, in which only residues 7 through 207 of Gag3 were cloned into the DNA-binding domain vector pMA424 (making pTM820) and

TABLE 1. CA-CA interactions

DNA-binding domain[a]	Interaction with the following activation domain[b]:							
	wt	Control[c]	Q86R	G87V	E90D	E90N	E90K	Q98R
wt	+	−	+	+	+	+	+	+
Q86R	+	−	ND[d]	+	+	+	+	+
G87V	−	−	−	−	−	−	−	−
E90D	+	−	+	+	+	+	+	+
E90K	+	−	+	+/−	+	+	+	+
Q98R	+/−	−	+	+/−	+	+	−	+

[a] CA fused to Gal4 amino acids 1 to 147.
[b] CA fused to Gal4 amino acids 768 to 881.
[c] Vector (Gal4 amino acids 768 to 881).
[d] ND, not determined.

the DNA activation domain vector (pGAD2F) (making pTM822). These clones were expected to encode almost the complete 26-kDa CA domain, since the mass of the predicted domain is 24 kDa. Control colonies which contained only one of these Gal4-Ty3 CA fusion plasmids were the same cream color as colonies containing no plasmids or vector plasmids alone. Colonies which contained both DNA-binding and DNA activation domain fusions with the CA-coding region were dark blue (Table 1). These results indicated that interactions predicted on the basis of the previous observation of particle formation by a truncated Gag3 protein (29) not containing NC residues included or consisted of interactions between isolated CA domains. Changes encoding mutations Q86R, G87V, E90D, E90K, and Q98R were introduced into the pMA424- and pGAD2F-CA fusion clones. A change encoding mutation E90N was introduced only into pGAD2F. The pMA424 and pGAD2F derivatives were transformed into strain GGY1::171 singly and in combination with each other and with the fusions expressing wt CA protein. Plasmids expressing fusions to the Gal4 DNA-binding domain were also introduced together with the Gal4p activation domain vector. None of the transformants containing the combination of plasmids expressing the Gal4p DNA-binding domain–CA fusions together with the Gal4p activation domain alone produced blue colonies when grown on X-Gal-containing medium, indicating that none of the mutant proteins reconstituted Gal4p activity or interacted with the Gal4p activation domain. The G87V and Q98R mutations abolished and reduced activity, respectively, when expressed as the DNA-binding domain fusion together with the wt CA in the activation domain fusion. However, no mutant expressed from the activation domain fusion resulted in a significant reduction in blue colony color when tested against wt CA in the DNA-binding domain fusion. All combinations of G87V expressed from the DNA-binding domain vector with other mutants expressed from the activation domain fusion abolished activity. Combinations of Q98R expressed from the DNA-binding domain fusion with G87V or E90K expressed from the activation domain reduced and abolished activity, respectively. When G87V was expressed from the activation domain, fusion activity was reduced with the E90K and Q98R but not with the Q86R and E90D DNA-binding fusion proteins. The apparent activity of these mutant proteins in at least one fusion context suggested, but did not prove, that these mutations did not produce changes which resulted in complete destabilization of the CA domain per se. Thus, some of the mutations, particularly, G87V and Q98R and potentially E90K, might affect interactions between CA domains. Fusions with the DNA-binding domain were more sensitive to the effects of the mutations, suggesting that CA interactions involving either the MHR or some other region of CA might also be attenuated in this fusion context.

