trans-Acting Inhibition of Genomic RNA Dimerization by Rous Sarcoma Virus Matrix Mutants

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The genomic RNA of retroviruses exists within the virion as a noncovalently linked dimer. Previously, we identified a mutant of the viral matrix (MA) protein of Rous sarcoma virus that disrupts viral RNA dimerization. This mutant, Myr1E, is modified at the N terminus of MA by the addition of 10 amino acids from the Src protein, resulting in the production of particles containing monomeric RNA. Dimerization is reestablished by a single amino acid substitution that abolishes myristylation (Myr1E[−]). To distinguish between *cis* and *trans* effects involving Myr1E, additional mutations were generated. In Myr1E.cc and Myr1E[−].cc, different nucleotides were utilized to encode the same protein as Myr1E and Myr1E[−], respectively. The alterations in RNA sequence did not change the properties of the viral mutants. Myr1E.ATG[−] was constructed so that translation began at the *gag* AUG, resulting in synthesis of the wild-type Gag protein but maintenance of the *src* RNA sequence. This mutant had normal infectivity and dimeric RNA, indicating that the *src* sequence did not prevent dimer formation. All of the *src*-containing RNA sequences formed dimers *in vitro*. Examination of MA-green fluorescent protein fusion proteins revealed that the wild-type and mutant MA proteins Myr1E.ATG[−], Myr1E[−], and Myr1E[−].cc had distinctly different patterns of subcellular localization compared with Myr1E and Myr1E.cc MA proteins. This finding suggests that proper localization of the MA protein may be required for RNA dimer formation and infectivity. Taken together, these results provide compelling evidence that the genomic RNA dimerization defect is due to a *trans*-acting effect of the mutant MA proteins.

All retroviruses incorporate two identical copies of their RNA genome into each virion. The genomic RNA molecules are linked near their 5′ ends by noncovalent interactions to form a stable structure possessing ordered secondary and tertiary structure. Although there are multiple contact points throughout the two parallel RNA molecules, the most stable linkage is called the dimer linkage structure. The dimer linkage structure can be visualized by electron microscopy and appears to be a region about 50 nucleotides (nt) in length near the 5′ end of the genome (centered around nucleotide 511 in Rous sarcoma virus [RSV]) (1, 20, 23). Dimerization is required for infectivity, although precisely how it contributes to the replication cycle remains poorly understood. Dimerization is believed to facilitate recombination during reverse transcription by enabling close approximation of the viral RNA molecules, leading to increased genetic diversity and improved viral fitness (15, 16, 26). The dimeric RNA structure has also been implicated in inhibiting the translation of unspliced viral RNA so that genomic RNA is available for packaging; however, there is little experimental evidence in support of this idea (26).

Because the RNA sequences that are important for dimerization overlap those required for RNA incorporation into virus particles, dimerization and packaging were postulated to be functionally associated. In support of this idea, there is evidence that dimerization is required for genomic RNA incorporation in murine leukemia virus and human immunode-

ficiency virus type 1 (HIV-1) (13, 14). Both of these viruses appear to initially package unstable dimers that mature into more thermostable complexes after protease activation (13, 14). However, for RSV, it is less clear whether dimerization occurs prior to packaging. When isolated by conventional methods, rapid-harvest and protease-defective RSV particles contain primarily monomeric RNA, although a small amount of dimeric RNA has been detected (25, 32; T. Cairns and R. Craven, unpublished results). Incubation of rapid-harvest virus results in conversion of monomers to dimers (2, 3). Thus, it is possible that monomers are initially packaged and dimerization begins later in the assembly process or even after budding. In contrast to these reports, Stoltzfus and Snyder found that the RNA from rapid-harvest B77 avian sarcoma virus particles exists as fragile dimers that readily dissociate upon extraction (33). These authors proposed that the dimers undergo stabilization after budding and that this RNA maturation process might be mediated by a viral protein. From the existing data, it has not been possible to determine with certainty whether RSV packages monomers or dimers.

To elucidate the *cis*- and *trans*-acting elements needed for dimer formation, *in vitro* assays for dimerization have been utilized. Studies of this type with RSV have identified sequences within the 5′ region of the genome that allow spontaneous dimer formation in the absence of any viral proteins. Initially, sequences at nt 485 to 530 and 531 to 634 were postulated to initiate dimerization *in vitro* (19). However, later results disputed this finding, instead reporting that an auto-complementary stem-loop structure (L3) between nt 258 and 274 in avian sarcoma-leukosis virus initiates dimer formation *in vitro* via a “kissing complex” formed by Watson-Crick base pairing between complementary bases in opposing loops (12).

