Evidence that a Central Domain of Nucleocapsid Protein Is Required for RNA Packaging in Murine Leukemia Virus

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We have analyzed RNA packaging by a series of mutants altered in the nucleocapsid (NC) protein of Moloney murine leukemia virus (Mo-MuLV). We found that mutants lacking residues 8 through 11 or 44 through 60 of NC package Mo-MuLV RNA with virtually the same efficiency as wild-type Mo-MuLV. In contrast, point mutants altered at the conserved cysteines in the cysteine array (residues 26 and 29) and a mutant lacking residues 16 through 23 packaged Mo-MuLV RNA with approximately 1% of the efficiency of wild-type Mo-MuLV. The deficiency in packaged RNA was observed not only in Northern (RNA) analysis but also in an RNA-PCR assay, which would detect degraded as well as intact RNA. One of the cysteine array mutants was also shown to be defective with respect to encapsidation of hygromycin phosphotransferase mRNA containing a Mo-MuLV packaging signal. We suggest that a central region of NC, consisting of the cysteine array and flanking basic residues, is required for RNA packaging in Mo-MuLV.

The mechanisms by which retroviral proteins selectively package viral RNAs during virus assembly are still poorly understood. A single sequence motif in the Gag polypeptide, the cysteine array within the nucleocapsid (NC) domain, has been linked to RNA packaging in a number of mutational studies of murine leukemia virus (MuLV), avian type C viruses, and human immunodeficiency virus (2, 8, 13, 15, 19–21, 31–33). In general, these studies have indicated that mutants altered within this domain are defective in RNA encapsidation. These observations suggested that the cysteine array plays an essential role in recognition and/or packaging of viral RNA during virus assembly.

In contrast to these studies of the cysteine array, relatively little attention has been paid to other regions of NC. Further, the interpretation of the experiments on the cysteine array is open to question, since nearly all of them used Northern (RNA) blot analysis in the RNA encapsidation assay. Unfortunately, this technique only detects intact viral RNA efficiently; fragmented or degraded RNA would not yield a discrete band of the expected electrophoretic mobility and might be overlooked in these assays. Indeed, a recent report by Aronoff et al. (4) has emphasized this uncertainty. Aronoff et al. suggested, on the basis of experiments with a cysteine array mutant of avian type C virus, that mutants altered in the cysteine array do package viral RNA efficiently but that it is degraded and thus undetectable by Northern analysis.

The results obtained by Aronoff et al. (4) lead to fundamentally different conclusions as to the function of cysteine arrays from those of prior studies. Aronoff et al. suggested that the arrays do not function in RNA encapsidation but rather in stabilizing or protecting the packaged RNA against degradative enzymes or fragmentation in the virion (or during RNA extraction). In an effort to gain further insight into the function of cysteine arrays, we have therefore reexamined Moloney

MuLV (Mo-MuLV) NC mutants for the presence of viral RNA, using a technique which can efficiently detect fragmented, as well as intact, viral RNA.

We have also assayed RNA packaging by several NC mutants which are altered outside the cysteine array. The results indicate that the MuLV cysteine array is indeed critical for RNA encapsidation, and partially delimit a region of NC on either side of the array which is required for this function. This region appears to consist of the cysteine array together with clusters of basic residues flanking it on either side.

New mutations analyzed in the present study are indicated in Fig. 1. They included two deletions, Δ 8-11 (deletion of residues 8 through 11) and Δ 16-23, on the N-terminal side of the cysteine array, and a truncation, R44*ter*, in which a TGA termination codon follows codon 43, on the C-terminal side. Stable transfectants were obtained by calcium phosphate transfection (22) of CHO cells and selection with G418 (Life Technologies, Inc., Gaithersburg, Md.). All virus preparations were from 24-h harvests of culture fluids.

Culture fluids from stable transfectants were initially tested for virus production by immunoblotting. Analysis of virus pellets with anti-p30^{CA} antiserum (40) showed that Δ 8-11 and R44*ter* mutants generated virus as efficiently as the wild type, while cultures containing the Δ 16-23 mutant produced only ~1/10 as much virus as those containing the wild type (data not shown). In all cases, the protein data also showed that the cultures were producing viruses with the expected phenotypes: immunoblotting with anti-p10^{NC} antiserum demonstrated that the NC proteins of the two deletion mutants were smaller than wild-type NC and that Δ 16-23 NC was slightly smaller than Δ 8-11 NC, while tests with anti-p30^{CA} antiserum showed that the R44*ter* mutant produced a Gag precursor slightly smaller than that of the wild type (data not shown). The Gag precursor in the R44*ter* mutant was not cleaved; this is presumably because the premature termination codon in this mutant is not followed by a signal for translational suppression (16, 41), so that this mutant fails to produce the viral protease.