Mutations in the Ty3 MHR domain affect accumulation of processed Ty3 VLP proteins and DNA. Results obtained in the two-hybrid assay suggested that conserved residues in some positions of the MHR might be involved in establishing interactions between CA domains. Alternatively, changes in MHR residues which affected interactions in the two-hybrid system could be changing the structures of other regions required for interaction, and thus these MHR residues might be only indirectly required for CA-CA interactions. However, changes of other conserved residues did not appear to have dramatic effects on the interactions between CA domains, suggesting either that the conserved residue was not required for the interaction between CA domains in this context or that the mutation did not significantly disrupt this function. In order to evaluate the effects of these mutations in the more complex but physiological context of the Ty3 Gag3 and Gag3-Pol3 proteins, the Q86R, G87V, E90D, E90N, E90K, and Q98R mutations were introduced into a Ty3 element carried on the low-copy-number helper plasmid pTM843. Because of the strong effect of the G87V mutation on two-hybrid interactions of CA, a second independent mutation, G87A, was also introduced for testing. These plasmids were transformed into yeast strain AGY-9 in order to test the effects of mutations of conserved residues of the MHR on Ty3 particle formation and DNA synthesis. This yeast strain is an *spt3* mutant and so does not transcribe Ty1, a distantly related retrotransposon. Although it contains endogenous Ty3 elements, these are not expressed at significant levels in the absence of pheromone induction (3). The levels of Ty3 Gag3 proteins in cells expressing wt and MHR mutant Ty3 elements were examined first. Cells were grown as described in Materials and Methods in medium containing galactose as a carbon source in order to induce Ty3 expression. Previous experiments had shown that comparable amounts of Ty3 RNA are produced from the mutant elements under these conditions, so none of these mutations affected Ty3 promoter activity or destabilized the Ty3 RNA (data not shown). Cells were harvested and broken open in the presence of the protease inhibitor aprotinin in whole-cell extract buffer. Protein levels were determined, and equivalent amounts of protein from cells expressing wt and MHR mutant Ty3 elements were analyzed by PAGE and immunoblot analysis. Samples were fractionated in a 12% polyacrylamide–SDS gel, transferred to a Hybond-ECL membrane, and probed with polyclonal antibodies against the 26-kDa CA protein. The results of this immunoblot analysis are shown in Fig. 2A. The wt Ty3 element produced detectable amounts of the 38-kDa (data not shown) and 26-kDa Gag3 proteins. The 38-kDa protein is the Gag3 precursor, and the 26-kDa protein is the mature CA species. In the cells expressing the wt element, the 26-kDa species predominates. Levels of Ty3 Gag3 proteins expressed from three of the seven mutants (the G87V, G87A, and E90K mutants) were greatly reduced, although E90K mutant expression resulted in slightly more of the 38-kDa protein than wt Ty3 expression.

Cells expressing wt and MHR mutant Ty3 elements were examined for reverse transcription of the Ty3 RNA into the full-length extrachromosomal Ty3 DNA. Cells induced for Ty3 expression were broken into nucleic acid extraction buffer as described in Materials and Methods. The total nucleic acid content was determined by fluorometric analysis. Equivalent amounts of DNA were treated with RNase to remove RNA, and the RNase was removed by phenol-chloroform extraction. DNA was fractionated by electrophoresis in 0.8% agarose, transferred to nitrocellulose, and probed with a ^{32}P-labeled,