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In the latter study, regions downstream of the *gag* initiation codon (nt 400 to 650) were found to be dispensable for in vitro dimerization. The only *trans*-acting factor of avian retroviruses that has a demonstrated role in dimer formation is the nucleocapsid (NC) domain of the Gag protein, which promotes in vitro dimerization at low concentrations of RNA (6, 9, 11, 21). The NC region acts as a nucleic acid chaperone to facilitate maturation of the dimer structure and also catalyzes the annealing of the tRNA primer to the primer binding site (5, 6, 11, 29).

The matrix (MA) protein of RSV was recently implicated as another possible factor involved in genomic RNA dimerization (27). In that study, we found that adding 10 codons of the *v-src* gene as a 5' extension of *gag* allowed normal virus particle release but led to a loss of infectivity. Mutant virus particles were normal in their levels of incorporation of Env, Gag, and Gag-Pol, and the proteolytic processing of these polyproteins appeared normal. However, genomic RNA packaging was mildly reduced (in the range of two- to fourfold) and the RNA isolated from virus particles was in the form of monomers. It seemed most likely that the loss of dimer formation was due to the change in MA rather than to the alteration in the RNA, because a point mutation, which led to the loss of myristylation of the Src sequence (and thus its membrane binding activity), restored RNA dimerization and infectivity (27). However, because the mutation was located within the portion of the genome that contains important regulatory elements for RNA packaging and dimerization, it was important to determine whether *cis*-acting elements could be responsible for the defect. In the present study, we constructed new mutants to distinguish between RNA and protein effects. Here we provide definitive evidence that it is indeed the mutant MA protein (rather than the altered RNA sequence) that affects dimerization. Moreover, our experimental results suggest that the intracellular distribution of MA may be linked to genomic RNA dimerization.

MATERIALS AND METHODS

Viruses and cells. The proviral RSV constructs utilized in these studies all contain the RSV Prague C *gag* gene from pATV8. The wild-type, infectious proviral construct pRC.V8 is a derivative of pBH.RCAN.HiSV (pRCAN) (4, 7). Mutants pRC.Myr1E, pRC.Myr1E- (27), pRC.HB12, and pRC.T14K.B1c (28) have been described previously. The pRCASBP(A)/GFP proviral construct (31), a kind gift from Mark Federspiel, Mayo Foundation, Rochester, Minn. (30), has the gene encoding green fluorescent protein (GFP) in place of the *src* gene. The chemically transformed QT6 quail fibroblast cell line was utilized for all experiments and was maintained as described previously (4, 22).

Site-directed mutagenesis of the *gag* gene. Oligonucleotide-directed mutagenesis was performed as previously described (17, 36). The oligonucleotides utilized were 5'-CCCGGTGGATCAAGCATGG(G/A)AAGTAGTAAGTCAAACCTAAGGACAGCGAAGCCGTCATAAAGGTGAT for *myr1e.cc* and *myr1e-.cc* and 5'-GGTGGCCGGTGGATCAACTTAAGGAAGTAGTAAGTCAAACCTAAGGACAGCATGGAAGCCGTCATAAAG for *myr1e.atg-*. The designation "cc" is an abbreviation for "changed codons." Replicative-form DNAs were digested with *Sst*I (nt 255) and *Hpa*I (nt 2731) and subcloned into pRCASBP(A)/GFP by restriction fragment exchange. A PCR-based strategy was used to make *myr1e.DMB*, using oligonucleotide primers 5'-CTCAGAAGTCGACGAGCTCTACT (USP19.263) and 5'-GGACTAGTGGGACGAAATCACCTTTATGACGGCTT with pRC.Myr1E as the template. The PCR product was digested with *Sst*I and *Spe*I and cloned into pRCAN.ΔMA6S (24). All mutations were confirmed by dideoxy sequencing, and two independent clones of each *gag* allele were used in each assay described below.

Radioimmunoprecipitation. QT6 cells were transfected using the calcium phosphate precipitation method and metabolically labeled with [³⁵S]methionine,

and RSV proteins were immunoprecipitated with a polyclonal anti-RSV serum (34), separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and detected by autoradiography as described previously (28).

Immunoblot analysis. Virus particles harvested from culture supernatants of QT6 cell lines were concentrated by ultracentrifugation and normalized according to reverse transcriptase (RT) activity (as above), and viral proteins were separated by SDS-PAGE and subjected to immunoblotting with anti-RSV and anti-MA sera as described previously (4, 27).

