Packaging of host mY RNAs by murine leukemia virus may occur early in Y RNA biogenesis

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Running Title: Host mY RNAs in murine leukemia virus

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ABSTRACT

Moloney murine leukemia virus (MLV) selectively encapsidates host mY1 and mY3 RNAs. These noncoding RNA polymerase III transcripts are normally complexed with the Ro60 and La proteins, which are autoantigens associated with rheumatic disease that function in RNA biogenesis and quality control. Here, MLV replication and mY RNA packaging were analyzed using Ro60 knockout embryonic fibroblasts, which contain only ~3% as much mY RNA as wild type cells. Virus spread at the same rate in wild type and Ro knockout cells. Surprisingly, MLV virions shed by Ro60 knockout cells continued to package high levels of mY1 and mY3 (about 2 copies of each) like those from wild type cells, even though mY RNAs were barely detectable within producer cells. As a result, for MLV produced in Ro60 knockout cells, encapsidation selectivity from among all cell RNAs was even higher for mY RNAs than for the viral genome. Whereas mY RNAs are largely cytoplasmic in wild type cells, fractionating knockout cells revealed that the residual mY RNAs were relatively abundant in nuclei, likely reflecting that most mY RNAs were degraded shortly after transcription in the absence of Ro60. Together these data suggest that these small labile host RNAs may be recruited at a very early stage of their biogenesis, and may indicate an intersection of retroviral assembly and RNA quality control pathways.
INTRODUCTION

Retroviruses are ribonucleoprotein (RNP) complexes that assemble at host cells’ plasma membranes. Their predominant RNA component is the unspliced retroviral genome, but virions also contain a number of host cell noncoding RNAs (3, 38, 45). Subsets of these RNAs are over-represented in virions relative to their abundance in host cells (38). Other than the primer tRNA, which anneals to the primer binding site on viral genomic RNA (gRNA) and initiates minus-strand DNA synthesis, the manner of recruitment of these RNAs and whether or not they function in retrovirus biology is unknown (5, 29, 30). Virion assembly does not require viral gRNA, but RNA of some sort is required for assembly, thus suggesting host RNA can serve this role (27, 33). Although prevailing notions suggest nonspecific RNA interactions drive retrovirus assembly (20), the concentration within virions of particular subsets of host cell noncoding RNAs suggests that their recruitment may have functional significance for viral assembly or other replication processes.

Among the most highly recruited noncoding cellular RNAs in MLV are mY1 and mY3 (38). These RNAs are enriched in MLV particles to a similar degree as the highly packaged 7SL RNA (7S or SRP RNA) (38). Packaging of 7SL RNA, the scaffolding RNA of host cell signal recognition particles, is observed for a number of retroviruses including Rous sarcoma virus (RSV) (4), MLV (11, 38, 41) and HIV-1 (39). In MLV, 7SL is present at three- to four-fold molar excess to gRNA, and therefore packaged at approximately six to eight copies per virion (38). HIV-1 particles contain roughly 10 to 14 molecules of 7SL per gRNA dimer (39). Like MLV, HIV-1 also packages at least some Y RNAs, albeit at a lower level of enrichment than 7SL (1, 25, 57).
mY1 and mY3 are host RNA polymerase III transcripts of ~100 nucleotides. Both these mouse RNAs fold into similar structures consisting of 5’ and 3’ ends joined in a base-paired stem surrounding an internal, largely single-stranded, loop (8). Within cells, most mY RNAs are complexed with the host cell protein Ro60, which appears to function in quality control of misfolded noncoding RNAs (7, 36, 44). Structural analysis suggests Y RNA binding may inhibit Ro60 access to misfolded RNAs, as Ro60 binding sites for Y RNAs partially overlap those for misfolded RNAs (18, 54, 62). In agreement with this hypothesis, a bacterial Y RNA inhibits the function of its Ro orthologue in 23S rRNA maturation (9). It has been suggested that Y RNA binding might sequester Ro60 in the cytoplasm, thereby preventing Ro60’s interaction with nascent nuclear transcripts (36, 42). Consistent with this view, the mouse Ro protein was recently shown to contain a signal for nuclear accumulation that is masked by Y RNA binding (50).