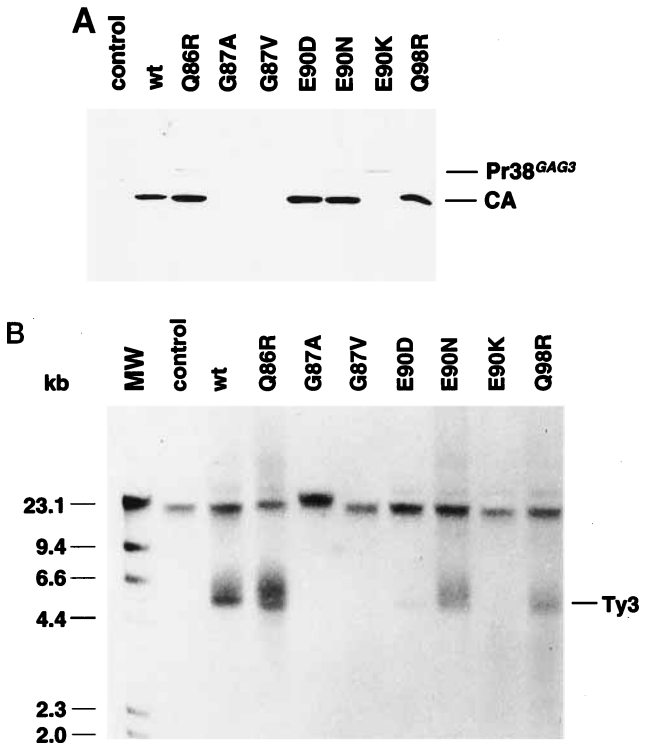
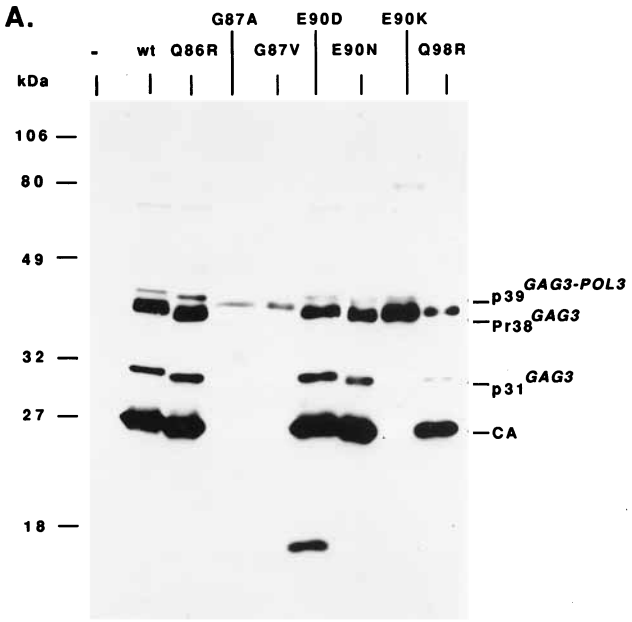


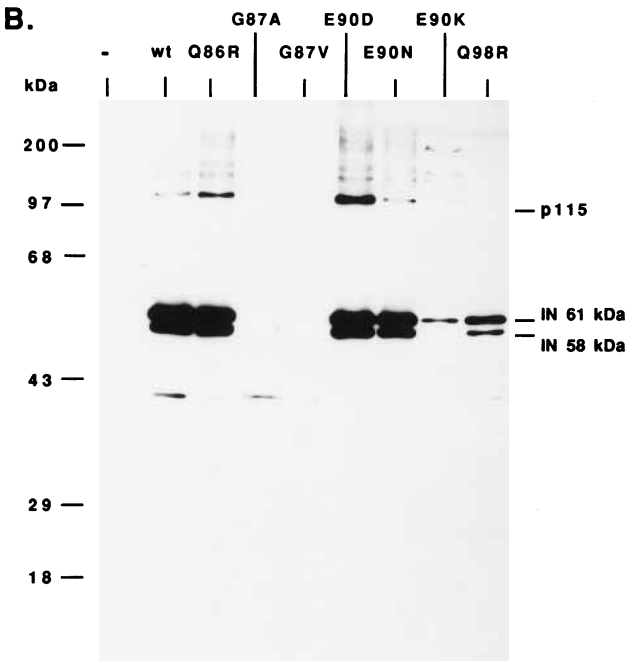
FIG. 2. Effects of mutations in the Ty3 MHR on intracellular levels of Ty3 proteins and DNA. Whole-cell extracts were prepared from AGY-9 cells expressing wt and mutant Ty3 from low-copy-number plasmids under galactose control. (A) Expression of CA-containing domains. Ten micrograms of protein from cells containing wt or mutant Ty3, prepared as described in Materials and Methods, was loaded per lane. The control was protein from cells containing no Ty3. Proteins were fractionated by electrophoresis on a 12% polyacrylamide–SDS gel, and Western blot (immunoblot) analysis was performed as described in Materials and Methods with polyclonal antibodies to CA. (B) Reverse transcription of Ty3 RNA into DNA. AGY-9 cells expressing wt and mutant Ty3 elements were extracted for nucleic acid. Equivalent amounts of DNA were loaded and fractionated by electrophoresis on agarose gels, transferred to Duralon-UV membranes, and probed with a ^{32}P-labeled, Ty3-specific probe. Lane MW, size markers (*Hin*dIII-digested λ DNA). The control was from cells containing no plasmid-borne Ty3.