Nondenaturing Northern blotting. Virus particles produced from the stable lines or after transient transfection were collected by ultracentrifugation and normalized for RT content, and RNA was isolated, separated by electrophoresis, and blotted to nylon membranes as described previously (14, 27). The blots were hybridized using an antisense *gag* riboprobe and autoradiography was performed (27).

In vitro dimerization. The sequence from nt 1 to 845 in pRC.V8 was PCR amplified using primers 5'-CAACCGGACGTCGCCATTTGACCATTACACACA and GTCCGGTACCATAGCAGGATGTGCCAAC (P89). The PCR product was digested with *Aat*II and *Asp*718 and transferred into pGEM7Zf(+) (Promega). The resulting plasmid, pGEM.RSVLTR/MA, was used to exchange *Sst*I-*Bsp*EI fragments to introduce the *myr1e*, *myr1e-*, *myr1e.cc*, *myr1e-.cc*, and *myr1e.atg-* *gag* alleles into the pGEM expression vector. The pGEM.RSVLTR.15.4 deletion was made by digesting pGEM.RSVLTR/MA with *Sst*I followed by *Bal* 31 endonuclease digestion to create a 77-bp deletion between nt 219 and 296. All constructs were confirmed by dideoxy sequencing. In vitro-transcribed viral RNAs were made using the Ribomax System (Promega) following linearization of each of the pGEM.RSV vectors described above with *Hind*III. The RNAs were purified using Chromaspin-100 columns (Sigma), and 1.05 μg of RNA was used for each in vitro dimerization reaction following the protocol described by Fosse et al. (12). The products of the reaction were subjected to electrophoresis on a 1.8% Metaphor agarose gel (FMC BioProducts), stained with ethidium bromide, visualized by UV light, and photographed.

RNAse protection assay (RPA). Virus particles were collected and concentrated by ultracentrifugation and analyzed for RT activity, and RNA was isolated by the method of Fu and Rein (14, 27). RT activity was used as an estimate of the relative number of virus particles, and each mutant was normalized to the wild type so that equivalent amounts of RNA per particle were used. Serial 1:2 dilutions of RNA were hybridized to an antisense *gag* riboprobe (approximately 550 bp in length) using the Ambion RPA III kit. The probe had been labeled with [³²P]CTP during in vitro transcription (Promega T7 Riboprobe System). The RNAs were separated on a 5% acrylamide-8 M urea denaturing polyacrylamide gel and analyzed by autoradiography and using a PhosphorImager (Molecular Dynamics).

Virus infectivity assays. QT6 cells were transfected with wild-type or mutant proviral constructs (as above), and growth medium was collected from the cells 1 day after transfection and then once or twice weekly. The medium was pelleted through a 25% sucrose cushion at 100,000 × g for 45 min, resuspended in phosphate-buffered saline, and stored at -60°C. At the end of the collection period, all of the samples were tested for RT activity in triplicate (4).

MA-GFP fusion proteins and confocal microscopy. The pEGFP.N2 plasmid (Clontech) was utilized to make MA-GFP fusion proteins. PCR amplification of the wild-type MA sequence from pRC.V8 was performed using primers USP19.263 and P89. The PCR product and pEGFP.N2 were digested with *Asp*718, filled in with Klenow, and cut with *Sst*I to create pMA-GFP. Mutations *myr1e*, *myr1e-*, *myr1e.cc*, *myr1e-.cc*, and *myr1e.atg-* were cloned into pMA-GFP by restriction fragment exchange utilizing *Sst*I and *Bsp*EI and were confirmed by dideoxy sequencing. The resulting plasmids were used to transfect 35-mm-diameter dishes of QT6 cells for 6 to 12 h using the calcium phosphate method. Approximately 6 to 18 h later, the cells were washed twice in Tris-buffered saline and examined using a Zeiss confocal LSM 10 BioMed microscope.