Here, we demonstrate that mY1 and mY3 are recruited into MLV virions without the cellular protein Ro60. Ro60 is necessary for the cellular stability of mY1 and mY3, and therefore Ro60 knockout cells contain only very low levels of these RNAs. Surprisingly, MLV packaged high levels of mY1 and mY3 when it replicated in Ro60 knockout cells. These findings demonstrate a remarkable degree of selectivity for mY RNA encapsidation into MLV particles and suggest mY RNAs are recruited for MLV packaging from a very early stage in their biogenesis.
MATERIALS AND METHODS

Cells and virus. NIH/3T3 and derivative cell lines were maintained in Dulbecco’s modified Eagle medium (DMEM; Invitrogen) supplemented with 10% bovine serum (Invitrogen). Wild type mouse embryonic fibroblasts (MEFs) and Ro60 -/- MEFs were maintained in Dulbecco’s modified Eagle medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (Gemini). Wild-type and Ro60 -/- MEFs were prepared by backcrossing 129/Sv x C57BL/6 Ro -/- mice (63) with C57BL/6 mice for six successive generations (50). Embryonic fibroblasts were prepared and immortalized by repeated passage (58). Wild-type MLV particles were obtained by collecting supernatants from 70% to 100% confluent NIH/3T3 cells, wild type MEFs, and Ro60 -/- MEFs chronically infected with wild type MLV at 8-16 hour intervals.

Plasmids. All riboprobe templates were derivatives of pBSII SK(+) (Stratagene). pEG604-1 was constructed with synthetic oligos for 95nt of mY1 (nts. 1-95) with SalI and EcoRI sticky ends and 65nt of mouse 7SL RNA (nts. 125-189) with PstI and NotI sticky ends. The insert in pEG467-10, which was generated by PCR and subcloned into the EcoRV site, included complementary portions to both the MLV 5’ untranslated region (nts. 55-214) and 100nt of 7SL RNA (38).

Viral and cellular RNA extraction. All supernatants were filtered using 0.2µm MCE syringe filters (Fisher Scientific), and stored at -70 ºC prior to use. Virus was concentrated at 4 ºC by centrifugation at 25,000 rpm for 90 min using the AH629 rotor in a Sorvall discovery ultracentrifuge. Viral pellets were resuspended in TRIzol (Invitrogen), and RNA extracted following the manufacturer’s instructions. RNA from chronically infected cells was also
extracted with TRIzol. Samples were resuspended in either DEPC-treated ddH₂O or TENS (10 mM Tris [pH 8.0], 1 mM EDTA, 1% SDS, 100 mM NaCl).

**Northern blots.** Northern blots were used both to visualize RNAs and as controls to normalize protein samples for western blotting. Hybridization probes were oligonucleotides complementary to the RNAs of interest, 5' end labeled using γ-³²P ATP (Perkin-Elmer) and T4 polynucleotide kinase (NEB). Labeled oligonucleotides were separated from unincorporated nucleotides on G-25 sephadex columns (Roche). Oligonucleotides included 5’-