Ty3-specific probe as described in Materials and Methods. The results of this analysis are shown in Fig. 2B. Inspection of the pattern of the sample from cells expressing the wt Ty3 element showed the presence of a small amount of cellular genomic DNA trapped near the wells, a low-mobility species representing the Ty3-containing plasmid, and a higher-mobility species representing the replicated, extrachromosomal Ty3 element. The levels of plasmid DNA in total nucleic acid samples were similar in cells expressing different mutant Ty3 elements, indicating that the mutations did not affect plasmid maintenance. A comparison of this pattern with that obtained for DNA samples from cells expressing mutant elements showed that the Q86R mutant produced at least wt levels of Ty3 DNA and that the E90N and Q98R mutants produced slightly lower levels of DNA. The E90D mutant produced DNA, but the level was severely reduced compared with that of the wt. The G87V, G87A, and E90K mutants did not produce detectable levels of extrachromosomal Ty3 DNA. Thus, all of the mutants except Q86R had reduced amounts of the product of reverse transcription, and, as was the case for the protein analysis, the G87V, G87A, and E90K mutants were the most severely affected.

The effects on protein processing and DNA production could be explained by either a failure to produce particles, which are assumed to be required for proteolytic processing and reverse transcription, or a loss of protease (PR) and reverse transcriptase (RT) activities caused by incorrect tertiary structure of particles. In order to determine which was the better model to explain the effects of the Ty3 MHR mutations on Ty3 particle morphogenesis, VLPs were prepared from cells induced as described above for Ty3 expression from low-copy-number plasmids bearing wt and mutant Ty3 elements and analyzed for Ty3 protein and DNA. The results of this analysis are shown in Fig. 3. VLPs were harvested and processed as described in Materials and Methods. VLPs were concentrated in the 70%–30% interface of 70%–30%–20% sucrose step gradients, and samples were divided in half. One half was processed for protein analysis, and one half was processed for DNA analysis. For Gag3 and Gag3-Pol3 protein analysis, each sample was further concentrated by centrifugation at 83,000 × *g* for 3 h, and samples representing equivalent amounts of protein were combined with Laemmli sample buffer and fractionated by electrophoresis on 12 and 10% polyacrylamide–SDS gels, respectively. Proteins fractionated on the 12% gels were transferred to Hybond-ECL membranes and probed with primary rabbit polyclonal antibodies against the CA domain (Fig. 3A). Proteins fractionated on the 10% gel were treated similarly but were reacted with primary rabbit polyclonal antibody raised against the Ty3 IN protein (Fig. 3B). Concentration of Ty3 VLPs showed that although some MHR mutants had reduced levels of Ty3 Gag3 proteins (Fig. 2A), some amount of particle protein was present in each case. Since the region of the gradient which contains wt particles is quite broad, even on linear sucrose gradients (24), and also contains particles composed exclusively of truncated Gag3 (CA) protein (29) when this protein is expressed alone, the presence of mutant protein in this fraction does not demonstrate resemblance to wt Ty3 VLPs. Inspection of the patterns produced in the mutants showed that particles were formed that included unprocessed protein observed in whole-cell extracts for the E90K mutant. Although equivalent amounts of particulate fraction protein were analyzed, differences in the levels of mutant proteins were apparent (presumably because of the compensating greater representation of non-Ty3 proteins in cases in which the levels of Ty3 proteins was extremely low). Expression of both mutants containing changes at position 87 resulted in extremely low levels of unprocessed VLP proteins. Expression of the E90K mutant resulted in higher levels of precursor proteins. The mature CA 26-kDa protein was not detectable in these three samples. Some 39-kDa protein, which is derived from the Gag3-Pol3 precursor (36), was detected in the E90K sample but could not be detected in G87V and G87A mutant VLPs. Interestingly, several bands potentially representing Gag3-Pol3 processing intermediates were absent in these three mutants. The Q86R and E90D mutations, which did not appear to significantly affect the relative levels of Gag3 proteins, resulted in some additional low-molecular-mass species which reacted with the anti-CA antibody and which were apparent upon overexposure of the autoradiogram (data not shown). An especially prominent band in the position of a protein with an apparent molecular mass of 16 kDa in the VLPs from the E90D mutant was observed in only one of three preparations (Fig. 3A).