RESULTS

We previously described a mutation in the RSV MA coding sequence that disturbs viral RNA dimer formation, raising the intriguing possibility that the MA protein may play a role in dimerization. This mutant, Myr1E (Fig. 1), has an extension of the 10-amino-acid Src membrane binding domain at the Gag N terminus. We hypothesized that the additional membrane binding domain (that of Src plus the RSV Gag membrane binding domain) on the Myr1E Gag protein could be respon-

FIG. 1. Schematic diagram of wild-type and mutant MA sequences. The RSV Gag protein is shown at the top, with the MA, p2, p10, CA, NC, and PR domains indicated. Numbers above the protein indicate the amino acid residues for MA and full-length Gag. The N-terminal sequence of wild-type MA is shown below the diagram. In the middle panel, substitutions in the RSV MA sequence are illustrated. The wavy line represents myristic acid attached to the N terminus of the protein. Dashed and dotted lines indicate amino acid residues that are identical to wild type. Myr2/HB12 contains a substitution of basic residues from the HIV-1 M domain (KKKYKLK 28). Myr2/T14K.B1c has a single substitution at position 14 and deletion of residues 74 to 98, as indicated. In the lower panel, N-terminal extension mutants are shown, along with their nucleotide sequences. The boxed sequence (GSSKSKPKD) is derived from the v-Src protein. Blank spaces within the sequence signify a deletion. Nucleotides indicated in bold italic type have been altered from the original *myr1e* sequence. Ovals indicate the initiation sites for protein synthesis. WT, wild type.

sible for inhibiting RNA dimerization (27). However, it was also possible that the dimerization defect was due to changes in RNA-RNA interactions arising from the nucleotides inserted in *myr1e*, and thus changes at the protein level could be irrelevant. To distinguish between these *cis* and *trans* effects, additional MA mutants were examined.

RNA dimerization defects in additional MA mutants. In earlier studies, we described other MA mutants that lacked infectivity despite being competent for particle assembly (28). In mutant Myr2.HB12, a myristic acid addition site was created by an E-to-G change at position 2 and a cluster of basic residues from the HIV-1 MA protein (KKKYKLK) was substituted for RSV MA residues 12 to 18 (Fig. 1). The Myr2.HB12 Gag protein directs particle assembly efficiently and is insensitive to a downstream deletion (residues 74 to 98, designated B1c) within MA that normally inhibits budding (28). This finding suggested that the membrane binding activity of the mutant Gag protein was increased compared to that of the wild type. We also found that a single substitution within MA (T to K at position 14) was sufficient to restore budding to the same downstream deletion (B1c) as long as myristate was present at the N terminus (mutant Myr2.T14K.B1c [Fig. 1]). For both mutants, the virus particles appeared normal with respect to Gag and Gag-Pol content and processing, but viral RNA had not been examined.

We also constructed a new mutant, Myr1E.ΔMB, which contains the Src N-terminal extension and a large, internal deletion within MA (Fig. 1). This mutant has only a single functional membrane binding domain, that of Src, since the RSV membrane binding domain is inactivated by the deletion. If it were the presence of two competing membrane binding domains in Myr1E that interfered with dimerization, then eliminating one of them might restore normal RNA structure.

To determine whether these additional MA mutants had the same RNA dimerization defect as Myr1E, virus particles were collected following transfection of QT6 cells with proviral plasmids and viral RNA was isolated and subjected to Northern blotting under nondenaturing conditions. Hybridization with an antisense *gag* riboprobe revealed that the wild-type virus, RC.V8, contained dimeric RNA, as expected (Fig. 2). However, RNA isolated from mutants Myr2.HB12, Myr2.T14K.B1c, and Myr1E.ΔMB was detectable only in monomer form. Thus, three additional mutations with different types of changes within the MA coding sequence also have impaired genomic RNA dimerization, supporting the idea that the mutant MA proteins rather than the mutations in the viral RNA interfere with dimerization. However, because the 5' end of the genome is known to be the site of viral RNA dimerization, it is conceivable that numerous types of nucleotide changes could perturb RNA-RNA interactions and result in a

FIG. 2. Nondenaturing Northern blot analysis of viral RNA. Viral RNA was extracted from virus particles produced after transfection of QT6 cells with mutant or wild-type (WT) proviral plasmid DNAs. After the RNA was blotted to a nylon membrane, an antisense radiolabeled *gag* riboprobe was used to detect viral RNA. The positions of genomic RNA dimers (D) and monomers (M) are indicated by arrows.

lack of RNA dimer formation. Therefore, it was imperative to determine definitively whether the RNA structure was impaired or whether the mutant MA proteins had an effect on genomic RNA dimer formation. Several new mutants were studied to address this question.