TGCTCCGTTCCGACCTGGGCCGGTTACCCCCTTCTTT-3’ for 7SL; 5’-

CTGACT GTGAACAATCAATTGAGATAA CTACACTAC-3’ for mY1; 5’-

TAAC TGGT GTGATCCAATAGTTGTA AACC ACTAC TC-3’ for mY3; 5’-

CGT GTCAT CACTTGCGCAC GGGGACTGCTA ATCTT CTTGTA -3’ for U6; 5’-

GAGTCCACGCTCTACAAACTGAGCTAGCTG-3’ for tRNA⁰; and 5’-

TGCTCCGTTCCGACCTGGGAAAAACTGACTGTGAACAATCAATTGAGATAA CTACACTAC-3’ as the chimeric 7SL-mY1 probe. Viral and cellular RNAs were separated by 5% polyacrylamide-8M urea gel electrophoresis in 1X TBE. For high resolution denaturing northerns, viral and cellular RNAs were separated on 8% polyacrylamide-8M urea gels (0.4 mm thickness) in 1X TBE using glass plates pre-treated with Sigmacote® (Sigma) to facilitate separation from the glass plates prior to transfer. RNAs were subsequently transferred by electroblotting to Zeta-probe GT Nylon membranes (Bio-Rad) in 0.5X TBE. Membranes were air dried, UV crosslinked (Stratalinker; Stratagene), and prehybridized at 45°C in 6X SSC-5X Denhardt’s solution-0.5% SDS-0.025M sodium phosphate-625µg/ml denatured salmon sperm DNA. Oligonucleotide probes were denatured at 85°C for 5 minutes before adding to the membranes, and hybridization was at 45°C. Blots were washed under the following conditions:
Figures 2A and 2B were washed first in 2X SSC-0.1% SDS at 25 °C, and then in 0.5X SSC-0.1% SDS at 25 °C; Figure 3A was washed first in 2X SSC-0.1% SDS at 25 °C, and then in 0.33X SSC-0.1% SDS at 55 °C; Figures 4A, 4B, 5A, and 5B were washed first in 2X SSC-0.1% SDS at 52 °C, and then in 0.33X SSC-0.1% SDS at 52 °C. Damp blots were wrapped in plastic wrap and exposed to phosphorimager screens and/or film. For re-probing, blots were stripped by at least 2 washes in 0.1% SDS at 80 °C, then prehybridized and probed as above.

Ribonuclease protection assays. To detect mY1 and 7SL, pEG604-1 was linearized with XhoI and transcribed with T3 RNA polymerase (Promega) and [α-32P] rCTP to create a 217 nt transcript which protected 95 nt of mY1 and 65 nt of 7SL RNA. To detect 7SL and MLV genomic RNA, pEG467-10 was linearized with HindIII and transcribed with T3 RNA polymerase to create a 364 nt transcript which protected 160 nt of MLV genomic RNA and 100 nt of 7SL RNA. Previously described RPA approaches (38) were modified by extending hybridization times to 16h and by digesting with a 5-fold excess of RNase T1 (Applied Biosystems). Bands were quantified by PhosphorImager analysis using a Typhoon for detection and ImageQuant TL for analysis. Bands were adjusted for the number of radiolabelled Cs incorporated. In the case of Figure 3 data, product bands in two separate RPAs like the one represented in Figure 3B were corrected for the 18 Cs in the mY1 protected fragment and 25 for 7SL, then multiplied by 7.75 7SL RNAs per virion as determined by two separate RPAs like that in Figure 3C. The product bands in Figure 3C were corrected for 53 labeled C residues in the fragment protected by MLV gRNA and the 35 Cs the 7SL protected fragment. These data were consistent with direct MLV gRNA per mY1 quantification by RPAs performed in triplicate (data not shown).
**Exogenous RT assay.** Media supernatants were harvested, filtered through 0.2 µM MCE syringe filters (Fisher Scientific) and stored at -70 ºC. RT assays were based on a protocol by (21) as described previously (56). Briefly, 3 µl of viral supernatant was incubated with 12 µl of a 1.2x solution (60 mM Tris [pH 8.3], 24 mM dithiothreitol, 0.7 mM MnCl$_2$, 75 mM NaCl, 0.06% NP-40, 6 µg/ml oligo(dT), 12 µg/ml poly(rA), 10 µCi/ml [α-32P]TTP at 3 Ci/mmol) at 37ºC for 2 h. Following incubation, 3 µl of reaction mix was spotted onto DEAE paper, dried, washed with 2xSSC followed by 95% ethanol, and once again dried. Spots were quantified by PhosphorImager analysis.

**Cellular fractionation.** Wild type and Ro60 -/- MEFs were fractionated using a modification of the procedure of Siomi et al. (53). Briefly, nuclear and cytoplasmic fractions were obtained by washing adherent cells twice with ice cold 1x phosphate buffered saline (PBS) followed by one wash with buffer RSB100 (10 mM Tris-HCl pH 7.4, 2.5 mM MgCl$_2$, and 100 mM NaCl). Adherent cells were permeabilized by incubation with 4 mls RSB100 supplemented with .01% (w/v) digitonin (Sigma) on ice for 5 min. The RSB100 buffer-digitonin mix was removed from the adherent cells and spun at 200 x g for 5 min to pellet residual cell debris. The RSB100 buffer supernatant served as the cytoplasmic fraction, and RNA was extracted from this aqueous mix with TRIzol LS (Invitrogen) according to the manufacturer’s recommended protocol. The remaining nuclei were washed once with ice cold PBS before the addition of 1 ml of TRIzol (Invitrogen) and RNA extraction according to the manufacturer’s protocol.
RESULTS