Pellets from culture fluids were initially assayed for viral RNA by Northern analysis. As shown in Fig. 2, the Δ 8-11 mutant and the R44*ter* mutant both packaged viral RNA at

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FIG. 1. Sequence of Mo-MuLV NC (38), showing regions modified by mutations in the present work. An infectious molecular clone of Mo-MuLV in the selectable vector pGCcos3neo, described in reference 20 and designated pRR88, was the parental clone used in these studies. Oligonucleotide-directed mutagenesis was performed as described previously (20). In every case, the mutated restriction fragment used in the construction of the full-length mutant genome was sequenced in its entirety and was wild type except for the desired change. The new mutants generated here were $\Delta 8-11$, in which residues 8 through 11 were deleted; $\Delta 16-23$; and $R44ter$, containing a TGA termination codon following codon 43. The cysteine array, consisting of residues 26 through 39, is highlighted. C26S, in which the first cysteine has been changed to serine, and C26S/C29S, in which the first two cysteines have both been replaced with serine, have been described previously (20).

levels similar to that in the wild-type control. (In fact, the $\Delta 8-11$ mutant appears to be nearly as infectious as the wild type [data not shown].) In contrast, no RNA was detected by this analysis in the $\Delta 16-23$ mutant. These results show that neither residues 8 through 11 of NC nor the extreme C-terminal 17 residues of NC are essential for RNA packaging. They suggest, however, that residues removed in the $\Delta 16-23$ mutant are required.

As discussed above, Northern analysis is actually appropriate only for detection of intact RNA. Mutants which appear to be negative for RNA by Northern analysis may package viral RNA, but this RNA may be partially degraded in the virion or during extraction. We therefore developed a different assay for viral RNA. This assay relies on PCR following reverse transcription (RT-PCR). It should detect fragmented, as well as intact, RNA, since the primers used are <300 nucleotides apart in the viral genome. This assay was used to test for viral RNA in virus preparations of cysteine array mutants and the

$\Delta 16-23$ mutant, since these mutants had previously (20) (Fig. 2) given negative results by Northern analysis.

RNA was isolated from pellets of wild-type and mutant particles, and serial dilutions of the RNA were analyzed for viral RNA by RT-PCR. These assays were performed as follows. RNA was extracted from pelleted virus by proteinase K digestion followed by phenol-chloroform extraction, as described elsewhere (17). Extracted, ethanol-precipitated RNA was redissolved in 1/1,000 of the original volume. Serial dilutions of the extracted RNA were made in RNase-free water (Promega, Madison, Wis.) and were heated to 65°C for 5 min immediately before use. A 5- μ l volume of each dilution was then added to 45 μ l of a reverse transcription mixture containing first-strand reverse transcription buffer (Life Technologies); 0.5 mM each dATP, dCTP, dGTP, and dTTP; 5 μ g of p(dN)₆ random primer (Pharmacia, Piscataway, N.J.); 40 U of RNasin (Promega); and 500 U of Superscript II RNase H⁻ Mo-MuLV reverse transcriptase (Life Technologies). These reactions were allowed to proceed at 37°C for 60 min and were then heated to 65°C for 10 min. They were then supplemented with 0.025 A_{260} units of each primer and 5 U of *Taq* DNA polymerase (Life Technologies). The primers were nucleotides 2446 to 2469 (plus strand) and 2686 to 2661 (minus strand) of Mo-MuLV (38). The reaction mixtures were incubated at 93°C for 3 min and then subjected to 35 cycles of 1 min at 94°C, 1 min at 55°C, and 3 min at 72°C. The final cycle was followed by a 15-min incubation at 72°C. One-third of each reaction mixture was then analyzed by electrophoresis in 2.5% Meta-phor (FME, Rockland, Maine) in the presence of 1 μ g of ethidium bromide per ml. Levels of RNA species in different samples were estimated by determining the dilution at which the DNA product was no longer visible by ethidium bromide fluorescence.

Results of these experiments are shown in Fig. 3. Each lane represents the amplification product from RNA in 1.7 ml of culture fluid or from a dilution of this RNA, as indicated. Figure 3A shows a comparison of RNA from wild-type virus with that from two previously described cysteine array mutants, C26S (a mutant with a C-to-S change at position 26) and C26S/C29S (20). The amplification product is detectable in the 10^{-3} dilutions of the mutants (representing 0.0017 ml of culture fluid) but not in the 10^{-4} dilutions. In contrast, the endpoint for detection of the product from the wild-type sample was between the 10^{-5} and 10^{-6} dilutions (representing 0.000017 and 0.0000017 ml, respectively). Thus, the two mutants evidently package Mo-MuLV genomic RNA with approximately 1% of the efficiency of the wild type. This conclusion is quite consistent with our previous results obtained by Northern analysis (20). As an additional test of the ability of these mutants to package RNA, we analyzed them for Mo-MuLV RNA by filter hybridization. As in the RT-PCR assays, they were both deficient by these tests (data not shown).