Alterations in the nucleotide sequences of *src*-modified *gag* alleles. Proviral mutants were constructed which altered the RNA coding sequence but maintained the protein sequences of wild-type Gag, Myr1E, and Myr1E- (Fig. 1). In Myr1E.ATG-, the RNA coding sequence for the Src peptide was inserted just upstream of the *gag* AUG; additionally, the initiation codon for Src was changed to UAA to prevent translation from beginning there, and instead translation initiated at the *gag* AUG. Three extra bases (AGC) were inserted immediately preceding the *gag* initiation codon to match the context of the wild-type translation start site in an attempt to maximize translation initiation from this site. In Myr1E.cc and Myr1E-.cc, the RNA sequences were altered but the protein sequences were identical to those of Myr1E and Myr1E-, respectively, except for the addition of a serine residue (amino acid 11) resulting from the extra AGC inserted to correspond to the sequence of Myr1E.ATG- (Fig. 1).

To ensure that the mutations did not interfere with Gag protein synthesis or with budding, transfected QT6 cells were metabolically labeled with [³⁵S]methionine and viral proteins were immunoprecipitated from cell lysates and growth media with polyclonal RSV antiserum. As shown in the left panel of Fig. 3A, the Gag polypeptide (Pr76^{gag}) and cleavage products including capsid (CA), MA, and protease (PR) were detected in cell lysates for the wild type (RC.V8, lane 5) and for each of the mutants (lanes 1 to 4). There was release of mature Gag proteins into the culture medium in each case, indicating efficient virus particle production (Fig. 3A, right panel). Of note, less Pr76^{gag} was detected in the Myr1E and Myr1E.cc lanes after a 3-h labeling period (Fig. 3A) (27), although pulse-labeling for 15 min revealed abundant synthesis of the Src-containing precursor proteins (data not shown). This phenomenon was seen for all of the Src-Gag chimeras we have studied and is due to more efficient plasma membrane targeting and an

FIG. 3. Virus particle production in avian cells. (A) Immunoprecipitation of RSV Gag proteins. Following transient transfection of QT6 cells with proviral DNAs bearing the indicated MA mutants, cells were metabolically radiolabeled and lysed, and Gag proteins were immunoprecipitated with polyclonal anti-RSV serum. The full-length Gag protein (Pr76^{gag}), CA, MA, and PR cleavage products are indicated at the left. (B) Immunoblot analysis. Virus particles were concentrated by sedimentation through a sucrose cushion and analyzed by Western blotting using a combination of anti-RSV and anti-MA serum to detect processed p23 (MA + p2) and p19 (MA) proteins, as shown by brackets. WT, wild type.

enhanced rate of budding associated with the Src membrane binding domain (references 27, 35, and 36 and data not shown).

Because wild-type, Myr1E, and Myr1E- mature MA proteins have distinctive migration patterns by SDS-PAGE analysis (27), we tested the new mutants to determine whether their MA proteins appeared as predicted. Particles were collected for 48 h posttransfection, pelleted through a sucrose cushion, and detected by immunoblotting using a combination of polyclonal anti-RSV and anti-MA sera (27). Myr1E.ATG- produced a doublet that was identical in appearance to wild-type MA, as expected (Fig. 3B, lanes 6 and 7). The upper band of the doublet is due to phosphorylation of a subpopulation of

FIG. 4. Genomic RNA analysis. Genomic RNAs isolated from virus particles produced by transfection of QT6 cells with the indicated mutant and wild-type (WT) DNAs were subjected to electrophoresis under nondenaturing conditions as described in the legend to Fig. 2. The positions of dimers (D) and monomers (M) are shown on the left.

MA molecules that causes it to migrate more slowly in the gel (10, 24a). The Myr1E.cc and Myr1E MA proteins appeared as a single band, probably due to the increased positive charge of the Src peptide, and phosphorylation was unaffected, as shown before (lanes 2 and 3) (28). The Myr1E-.cc and Myr1E- MA bands were also identical and migrated as a doublet, but they migrated more slowly in the gel than did the wild-type bands, as we previously demonstrated for Myr1E- (lanes 4 and 5) (27). Thus, it appears that all of the Gag proteins are properly synthesized and the newly constructed mutants are assembly competent.

Dimerization state of the viral RNA. To determine whether the *src* nucleotide insertion upstream of *gag* was responsible for disrupting genomic RNA dimerization, viral RNAs from particles synthesized by each mutant were analyzed by nondenaturing Northern blot analysis as previously described (13, 27). If the *src* mutation had a *cis*-acting effect, we would expect that mutant Myr1E.ATG-, which has the *src* nucleotide sequence but encodes a wild-type Gag protein, would be defective in RNA dimer formation. However, this was not the case (Fig. 4). Despite the presence of the *src* sequence, Myr1E.ATG- particles contained dimeric RNA just like wild-type particles. Further support for a protein effect was obtained from examining Myr1E.cc and Myr1E-.cc. Both of these constructs gave the same results as their unmodified counterparts. That is, Myr1E.cc, like Myr1E, produced particles containing monomers, and Myr1E-.cc, like Myr1E-, produced dimers. Therefore, these changes in primary nucleotide sequence are not critical for dimer formation, and the *src* RNA sequence does not prohibit RNA dimerization in vivo.