**MLV replication is unaltered in Ro60 -/- cells.** Ro RNP RNAs mY1 and mY3 are among the most highly enriched host RNAs in MLV particles (38). Within cells, most Y RNAs exist in RNPs containing the Ro60 cellular protein, and most Ro60 protein is associated with Y RNAs (40, 61). When Y1 RNA levels were used to normalize parallel viral and cellular protein extracts, the Ro60 protein in virus was below the limit of detection by western blot (37). To test the inference that Y RNAs were not recruited as parts of Ro RNPs, the kinetics of viral spread and Y RNA recruitment were analyzed in Ro60 knockout cells.

Ro60 binding is necessary for the stable accumulation of Y RNAs (6, 28, 63). Accordingly, embryonic fibroblasts prepared from Ro knockout mice contain only very low levels of mY1 and mY3 (50). Thus, studying MLV infectivity in Ro60 knockout cells allowed an examination of the effects of limiting mY RNA availability on viral replication, as well as possible roles of Ro60 itself. Ro60 -/- mouse embryonic fibroblasts (MEFs) and isogenic wild type cells were infected with MLV, and infected and control uninfected cells were serially passaged. Culture media were collected and assayed for reverse transcriptase (RT) activity at various time points post-infection to monitor virus spread (Figure 1). Control uninfected cells retained only background levels of RT activity throughout the time course. For media samples from wild type and Ro60 -/- infected MEFs, the levels of RT activity rose above background on the same day (day 7 in Figure 1). Thereafter, the kinetics of virus spread in infected wild type and Ro60 -/- cells remained approximately equal, with infected Ro60 -/- supernatants containing levels of RT activity that differed by less than 2 fold from those of infected wild type MEFs (not shown). Thus, Ro60 was dispensable for infectious MLV particle assembly and infectivity.
MLV virions from both wild type and Ro60 -/- MEFs package high levels of mY1 and mY3. Because MLV replicated normally in cells containing very low levels of mY RNAs, it initially seemed likely that virions shed from Ro knockout cells would contain correspondingly low levels of mY RNAs. To address directly whether or not reduced mY RNA levels in Ro60 knockout cells led to diminished mY RNA recruitment by MLV, mY1 and mY3 in viral supernatants shed by wild type and Ro60 -/- MEFs were compared to their intracellular levels by northern blot (Figure 2). Quantifying virus by RT assay and northern blotting confirmed previous findings that the host cell non-coding RNA 7SL is enriched in MLV in proportion to viral proteins (38). Thus, 7SL served as a loading control. Consistent with previous studies (7, 50), both infected and uninfected Ro60 -/- MEFs contained 30-fold less mY1 and mY3 than wild type cells (Figure 2A and B, lanes 1-4). Surprisingly, despite the very low levels of mY RNAs in R060 -/- cells, MLV virions produced from these cells continued to package high levels of mY RNAs (Figure 2A and B, lanes 7 and 8). When normalized to the co-packaged 7SL RNA, which is undiminished in Ro60 -/- cells, virus produced from the Ro60 -/- cells contained nearly the same amount of mY RNA per virion as virus from wild type cells (Figure 2A and B, lanes 7 and 8).

Because the Y/7SL RNA ratios in virus vs. those in cells were the same for both the mY1 and the mY3 panel in Figure 2, this suggests that each MLV virion encapsidates the same number of mY1 molecules as mY3s. However, because the blots shown in Figure 2 resulted from co-probing with two separate oligonucleotide probes—one for mY RNA and the other for 7SL—the absolute number of Y RNAs per virion could not be addressed by the radioactive signals observed in Figure 2. Thus, to ensure uniformity in probe specific activities and estimate the number of mY1 RNAs packaged per virion, virus and cell RNA samples were subsequently...
probed with a single radiolabeled oligonucleotide complementary to both 7SL and mY1 (Figure 3A). The results indicated that approximately two-fold less mY1 than 7SL RNA was encapsidated into MLV produced by wild type cells (Figure 3A, lane 1), and slightly less mY1 was packaged into MLV produced from Ro60 knockouts (Figure 3A, lane 2). Ribonuclease protection assays (RPA) for mY1 and 7SL RNA confirmed this quantification and revealed a near 2:1 ratio of 7SL:mY1 in virus from wild type cells (Figure 3B, lane 7). When normalized to 7SL, a detectable but less than two-fold decrease in mY1 packaging was observed in virus from Ro60 knockouts (Figure 3B, lane 8). The gRNA to 7SL ratios in virus from both cell types (Figure 3C, lanes 3 and 4) were consistent with previously established levels of 7SL packaging (three- to four-fold molar excess to gRNA (38)). Taken together these results indicated that each MLV virion from wild type MEFs contained four to five copies of both mY1 and mY3, and virions from Ro60 knockouts contained approximately 2 copies apiece of mY1 and mY3.