FIG. 2. Northern analysis of RNA packaging by NC mutants. RNA was extracted from viral pellets and analyzed by Northern blotting with a Mo-MuLV-specific probe as described previously (20). In these experiments, RNA was extracted from virus preparations with phenol, without prior proteinase digestion. (A) Analysis of deletion mutants altered on the N-terminal side of the cysteine array. The $\Delta 16-23$ lane contained RNA from 10 times as much culture fluid as used for the other lanes in panel A. (B) Analysis of truncation mutant $R44ter$. Equal volumes of each culture fluid were tested. WT and (-) represent culture fluids from cells transfected with the wild-type clone and pGCcos3neo, respectively.

FIG. 3. Analysis of Mo-MuLV RNA packaging by RT-PCR. RNA in virus particles was analyzed as described in the text. (A) Comparison of C26S, C26S/C29S, and wild-type particles. (B) Comparison of Δ16-23 and wild-type particles.

We also used the RT-PCR assay to analyze pellets of the Δ16-23 mutant for viral RNA. As shown in Fig. 3B, these results showed that these pellets contained $\leq 1\%$ of the level of viral RNA in wild-type pellets; this result is also fully consistent with the negative results obtained by Northern analysis (Fig. 2B).

One difference between the study of Aronoff et al. (4) and previous studies of cysteine array mutants is that Aronoff et al. did not measure packaging of authentic genomic RNA, but rather of a much shorter mRNA containing a viral packaging signal in its 3' untranslated region. It seemed possible that cysteine array mutants are actually defective in packaging viral RNA but not in packaging small mRNAs containing viral packaging signals. To test this possibility, we constructed a plasmid, pCMVhygMoψ, which expressed the hygromycin phosphotransferase (hph) gene. Hph mRNA molecules encoded by this plasmid contained the Mo-MuLV packaging signal in their 3' untranslated regions. This plasmid was transfected into cells containing wild-type Mo-MuLV or the C26S mutant. RNA extracted from the virions produced by these stably transfected cultures was then assayed simultaneously for hph RNA and Mo-MuLV RNA by using RT-PCR with a pair of hph-specific primers in the same reactions with a pair of Mo-MuLV primers.

The pCMVhygMoψ plasmid was constructed as follows. pCMVneoAva, a modification of pCMVneo (29), was a kind gift of Maxine Linial (Fred Hutchinson Cancer Research Center, Seattle, Wash.); when transfected into mammalian cells, this plasmid expresses aminoglycoside phosphotransferase mRNA, inducing resistance to neomycin analogues. A large double-stranded synthetic oligonucleotide, containing

several restriction sites and promoters for RNA polymerases, was inserted in the plasmid between the *Ava*I and *Bst*EII sites; this insert is in the 3' untranslated region of the mRNAs encoding aminoglycoside phosphotransferase. The aminoglycoside phosphotransferase coding region was then removed by digesting the plasmid with *Hind*III and *Sal*I (*Sal*I cuts in the inserted oligonucleotide). It was replaced with the hph coding region, which had been isolated from pSV2hph (obtained from the American Type Culture Collection, Rockville, Md.) after digestion with *Hind*III and *Ava*I. The resulting plasmid, termed pCMVhyg, induces resistance to hygromycin in mammalian cells. Finally, a Mo-MuLV packaging signal was added: a fragment from pRR88, extending from the *Xba*I site in U3 (nucleotide -151) to the *Xho*I site in *gag* (nucleotide 1560) (38), was inserted between the *Xba*I and *Xho*I sites in the oligonucleotide. The product of this last step was designated pCMVhygMoψ. Further details of the plasmid construction are available on request.

CHO cells which had already been stably transfected with the wild type or the C26S mutant were transfected with these hph plasmids (22), and pools of stable transfectants were selected with 900 U of hygromycin B (Calbiochem-Novabiochem, La Jolla, Calif.) per ml. Viral pellets were analyzed by RT-PCR as described above (Fig. 3). The Mo-MuLV PCR primers were nucleotides 2446 to 2469 (plus strand) and 2737 to 2714 (minus strand) (38), and the hph primers were nucleotides 264 to 290 (plus strand) and 519 to 493 (minus strand) (23). The 292-bp Mo-MuLV amplification product was resolved from the 256-bp hph product by agarose gel electrophoresis.