RNA dimerization in vitro. Although it is clear that viral RNAs isolated from Myr1E and Myr1E.cc do not dimerize, we wondered whether these RNAs could form dimers under in vitro conditions. If not, it would be likely that a *cis*-acting element prevents the RNAs from forming a stable secondary structure. However, if all of the viral RNAs form dimers in vitro, there must be a factor other than the RNA sequence (e.g., MA) that is involved in the defect seen in Myr1E. We generated subviral RNAs (nt 1 to 848) for the wild type and for each mutant by in vitro transcription. Purified RNAs were denatured by heating to 90°C and then incubated at 20°C

FIG. 5. In vitro dimerization assay. In vitro-transcribed viral RNA was denatured (90°C) or incubated at 20 or 50°C and separated by native agarose gel electrophoresis as described in Materials and Methods. RNA molecular size markers (in kilobases), are indicated on the left. The positions of RNA dimers (D) and monomers (M) are indicated by arrows to the right of the gel. WT, wild type.

(monomer conditions) or 50°C (dimer conditions), following an established protocol (12). As a negative control for dimerization, we deleted nt 219 to 296 (pGEM.RSV.15-4), a region shown to be essential for in vitro dimerization (12). This mutation abrogated dimer formation (Fig. 5, upper panel), as expected. Wild-type viral RNA formed a mixture of monomers and dimers at 20°C and was fully dimerized at 50°C (Fig. 5, lower panel), as shown by others (12).

RNA derived from *myr1e.ΔMB* contains the *src* sequence followed by deletion of nt 410 to 675 in the MA coding sequence. Lear et al. (19) showed that nt 485 to 634 were important for in vitro dimerization, although Fosse et al. (12) showed the same region to be dispensable. In our experiments, deletion of these nucleotides in *myr1e.ΔMB* did not impair in vitro dimerization, although the RNA did migrate faster in the gel due to the large deletion (Fig. 5, lower panel).

All of the remaining *src*-containing viral RNAs also formed exclusively dimers at 50°C and showed partial dimerization at 20°C (Fig. 5). Even RNAs having the *myr1e* and *myr1e.cc* mutations, which are not dimeric in virus particles, efficiently dimerized in vitro. These results directly show that the *src* sequence does not preclude viral RNA dimerization. However, because the *src* mutation lies within the region of the genome that is also involved in RNA packaging, we needed to examine the levels of genome incorporation for each of the *src* extension mutants.

Genomic RNA incorporation into virus particles. In our earlier work, we found that Myr1E, which contains only monomers, had a mild decrease in genome incorporation, at the level of two- to fourfold when measured by Northern slot blot analysis (27). To determine how efficiently genomic RNA was packaged for each of the MA mutants, RPA was performed as a sensitive and quantitative method. The amount of RNA used

FIG. 6. Levels of viral RNA packaging. (A) RPA. Following transfection of QT6 cells with proviral constructs, RNA was isolated from virus particles. The amount of RNA used for each mutant was normalized compared to the wild type (WT) using RT activity measured prior to RNA extraction. Twofold dilutions of RNA were used for each mutant, as indicated by the triangular box above each set of lanes. After hybridization of the RNA with a 550-nt ³²P-radiolabeled antisense riboprobe and digestion of the unprotected fragment with RNase, the RNA was separated by electrophoresis and the amount of radioactivity present in the protected band was quantified using PhosphorImager analysis. (B) Relative packaging efficiencies. The results of replicate RPA experiments were obtained as described in panel A, and the mean for each mutant is shown (two to four independent experiments were performed for each RNA sample). The packaging efficiency for the wild type was assigned a value of 1.0, and the mutants are expressed as a ratio compared to the wild-type value.

for each mutant was normalized according to RT activity measured from virus particles prior to RNA extraction (as described in Materials and Methods). As shown in Fig. 6A, serial twofold dilutions of each genomic RNA sample could be detected and the assay was performed in the linear range. Wild-type virus, Myr1E⁻.cc, and Myr1E.ATG⁻, all of which contain dimeric RNA, packaged similar amounts of RNA per virus particle. In contrast, there was a mild decrease in RNA packaging for Myr1E.cc. When we performed RPA using a riboprobe designed to differentiate between spliced and unspliced viral RNAs, we found that the intracellular ratio of unspliced to spliced RNA was about the same for Myr1E, Myr1E⁻, and wild type; furthermore, only unspliced genomic RNA was detected within the mutant and wild-type virus particles (data not shown).