**The subcellular distribution of mY1 RNA is altered in Ro60 -/- cells.** Ordinarily, most Y RNA localizes to the cytoplasm, where it is bound to Ro60 in RoRNPs (35, 40). The reduced levels of mY RNAs in Ro60 -/- cells are believed to reflect their decreased intracellular stability when their cognate RNP protein is not present (6, 28, 63). Thus, a reduction in mY RNA stability in Ro60 -/- cells would likely be accompanied by a more severe deficit of mY RNA in the cytoplasm than in the nucleus.

To determine the subcellular distribution of the residual mY1 RNA in Ro60 -/- cells, RNA was extracted from total, nuclear and cytoplasmic fractions of knockout and isogenic wild type MEFs (Figure 4). Northern blots were probed for mY1 or co-probed for nuclear U6 snRNA and cytoplasmic tRNA\(^{\text{Lys1}}\) to control for fractionation efficiency. The results indicated that the cytoplasm of wild type cells contained at least four-fold more mY1 RNA than their nuclei.
(Figure 4A, lanes 3 and 5). In contrast, nuclear and cytoplasmic fractions of Ro -/- MEFs contained similar amounts of mY RNA (nuclear fraction lane contains 10% more Y1 than cytoplasmic fraction; Figure 4A, lanes 4 and 6). Compared to wild type cells, knockout cell cytoplasmic amounts of mY1 were reduced about 20-fold, and nuclear amounts were reduced about four-fold. Because the nuclear levels of the control RNA U6 are not reduced in knockout cells, the four-fold decrease of nuclear Y1 in the knockouts suggest at least some of the RNA degradation associated with the absence of Ro60 may initiate in the nucleus. These findings confirm a marked alteration in the subcellular location of mY RNAs, from their cytoplasmic prominence in wild type cells to a far greater depletion from cytoplasm than from nucleus for the residual mY RNAs in Ro60 knockout cells.

The results here showed mY RNAs were recruited at wild type levels from Ro60 knockout cells, despite 20 to 30-fold reductions in mY RNA intracellular levels. When mY to gRNA ratios in wild type cells and virus are compared, mY RNAs are selected for packaging 3 to 4 fold less well than MLV gRNAs (38). Thus, whereas virions produced by wild type cells package mY RNAs slightly less effectively than viral gRNAs, when MLV replicated in Ro knockout cells, mY1 and mY3 displayed a roughly 5- to 10-fold higher packaging selectivity than gRNAs.

**mY RNA packaging is independent of Y RNA processing step.** The biosynthesis of host ribonucleoprotein complexes involves a series of RNA processing steps and alternate protein associations. Significant uncertainty remains about both temporal and spatial aspects of these steps in Ro RNP assembly. Nonetheless, it is clear that transcription of Y RNAs, as is the case for all RNA polymerase III transcripts, terminates in a run of uridines, and that the resulting 3’ ends are bound by the La protein, which recognizes RNAs ending in three or more uridines.
Subsequent end-trimming by exonucleases removes some of the terminal uridines, resulting in a population of RNAs slightly shorter than the initial transcription products; some of these no longer possess La binding sites. Neither the identity of the exonuclease nor its subcellular location is known. Ro travels to the nucleus prior to Ro RNP assembly, and binds the bulged Y RNA stem region (22, 51). Although La has been observed to shuttle between the nucleus and cytoplasm (15), La binding retards nuclear export of Y RNAs (23, 52). Thus, Y RNA 3’ end shortening and elimination of the La binding site may occur prior to nuclear export (24, 46). Ro and La can bind a single Y RNA simultaneously. In unstressed wild type cells, most Y RNAs exist in the cytoplasm in a shorter, matured form, in an RNP complex containing Ro but not La (shorter mY1 RNAs lack the 3’ La binding site (Figure 4A) and (31, 60)).