Proteins fractionated on a 10% polyacrylamide gel and analyzed with anti-IN antibodies showed the wt IN species of 61 and 58 kDa (Fig. 3B). Other reactive species were apparent upon overexposure of the autoradiogram (data not shown). The most severely affected mutants, as in the case of the Gag3 species, were ones containing a mutation at position 87. Even





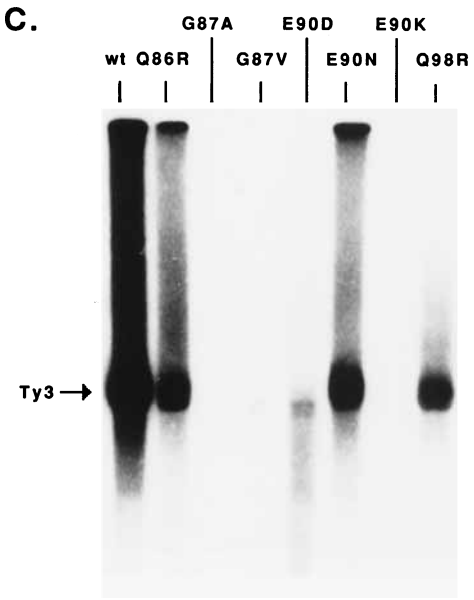


FIG. 3. Effects of mutations in the Ty3 MHR on Ty3 VLP proteins and DNA. The lanes show the pattern of protein or nucleic acids from nontransformed AGY-9 cells and from AGY-9 cells transformed with Ty3 elements encoding wt CA and the Q86R, G87A, G87V, E90D, E90N, E90K, and Q98R CA mutants. (A and B) Fifteen-microgram aliquots of protein concentrated from the 70%–30% interface of sucrose step gradients were fractionated by SDS-PAGE with 12% (A) or 10% (B) polyacrylamide, transferred to Hybond-ECL membranes, and probed with anti-CA (A) or anti-IN (B) antibodies. (C) For analysis of nucleic acids, equal volumes representing 2.7 to 5.1 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 0.8% agarose gels, transferred to Duralon-UV membranes, probed with a ^{32}P-labeled, Ty3-specific DNA, and processed as described in Materials and Methods for Southern analysis.

high-molecular-mass species were not represented in VLPs from these mutants. In both cases, the 61-kDa species was barely detectable in very long exposures. The E90K mutant, which had Gag3 protein but no processed species, displayed reduced levels of IN protein and only the 61-kDa species. In contrast to the mutants with changes at position 87, the E90K mutant did have detectable levels of higher-molecular-mass species. The Q86R, E90D, E90N, and Q98R mutants had patterns of IN protein which contained both 61- and 58-kDa IN species.

Nucleic acid was extracted from VLPs in order to further examine the effects of these mutations on production of Ty3 DNA. Even in these concentrated VLP samples, no DNA was apparent for the G87V, G87A, and E90K mutants. DNA was barely detectable for the E90D mutant; this was surprising because this mutant appeared to produce close to wt levels of processed proteins. As anticipated on the basis of whole-cell extracts, the Q86R, E90N, and Q98R mutants each contained substantial amounts of DNA. Thus, the mutants which failed to process significant amounts of Gag3 protein also failed to process Gag3-Pol3 protein and to produce Ty3 DNA.