The results of replicate RPA experiments were combined and plotted in graphic form in Fig. 6B. The amount of viral RNA packaged per virion for the wild type was assigned a value of 1.0, or 100% packaging efficiency. For Myr1E⁻, Myr1E⁻.cc, and Myr1E.ATG⁻, the amount of RNA per particle was nearly the same as for the wild type (76, 98, and 90% of the wild-type level, respectively), while for Myr1E.cc and Myr1E, the amounts were reduced to 45 and 40% of the wild-type level, respectively. Thus, both mutants that package monomeric RNA have about half as much genomic RNA as normal. An attractive explanation for this finding is that only a single viral RNA molecule is contained within each virus particle for Myr1E and Myr1E.cc. If so, then monomers are very efficiently packaged into the mutant virions and it is unlikely that the dimeric structure is required for viral RNA incorpo-

ration. However, we cannot rule out the possibility that half of the particles contain two monomeric viral RNA molecules while the other half contain none.

Infectivity of the *src*-containing proviruses. To determine whether the infectivity of the new mutants corresponded to our previous observations for Myr1E and Myr1E⁻, replication in avian cell culture was assessed. Proviral DNAs were transfected into QT6 cells and virus particles were collected at 1 day posttransfection and then once or twice weekly. At the end of the collection period, RT activity was measured for all of the samples. The mean RT activity from two independent experiments is shown in Fig. 7. The time points of particle collection varied somewhat between experiments, so similar time points were combined. On day 1 posttransfection, virus particles were released into the media for all of the constructs, as expected. The wild-type virus and Myr1E.ATG⁻ (both of which encode the wild-type Gag protein) quickly spread throughout the culture, and high levels of virus production continued for the duration of the experiment. RT activity for Myr1E⁻ and Myr1E⁻.cc increased over time but did not reach full wild-type levels. Thus, Myr1E⁻ and Myr1E⁻.cc are both infectious but have a mildly reduced ability to replicate, with Myr1E⁻.cc being more severely affected. As anticipated, Myr1E and Myr1E.cc were not infectious and their levels of RT activity decreased to nearly background after the initial burst of particles produced posttransfection (compare with the mock-infected sample [Fig. 7]). Thus, the *src* nucleotide sequence in Myr1E.ATG⁻ did not impair infectivity and the differences in codon usage between Myr1E⁻ and Myr1E⁻.cc had minor effects on their overall patterns of infectivity. The infectivity defects of Myr1E and Myr1E.cc are due to the change in MA, not the RNA sequence.

Localization of mutant and wild-type MA proteins. We wondered whether the mutant MA proteins might have different

FIG. 7. Infectivity in avian cells. QT6 cells were transfected with proviral constructs expressing the wild-type or mutant sequences and passaged every 3 to 4 days. Medium samples were collected once or twice weekly, and virus particles were concentrated by ultracentrifugation and stored at -80°C . At the end of the collection period, RT assays were performed in triplicate for each sample. The mean RT activity from two independent experiments is shown in counts per minute (cpm) on a logarithmic scale.

locations within the cell owing to their different membrane-targeting signals. If so, this difference might yield insight into the mechanism by which dimerization is impaired by the MA mutants. Wild-type and mutant MA-GFP fusion proteins were expressed in QT6 cells and examined by confocal microscopy. Representative images are shown in Fig. 8. Expression of GFP alone was diffuse throughout the cytoplasm and nucleus, without any predominant pattern (Fig. 8A). In contrast, the wild-

type MA-GFP fusion protein was present in both compartments but there was an enhanced concentration within the nucleus (Fig. 8B). The same intracellular distribution was observed for Myr1E.ATG- GFP and Myr1E-.cc GFP (Fig. 8C and D, respectively). However, expression of Myr1E GFP and Myr1E.cc GFP proteins, both of which contain an active Src membrane binding domain, revealed that these proteins were very efficiently targeted to the plasma membrane (Fig. 8E and

FIG. 8. Subcellular localization of wild-type and mutant MA-GFP fusion proteins. QT6 cells transfected with plasmid DNAs expressing wild-type or mutant MA-GFP fusion proteins were examined by fluorescent confocal microscopy 6 to 18 h posttransfection. Representative images are shown for each of the indicated proteins in panels A through F.