To address whether or not mY RNA encapsidation into MLV represented diversion of the RNAs from a specific stage in their maturation pathway, RNAs from virions produced by wild type and Ro60-/- cells were compared to those in cells for signatures of Y RNA processing intermediates. Consistent with the Y RNAs in the knockout MEFs representing newly synthesized RNAs, the residual Y RNAs in Ro60-/- cells migrated slightly slower than the majority of the Y RNAs in wild-type cells, and thus exhibited the pattern observed for nascent La-bound Y RNAs (Figure 4A and (7)).

The lengths of Y RNA processing intermediates were also examined by high resolution denaturing acrylamide gels (Figure 5A). These results confirmed that the spectra of mY RNAs in knockout cells was longer than that in wild type cells. The short length of mY RNAs in virions from wild type cells suggested that La binding was not necessary for mY RNA packaging. However, the encapsidated RNAs from knockout cells were longer than those from wild type.
cells. In both cell types, the RNA length distribution in virus was found to resemble that in the producer cells (Figure 5A), suggesting that packaging did not require mature 3’ end formation.

To further address possible packaging of specific Y RNA subsets, high resolution gels were used to examine mY1 processing species’ distribution in cytoplasmic and nuclear fractions (Figure 5B). Under the fractionation conditions used, the spectra of RNAs in knock-out cells’ cytoplasmic and nuclear fractions were indistinguishable (Figure 5B lanes 5 and 6). A subtle but reproducible slight bias toward more completely processed products was observed both in wild type cells’ cytoplasmic fractions (Figure 5B lane 3) and in virions (Figure 5A lane 6), compared to these cells’ nuclear RNA spectrum (Figure 5B lane 2).
DISCUSSION

Retroviruses like MLV encapsidate distinct subsets of cellular non-coding RNAs (3, 38). Data here demonstrated that mY1 and mY3 RNA were recruited into budding MLV at four to five copies apiece from wild type MEFs and about 2 copies apiece from Ro60 knockouts. Although most mY RNA in cells resides in Ro RNPs, these RNAs were recruited into MLV without their cognate RNP protein, Ro60.

Similar observations of host protein-independent packaging of RNP RNA have been made for the SRP RNA, 7SL, which is packaged without the 54-kd SRP protein in both MLV and HIV-1 ((25, 39) and unpublished). Because of its essential role in signal recognition particles, direct knock-down of 7SL RNA is not readily achievable ((1) and unpublished). Facilitating our analysis of MLV mY RNA packaging, viable Ro60 knockout mice have been generated. Their cells lack Ro RNPs and display vastly reduced levels of mY RNAs (63).

We therefore used Ro60 -/- cells to examine the intersection of mY RNA biogenesis with MLV replication. The normal spread of MLV in Ro60 -/- embryonic fibroblasts demonstrated that the Ro60 protein is not necessary for virus replication. Because mY1 and mY3 are highly labile in the absence of Ro60, knockout of Ro60 leads to a 30-fold reduction in these RNAs (63). Strikingly, this intracellular reduction was not accompanied by a proportional reduction in mY RNA packaging. Instead, mY1 and mY3 were so highly enriched that MLV’s selectivity for mY RNAs, from among all RNAs in Ro60 knockout cells, was 5- to 10-fold higher than selectivity for its own genome.

MLV’s high level of selectivity for mY RNAs led to our model in which Ro RNP and MLV assembly pathways intersect at an early step in Ro RNP biogenesis (Figure 6). In this model for the encapsidation of mY RNAs without Ro60, the pathways of mY RNA biogenesis
and MLV assembly intersect, and a virion-specifying factor diverts Ro60-free mY RNAs away from host cell RNA degradation machinery toward assembly sites on the plasma membrane (Figure 6).