TABLE 2. Transposition frequencies of Ty3 mutants

Ty3 element	No. of colonies assayed	Transposition events	
		Mean[a]	% of wt level[b]
wt	708	1.252 (1.120–1.400)	100
Q86R	503	0.158 (0.096–0.221)	12.5 (6.9–19.7)
G87A	405	0.003 (0–0.052)	Background
G87V	494	Background	Background
E90D	340	0.173 (0.099–0.250)	13.8 (7.1–22.3)
E90N	501	0.023 (0–0.071)	Background
E90K	663	Background	Background
Q98R	465	Background	Background

[a] Mean number of transposition events per colony (m) based on a P_0 analysis of the data, where P_0 (the proportion of colonies with zero transpositions) = e^{-m}. For strains that transpose at a frequency above the background level, the range of m that lies within the 95% confidence interval is shown in parentheses.

[b] Relative values of m compared with the wt value. The frequencies of transposition events for strains containing the E90N and G87A mutants were determined to be not significantly different from the background level by chi-square analysis of the data.

Mutations in the Ty3 MHR block Ty3 transposition. MHR mutants were tested in a transposition assay in order to determine the effect of each on intracellular replication. All of the Ty3 MHR mutants tested had transposition frequencies below that of the wt element (Table 2). The most severely affected were mutants with changes G87A, G87V, E90N, E90K, and Q98R. The transposition frequencies of these elements could not be distinguished from the background transposition frequency of cells grown on glucose. Mutants with changes Q86R and E90D had values of between 12 and 14% of the wt value. Thus, even mutations Q86R, E90N, and Q98R, which caused relatively modest defects in protein processing and DNA synthesis, produced significant defects in transposition.

DISCUSSION

Although CA proteins of different retroviruses are not highly conserved overall, they contain a region of about 20 residues of which 6 are conserved. A region of 19 amino acids within the Ty3 CA where five of these six residues are found could be aligned with the retroviral MHR by introducing a single-residue gap into the retroviral sequence. In Ty3 a truncated Gag3 protein containing no NC has been shown to be sufficient for particle formation (29); thus, Ty3 CA is potentially central in the formation of the nucleocapsid particle. In this study, we introduced a total of seven mutations into four positions of the Ty3 MHR to determine the requirement for this motif in Ty3 particle morphogenesis, replication, and integration and to determine how tightly the sequence of two of the six positions is constrained. The effects of the mutations can be sorted into three classes. Mutations at position 87 (G87V and G87A) resulted in severely reduced levels of particles and so must have decreased either particle formation or particle stability. The loss of activity of CA hybrid proteins containing these mutations is consistent with either of these models. Extended exposures of the immunoblots of the G-87 mutants showed the presence of precursor Gag3 species at very low levels. This indicated that protein can be expressed by these mutants. Formally, a lower level of mature species relative to precursor species could have resulted from a reduction in processing caused by a failure to incorporate Gag3-Pol3, a disruption of tertiary structure, or a more rapid turnover of mature compared to precursor species. In other Ty3 mutants, such as PR mutants, precursor species are unstable (29). Poor incorporation of Gag-Pol was noted for HIV-1 mutants with deletions in the MHR (46). Because the G87A change is relatively conservative, our results suggest that in the case of the Ty3 MHR, this position is highly constrained for a glycine residue.

Different phenotypes were produced by the three mutations, E90D, E90N, and E90K, introduced at position 90 of the Ty3 MHR. All three mutants, however, had higher levels of Ty3 CA and IN species than did the mutants with changes at position 87. Two of the mutations, E90D and E90K, caused changes in the patterns of proteins present in the VLP. These could be related to changes in susceptibility to cellular proteases or PR or to changes in PR activity. Not surprisingly, the most drastic change, E90K, produced the most severe mutant phenotype. The level of mature species was greatly decreased for this mutant, even in the particles which were formed. As noted above, this would be consistent with failure to form stable particles or failure to process. The E90K mutant did not produce detectable amounts of Ty3 DNA, which is consistent with the low level of RT activity previously observed in unprocessed particles.