The results of these experiments are presented in Fig. 4 and summarized in Table 1. Comparison of the upper (Mo-MuLV-specific) bands in the first two sets of lanes in Fig. 4 (i.e., lanes 1 through 6 and 7 through 14) shows that the level of Mo-MuLV packaging by the mutant was ≥ 100 times lower than that in the wild type, in agreement with the data shown in Fig. 3. Similarly, the lower (hph-specific) bands in these lanes also show an endpoint that is ~ 100 times lower in the mutant than in the wild type, indicating that the hph mRNA was also packaged far less efficiently by the mutant proteins than by the wild-type proteins.

The remaining lanes in Fig. 4 are a series of controls, measuring background signals in the RT-PCR assay used here. The samples in lanes 15 through 19 and 20 through 26 of Fig. 4 are the same as those in lanes 1 through 6 and 7 through 14, except that the hph mRNA expressed in these cultures does not contain a viral encapsidation signal. It can be seen that the absence of the signal had no detectable effect on the level of hph RNA packaged by the NC mutant, while it reduced the level packaged by the wild type ≥ 100 -fold. The amounts of Mo-MuLV RNA packaged by mutant and wild-type Mo-MuLV were approximately the same as those in the first and second sets of lanes of Fig. 4, respectively, as would be expected. Finally, lanes 27 through 31 of Fig. 4 represent another type of negative control: they show an assay of culture fluid collected from cells which contain an hph expression plasmid lacking a viral packaging signal (pCMVhyg) but which were not transfected with a viral clone at all. Remarkably, the level of hph RNA in this sample was nearly the same as that in the C26S samples (Fig. 4; lanes 1 through 6 and 15 through 19). A signal corresponding to the Mo-MuLV RNA RT-PCR product was also detected in this sample, despite the absence of any MuLV sequences in this culture; the level of this RNA in this sample is $\sim 1/100,000$ of that in the wild-type sample.

The data presented here can be summarized as follows. First, results from an RT-PCR assay (Fig. 3 and 4), which

FIG. 4. Analysis of Mo-MuLV (Mo) and hph RNA packaging by RT-PCR. RNA in wild-type (WT) and C26S particles was assayed as described in the text. All reaction mixtures were electrophoresed on a single gel and analyzed in a single photograph. M, *Hae*III digest of ϕ X174 DNA. Lane 32 contains the product of a control RT-PCR experiment using viral RNA from a culture transfected with pRR88 Mo-MuLV DNA but not with an hph plasmid. +, present; -, absent.

would detect fragmented, as well as intact, viral RNA, are completely consistent with earlier results (20) obtained by Northern analysis. Mutants which were negative for viral RNA by Northern analysis were found, by the far more sensitive RT-PCR technique, to contain $\sim 1\%$ of the level of viral RNA present in comparable amounts of wild-type virus. Second, a mutant lacking residues 16 through 23 of NC is, like cysteine array mutants, defective with respect to RNA packaging; in contrast, a mutant lacking residues 8 through 11 and a mutant lacking the C-terminal 17 residues package viral RNA with roughly the same efficiency as wild-type virus (Fig. 2).

The confirmation of negative Northern blotting results for cysteine array mutants and the $\Delta 16-23$ mutant by the RT-PCR assay (Fig. 3 and 4) argues against the idea that these mutants contain normal amounts of viral RNA which is degraded in the virus or during RNA extraction (4). For the cysteine array mutant C26S, the packaging defect was observed not only with genomic RNA but also with an mRNA containing a viral packaging signal in its 3' untranslated region (Fig. 4); this packaging substrate is similar to that used by Aronoff et al. (4). On the basis of these results, it seems likely that cysteine array mutants are, in fact, defective with respect to RNA packaging. (It should be noted, however, that there is a degree of degradation beyond which viral RNA could not be detected by the RT-PCR assay. If the mutants really package viral RNA with full efficiency, then random degradation to an average fragment size of ~ 50 nucleotides would produce the ~ 100 -fold

decrease in signal observed here with primers spaced 241 nucleotides apart.)