The similar Y RNA packaging observed in virions produced by cells with either high or very low intracellular Y RNA levels suggests that these RNAs are not recruited from RoRNPs, but from a separate intracellular pool of Ro60-free RNAs. Towards localizing this intracellular pool, cell fractionation suggested MLV recruitment from an early pool of nascent RNAs. Consistent with the role of Ro60 in stabilizing mY RNAs (6, 28, 63), as well as the likely importance of Ro60 binding to Y RNA nuclear export (52), cytoplasmic pools of mY RNAs decreased more than the residual pool of nuclear mY RNAs in Ro60 knockout cells. Because this redistribution did not result in a corresponding decrease in Y RNA recruitment by MLV, it suggests that recruitment occurs at an unaffected, and possibly earlier, step in Y RNA biogenesis.

Precisely where this occurs was not resolved by monitoring 3’ end modifications that accompany Y RNA maturation. In virus from wild type cells, encapsidated Y RNAs resembled the biased pattern of mature Y RNAs in the cytoplasm. However, encapsidated RNAs from Ro60 knockout cells, like the Y RNAs in these cells, resembled nascent RNAs that had not undergone 3’ end maturation. Thus, although we cannot rule out a cytoplasmic point of recruitment from a small pool of immature cytoplasmic Y RNA, these data suggest that recruitment into particles is independent of 3’ end maturation, which likely occurs prior to cytoplasmic entry for the bulk of Y RNA.

Assuming that mY RNAs are recruited at the same step of their biosynthesis in both cell types, these results suggest that recruitment for packaging occurs before 3’ end maturation is completed but does not preclude subsequent 3’ end maturation. Both La and Ro recognize and
act on RNA motifs in the Y RNA stem and 3’ tail region, while other factors such as nucleolin and hnRNP I are known to interact with some Y RNAs via the internal loop (12, 16, 22). Thus, these findings may indicate that recruitment of Y RNAs occurs via interactions with the loop region that prevent degradation of Ro-deficient RNAs but do not preclude 3’ end maturation (Figure 6). Speculatively, the slower migration of residual Y RNAs in knockout cells may be suggestive of a role for Ro60 in exposing Y RNA 3’ ends for completing their exonucleolytic processing by an as yet unknown mechanism.

Because they appear to be recruited early, one possibility is that mY RNAs may be selected for encapsidation into MLV from near their site of transcription in the nucleus (Figure 6). The possibility of nuclear recruitment is plausible, considering that known pools of protein-free Y RNAs localize to perinucleolar sites of early RNP assembly (32). Although assembly of MLV, as for all retroviruses classically described as type C, is first visualized at the plasma membrane (17), the notion that retroviral late replication phases may include a nuclear step is not unprecedented, as it has been shown that a portion of avian sarcoma virus Gag molecules transit through the nucleus prior to assembly (47-49). Although one study reported that 18% of MLV infected cell-associated Gag immunoprecipitated from nuclear fractions, it remains controversial whether or not retroviruses other than ASV share this step (34).

If mY RNAs are recruited in the nucleus, they may join a complex that includes MLV gRNA. The hypothesis that MLV gRNAs transit directly from the nucleus to sites of assembly is supported by the propensity of sibling MLV gRNAs, but not those of HIV-1, to self-associate for packaging (13). When sibling gRNAs are expressed from a single nuclear locus or proximal integration sites, they associate randomly (14, 26, 43). The impact of nuclear distance on gRNA dimer partner selection argues for an early association of gRNA siblings and an early formation
of a subviral RNP destined for encapsidation at the plasma membrane. If mY RNAs are recruited in the nucleus, they may join this hypothetical subviral RNP and accompany it to the plasma membrane, with the possibility that mY RNA binding may modulate the RNP’s intracellular trafficking, as it does for Ro RNPs (Figure 6) (50).

These findings of highly specific recruitment and enrichment of host RNP RNAs into MLV particles, even when the particles are produced by cells in which the RNAs are barely detectable, adds to growing evidence that the pathway of retroviral assembly—from nuclear provirus to plasma membrane released virion—may be less linear than previously believed (55). Whether the apparent intersection of host and viral RNP biogenesis pathways is a fortuitous convergence, represents a viral evolved reliance on host RNP biosynthetic machinery, or is indicative of an abortive attempt of the host RNA quality control circuitry to thwart viral attack is not clear. The comparable packaging of similar subsets of noncoding RNAs in several different retroviral species does not immediately differentiate between these possibilities (1, 25, 38, 57), although the argument for chance interactions may be weakened if some retroviruses include a nuclear preassembly step while others do not (10, 13). Accumulating evidence suggests that some viruses co-opt protein quality control machinery to aid their replication (59): the work here adds to evidence for a similar intersection between retrovirus assembly and cellular machinery associated with RNA quality control (2, 19).