The E90D mutation caused a phenotype intermediate between those caused by the other two mutations introduced at this site. The Gag3 and Gag3-Pol3 protein levels present in the cells approached those in cells expressing wt Ty3. However, in addition to the processed 31-kDa species and the 26-kDa species derived from Gag3, species of anomalous sizes were observed in some preparations. Whether these species resulted from aberrant PR cleavages or preferential cleavage by cellular proteases is not known. The PR processing site is not expected to contain a hydrophilic residue such as aspartate (28). Therefore, if cleavage is by the Ty3 PR, the E90D change may have affected the tertiary structure to present a novel processing site. In addition, however, minor novel bands also appeared in overexposures of the immunoblots of proteins reacted with the anti-IN antibody, suggesting that protease susceptibility was modified at multiple positions. Aberrant processing was not simply a Gag3-specific phenomenon. In contrast to the effect of the E90K mutation, the E90D mutation did not grossly affect the appearance of mature Gag3 and IN species but greatly reduced the level of Ty3 5.4-kb DNA. In the case of retroviruses, reverse transcription is triggered upon infection of the target host cell. For retroviruslike elements, which lack this life cycle step, it is not known whether reverse transcription is triggered by maturation of the particle or whether there are more-specific initiation signals for reverse transcription. It is known that reverse transcription of Ty3 is blocked when host cells are arrested in G_1 (34), so restrictions exist which can be imposed upon reverse transcription in particles which contain predominately mature proteins. It is possible that the E90D mutation affected reverse transcription by perturbing such signals.

The E90N mutant was relatively mildly affected in both Gag3 and Gag3-Pol3 protein processing patterns and produced an amount of DNA approaching that found in cells expressing the wt Ty3. Nevertheless, this mutant exhibited a transposition frequency indistinguishable from that in uninduced cells. Similarly, the Q98R mutation appeared not to grossly affect VLP function prior to and including DNA synthesis but resulted in a transposition frequency which could not be distinguished from the background level. These results, particularly considered together with those for the E90D mutant, which produced less DNA but had a transposition frequency about 14% of that of wt Ty3, argue that these two mutations produced defects primarily in some function subsequent to reverse transcription.

The role of the MHR in retroviruses is not completely understood. Mutations have been introduced into MHR motifs of M-PMV (47), HIV-1 (31, 46), and RSV (9). Some of the mutations introduced into the Ty3 MHR produced effects similar to those produced in retroviruses, while others produced defects which appeared to be qualitatively different. In the case of M-PMV, mutations were introduced into the positions corresponding to 89 (D153N), 90 (E154K), 91 (P155E), 92 (F156C), 94 (D158Y), 95 (F159Y), 97 (V160I), 98 (R162K), and 100 (L163H) in Ty3. Only the E154K mutation corresponds to a mutation used in this study (E90K). The phenotypes of the mutants were of two classes: defective in stable particle assembly and noninfectious. The mutations in M-PMV CA, i.e., E154K, F156C, D158Y, and L163H, blocked processing. As for changes at position 154 of M-PMV, disrupted processing was observed for two of the three changes introduced at position 90 of the Ty3 MHR. The M-PMV mutations D158Y and I164C reduced processing. The D158Y change also created a novel processing site and resulted in the appearance of a protein of 17 kDa. Our results are similar to these results in showing that the sequence of this region is constrained and

that mutations at several positions produce processing irregularities.