The results permit us to roughly define a domain within NC which is required for RNA packaging. Clearly, neither residues 8 through 11 nor amino acids distal to residue 43 are necessary. In contrast, the Northern blotting data of Gorelick et al. (20) and Méric and Goff (31) suggested that changes at many sites within the cysteine array (i.e., NC residues 26 through 39) produce an encapsidation defect; we assume that mutants altered at these sites, like the C26S and C26S/C29S mutants (Fig. 3 and 4), would also show this property in an RT-PCR assay. The finding that the $\Delta 16-23$ mutant is also defective in RNA packaging (Fig. 2 and 3) is in agreement with the report of Housset et al. (24), who presented Northern blotting data indicating that simultaneous replacement of the three consecutive arginine residues at positions 16 through 18 with neutral amino acids causes a defect in RNA encapsidation. On the C-terminal side of the array, Housset et al. also showed by Northern analysis that replacement of basic residues reduces RNA packaging.

Taken together, these results suggest that the cysteine array, along with clusters of basic residues on either side, is a unit which functions in RNA packaging. In contrast, large portions of NC on either side of this unit do not appear to be necessary for RNA encapsidation (Fig. 2). The results obtained by Housset et al. imply that a single basic residue in each of the flanking basic clusters is sufficient for packaging.

Recent biochemical studies have shown that retroviral cysteine arrays coordinate zinc ions in the virion (6, 9, 39). It seems significant that the functional unit proposed here, i.e., a zinc finger flanked on either side by basic residues, is conserved in all retroviruses (except the spumaretroviruses, which lack the zinc finger motif).

The NC proteins of many retroviruses contain two cysteine arrays. In these viruses, the N-terminal ("first") array is always surrounded on both sides by basic residues. We have previously presented Northern blotting data on human immunodeficiency virus type 1 mutants indicating that the presence of the first array in its proper position, i.e., flanked on both sides by basic residues, is important for RNA packaging (19). In a study of an avian type C retrovirus, Fu et al. (18) also showed that RNA encapsidation was reduced when a pair of lysine residues

TABLE 1. Levels of Mo-MuLV and hph RNA packaged by wild-type and C26S Mo-MuLV^a

Helper/ hph plasmid	RNA end point ^b	
	Mo-MuLV	hph
C26S/pCMVhygMo ψ	-3	-1
Wild type/pCMVhygMo ψ	-6	-3
C26S/pCMVhyg	-3	-1
Wild type/pCMVhyg	-5	-1
None/pCMVhyg	-1	-1

^a RNA in virus pellets was analyzed as shown in Fig. 4.

^b Highest $\log_{10}$ dilution at which the ethidium bromide-stained RNA band is visible.

on the C-terminal side of the first array were replaced by isoleucines. Thus, results with these other retroviruses are consistent with the idea that basic residues act in concert with the first cysteine array in RNA packaging.

It is interesting that a remarkable variety of interactions of NC proteins with nucleic acids *in vitro* have been described (e.g., see references 5, 7, 10–12, 25–27, and 34–37). In several cases, the results suggest that the basic residues are important in these interactions (11, 24, 34) while the native zinc finger structure is not (11, 25–27, 34). However, the relationships between these *in vitro* activities of NC and its functions *in vivo*, both before and after its release from the Gag polypeptide by proteolysis, are completely unclear at present.

As noted above, R44ter does not express the *pol* gene. Thus, the results shown in Fig. 2 indicate that Pol protein sequences are not required for RNA encapsidation, in agreement with earlier findings (for a review, see reference 30).

We also analyzed (Fig. 4; Table 1) the effect of inserted viral ψ sequences on the packaging of hph mRNA molecules. Packaging of these mRNAs by wild-type Mo-MuLV was ~100-fold more efficient when they contained the insert than when they did not; this result confirms the finding of Adam and Miller, obtained by Northern analysis of *neo* mRNA, that the 5' region of the viral genome is sufficient for packaging of nonviral RNAs (1). In contrast, the C26S mutant packaged this mRNA at a level only slightly higher than the background level "packaged" when no helper was transfected into the cells.

While we have not characterized the material producing the background in the RT-PCR assay for Mo-MuLV RNA, it may be RNA of one of the endogenous viruses which have been described in Chinese hamster cells (e.g., see references 3, 14, and 28). It should be noted that other, less sensitive assays would never encounter this background, which represents ~1/100,000 of the signal generated by wild-type Mo-MuLV in these cells (Fig. 4; Table 1).

In summary, our results, along with those obtained by several other laboratories, suggest that a central domain of NC, containing a zinc finger surrounded by basic residues, is required for RNA packaging in MuLV. This may be true of other retroviruses as well. The mechanism by which this domain participates in packaging and particularly the source of the specificity in packaging are unknown at present.

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ADDENDUM IN PROOF

Recent results using avian retroviral Cys-Hys deletion mutants suggest that psi-containing RNAs are present at 1 to 5% of wild-type levels in particles. Such findings are in agreement with the results presented here, suggesting a role for the NC Cys-His boxes in packaging (A. Yeo and M. Linial, personal communication).

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