Acknowledgements:

This work was supported by NIH grants R21 AI080276 to AT and R01GM073863 to SW as well as by a Gates Grand Challenges Exploration Grant to AT. SS was supported by a postdoctoral fellowship from the Arthritis Foundation.
The authors thank Michael Malim for constructive comments on the manuscript.

References:


**Figure legends**

**Figure 1. Time course of MLV spread in Ro60 -/- cells and wild type MEFs.** Wild type and Ro knockout cells were infected with identical amounts or MLV, and virus spread was monitored by assaying for RT activity at the indicated time points. (dpi designates days post infection)

**Figure 2. mY1 and mY3 RNA recruitment in the absence of Ro60.** Northern blot co-probed for 7SL and either (A) mY1 or (B) mY3. The left half of each blot shows cellular RNA samples: lane 1, from wild type Ro60 +/- MEFs; lane 2, Ro60 +/- MEFs; lane 3, Ro60 +/- MEFs infected with MLV; lane 4, Ro60 +/- MEFs infected with MLV (infected with MLV designated as MLV Expres for MLV expressing). Cell-free media/virus samples on the right are RNAs extracted from supernatants of the following: lane 5, uninfected Ro60 +/- cells; lane 6, uninfected Ro60 +/- cells; lane 7, MLV infected Ro60 +/- cells; lane 8, MLV infected Ro60 +/- cells. M indicates RNA size marker.

**Figure 3. Stoichiometric analysis of mY1 RNA packaging.** Northern blot of MLV RNA from Ro60 wild type and knockout MEFs probed with a chimeric oligo complimentary to both 7SL RNA and mY1 (A). Ribonuclease protection assay of cellular and supernatant/viral RNA for mY1 and 7SL RNA (B). Lanes show: undigested probe (P); RNA size markers (M); experimental samples indicated at top either Ro60 wt (+/+ or Ro60 knockout (-/-); and digested probe alone control (C). Ribonuclease protection assay (RPA) of supernatant/viral RNA for
gRNA and 7SL RNA (C). Quantification of 4 to 5 mY1 RNAs per virion (gRNA dimer) was determined as described in Materials and Methods.

**Figure 4. mY1 RNA redistribution in Ro60 -/- cells.** (A) Northern blot of RNA from total (T), nuclear (N), and cytoplasmic (C) fractions of both wild type Ro60 +/+ MEFs and knockout Ro60 -/- MEFs, probed for mY1. (B) Northern blot of RNA from total (T), nuclear (N), and cytoplasmic (C) fractions of both wild type Ro60 +/+ MEFs co-probed for U6 snRNA, a nuclear RNA control, and tRNA^{Lys1}, a cytoplasmic RNA control.

**Figure 5. mY1 RNA processing intermediates in cell and virus samples.** (A) Northern blot of RNA from total cell and viral samples from wild type Ro60 +/+ MEFs and knockout Ro60 -/- MEFs, separated on 8% acrylamide, 8M urea sequencing gel and probed for mY1. Note that whereas lanes 1 and 2 contain equivalent samples, 20 times as much -/- cell sample was loaded in lane 3 as +/+ cell sample in lane 1 to facilitate qualitative comparison; lane 4 and lane 5 are RNA samples from supernatants of uninfected cells – the same volume of cell-free media as was used in lane 6; and virus samples (lane 6 and lane 7) were normalized to 7SL. (B) Northern blot of RNA from total, nuclear, and cytoplasmic fractions from wild type Ro60 +/+ MEFs and knockout Ro60 -/- MEFs, separated on 8% acrylamide, 8M urea sequencing gel and probed for mY1.

**Figure 6. Ro RNP biogenesis and late stages of MLV replication.** A speculative model for their intersection based on observations reported here and Ro RNP assembly properties, as
described in the text. Light gray oval represents nucleus; gray box indicates the most parsimonious locations of mY RNA recruitment for packaging.