In the case of HIV-1, mutations were introduced at two positions analogous to those mutated in our study, Q-86 (Q155N) and E-90 (E159D). The HIV Q155N mutation blocked particle formation. In contrast, mutation of this Q to R in Ty3 did not severely disrupt particle formation and processing, although it blocked transposition. Nevertheless, each of two changes introduced at the neighboring glycine (converting it to alanine or valine) in our study, but not tested in the HIV-1 study, resulted in the loss of all stable particle protein. Three changes (D, N, and K) were introduced into a position also tested in the HIV study (E159D). This HIV mutant did not produce detectable particles. One of the changes (K) in Ty3 blocked formation of wt levels of particles and decreased levels of mature proteins significantly. However, two of the Ty3 mutants were apparently affected primarily at stages after particle assembly.

The phenotypes of RSV mutants having deletions spanning the MHR as well as point mutations within the MHR domain have been investigated by using expression of the *gag* vector pSV.Myr1 in COS-1 cells and of RSV proviral vectors in QT6 quail cells (9). A deletion of 69 amino acids, including the MHR, in the former case resulted in a 50% reduction in release of Gag-derived proteins into the medium in COS-1 cells. In addition, processing was diminished. Some mutations at Q-158, E-162, and L-171 also reduced particle formation, although by less than 50%. These results together with those of earlier studies argued that the MHR was not essential for particle formation. Virion formation in quail cells proved to be much more sensitive to mutations in the MHR. Mutations analogous to those in our study (Q86R, G87A, and E90) were introduced to change the conserved Q-158, G-159, and E-162 residues. In quail cells, changing Q-158 to L, R, or P or changing E-162 to G blocked particle formation. The change of G-159 to A did not have a significant effect even on infectivity. In the case of mutations which resulted in particle formation, the level of RT activity did not suggest a deficiency in the ability to incorporate Gag-Pol despite an abnormal morphological appearance. Thus, it appears that although mutations in this portion of the MHR can affect RSV and Ty3 particle formation, the sensitivities to changes at these two positions differ substantially. The carboxyl-terminal portion of the Ty3 MHR does not contain a conserved R. Mutation of the R in RSV to Q (which occurs at the adjacent position in the Ty3 MHR) did not block assembly but did block formation of infectious particles. Similarly, mutation of the Q to R in Ty3 resulted in a mutant which formed particles and synthesized DNA but was defective in transposition. Thus, the effects of mutations in retrovirus MHRs are a function of the virus and the nature of the mutation. Some of these effects parallel effects of mutations in the Ty3 MHR, while others appear to be qualitatively different.

The CA domain is also required for functions in addition to particle formation which are less well understood. In the case of N- and B-tropic MLVs, resistance in the host cell is determined by the *Fv-1* locus. The step at which this restriction occurs is after replication of viral DNA (27, 48). The differences between N- and B-tropic viruses have been identified by chimeric mapping to include positions 109 and 110 of the CA protein (10, 37). This region lies amino terminal to the MHR (amino acids 142 through 161 in MLV). N- and B-tropic viruses process the ends of replicated DNA (38), and integration from core particles prepared in resistant target cells can occur in vitro (38). It is intriguing that at least the Q86R, E90N, and Q98R mutants were able to produce significant levels of Ty3 DNA but transposed at levels equivalent (E90N and Q98R) or close (Q86R) to the background level. This domain may therefore provide determinants which either protect the virus from host proteases or are essential for development of an appropriate integration complex.

In summary, we have described an MHR in a retroviruslike element for the first time. Assembly and processing of the Ty3 VLPs are perturbed by mutations in this domain. One of these mutations affected the CA-CA interaction in the two-hybrid assay, suggesting that at least a portion of this region is directly involved in CA-CA interaction. However, some conservative changes in the MHR which did not grossly disrupt CA-CA interaction, processing, stability of Ty3 proteins, or production of DNA significantly reduced the level of transposition. These results suggest a role for the MHR at a step after particle formation. There are clear parallels between the phenotypes of our mutants and blocked particle morphogenesis and noninfectious phenotypes of some retrovirus mutants. These similarities prompt us to speculate that the MHR functions are independent of the envelope and of infection functions of retroviruses except as those are recapitulated by the intracellular retroviruslike life cycle.

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