F, respectively). Thus, the mutants that contain dimeric RNA and are infectious localize to the nuclear and cytoplasmic compartments while noninfectious mutants having monomeric RNA are strongly targeted to the plasma membrane. These striking differences in subcellular localization suggest that targeting MA too efficiently to the plasma membrane interferes with the dimerization process.

DISCUSSION

The involvement of *trans*-acting factors other than NC in retroviral RNA dimerization has not been previously described. In this study, we have characterized several mutants of the MA protein that are noninfectious and are unable to form stable dimeric RNA complexes within virus particles. Our data convincingly show that the defect in RNA dimer formation is not due to problems with RNA-RNA interactions arising from changes in nucleotide sequence but results from changes in the MA protein sequence itself. Although we have identified four distinct MA mutants with dimerization defects, the Myr1E mutant has been studied most thoroughly. This mutant has a functional Src membrane-targeting domain attached to its N terminus, leading to improper subcellular localization of the MA protein. Taken together, our data suggest that mistargeting of MA (in the case of Myr1E) results in the failure of viral RNA dimerization.

How might the MA protein sequence influence RNA dimer formation? We can envision several possible scenarios. If viral RNA molecules begin to dimerize early in assembly, then sending the assembling RNA-Gag complex along the wrong pathway—perhaps by way of an improper membrane-targeting signal, such as that of Src—might prevent necessary RNA-RNA interactions from occurring. During the normal assembly process, the wild-type Gag protein moves along an intracellular pathway to a specific plasma membrane site that includes the necessary conditions for dimerization. However, since the mutant Myr1E Gag protein has altered membrane-targeting properties, it might direct the RNA-protein complex to the wrong cellular localization or to the wrong location on the membrane, thereby disrupting dimerization. In this model, any changes that perturb the normal trafficking properties of the MA sequence would be expected to affect genomic RNA dimerization as well.

Alternatively, if dimerization does not occur until after budding in RSV, it is possible that the MA mutants influence the dimerization process within the virus particle. In this case, the Src membrane binding domain of Myr1E might associate too tightly with the viral membrane. This distortion of the usual MA-membrane interaction could disrupt interactions between MA and other structural proteins (CA or NC, for example), ultimately affecting the structure of the virion core and the integrity of the RNA located within it. These structural changes could either prevent dimerization from occurring or allow only very unstable dimers to form, perhaps by interfering with dimer maturation (14). This model does not require any direct contact between the MA protein and the viral genomic RNA, although how MA might interact with components of the core is unknown for RSV. It is important to keep in mind that RSV MA does have RNA binding activity (31), and so a

role in promoting dimer formation or stability within the virus particle remains a possibility.

A third possibility is that the MA protein serves to enhance dimer formation in an indirect way. For example, MA might promote the stability of newly synthesized genomic RNA, allowing dimerization to be more efficient. MA could target the Gag-RNA complex to an environment within the cell that protects the RNA from degradation. Recently it was reported that HIV-1 MA appears to play a role in enhancing RNA stability (18), and perhaps RSV MA plays a similar role. Alternatively, MA might interact with another cellular or viral factor (e.g., NC), which in turn promotes RNA dimerization. Additionally, it has been proposed that HIV-1 MA might affect RNA transport out of the nucleus, based on studies of a mutant displaying mislocalization of its viral RNA (8). This MA mutant also has a defect in genomic RNA dimerization, suggesting to us that there may be a link between RNA localization and dimerization. Thus, a potential role for MA in viral RNA dimerization and/or transport of the Gag-RNA complex might be a common feature among retroviruses.

We also found that the Myr1E mutants that contain monomeric RNA package about half as much unspliced viral RNA per particle as wild-type viruses do (Fig. 6). It is feasible that monomers are efficiently packaged into these virus particles. Therefore, the dimer linkage structure might not be required for RNA packaging, at least in RSV. In support of this idea, others have shown that rapid-harvest and immature viral RNA from RSV is largely monomeric (2, 3, 25, 32). However, we still must consider the possibility that some Myr1E particles contain unstable dimers while others contain no viral RNA at all. Future experiments will address this question.

Other questions that remain regarding viral RNA dimerization include when dimerization occurs, early or late in the assembly process. Also, it is not known if passage through a specific cellular compartment or a specific membrane site is required for dimerization *in vivo*. While we now have evidence that both the MA and NC proteins of RSV have significant effects on RNA dimerization, the molecular mechanisms of their actions need to be further investigated. Finally, it remains to be shown whether additional viral or cellular factors participate in the dimerization process.

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