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Effect of Polymerase Mutations on Packaging of Primer tRNA^{Pro} During Murine Leukemia Virus Assembly

JUDITH G. LEVIN* AND J. G. SEIDMAN

Laboratory of Molecular Genetics, National Institute of Child Health and Human Development, Bethesda, Maryland 20205

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The role of reverse transcriptase in selective encapsidation of the murine leukemia virus (MuLV) tRNA primer, tRNA^{Pro}, was investigated by examining the tRNA composition of several nonconditional *pol* mutants. One mutant, clone 23, which contains an altered polymerase about 40% smaller than the wild-type enzyme (B. I. Gerwin et al., J. Virol. 31:741–751, 1979) had a typical viral tRNA pattern, including normal levels of tRNA^{Pro} in free and 70S-associated 4S RNA. Another class of mutants, produced by Moloney murine leukemia virus-infected cell clone M13 and subclone M13/1, does not contain any detectable polymerase protein (A. Shields et al., Cell 14:601–609, 1978) and was found to have reduced amounts of tRNA^{Pro} in free 4S RNA. However, the level of tRNA^{Pro} associated with the genome was normal in the mutant virions. These results suggest that the reverse transcriptase protein is involved in the initial selection of tRNA primer during virus assembly, but not in the subsequent association of this tRNA with genomic RNA.

The 4S RNA found in RNA tumor virus particles represents a selected class of host tRNA molecules (5, 11, 15, 29, 36, 38, 41, 44, 46, 47). Since the tRNA composition of different retroviruses diverges much more than that of their corresponding hosts (40, 47), it seems clear that tRNA selection is a virus-specified function (29, 40). In a previous study concerning the mechanism of selective encapsidation, we used actinomycin D virions which lack 35S RNA (27) to demonstrate that genomic RNA is not required for this process (29). These findings suggested that tRNA selection is mediated by a viral protein, for example, one or more of the viral RNA binding proteins (29). In particular, based on the known interactions of viral reverse transcriptase with primer tRNA's (1–3, 7, 18, 22, 23, 34), we suggested that the polymerase protein may be important in determining the relative amount of primer tRNA assembled into virions (29). The present study provides evidence which supports this hypothesis.

Experimentally, this problem was approached by screening several nonconditional murine leukemia virus (MuLV) *pol* mutants in an effort to determine whether a mutation in the *pol* gene affects the amount of MuLV tRNA^{Pro} primer (20, 36) incorporated into virions. Two classes of mutants were studied. (i) Mutant clone 23 virions are noninfectious B-tropic MuLV particles containing 35S genomic RNA and normal levels of MuLV structural proteins, but only 2 to 5% of wild-type reverse transcriptase activity (17). This low level of enzymatic activity is associated with an altered polymerase molecule approximately 3/5 the size of the normal enzyme (17). Intracellular precursor molecules of 147,000 daltons (147K) and 114K are found in infected cells (17) but the normal 180K *gag-pol* precursor (24) is not detected (17). Wild-type particles produced by a parallel cell clone are designated clone 32 virions (17). Clones 23 and 32 were generously provided by Alan Rein and Brenda Gerwin (National Cancer Institute). (ii) Mutant M13 and M13/1 virions are produced by Moloney MuLV-infected cell clone M13 and subclone M13/1 and are noninfectious (43). They contain 35S genomic RNA and standard MuLV structural proteins as well as elevated levels of Pr65^{gag} (43). No polymerase protein is detectable in virions (43), and the 180K *gag-pol* precursor (24) is absent in infected cells (43). Enzymatic activity in the supernatant fluids is less than 1% of normal (J. G. Levin, unpublished data). The corresponding wild-type virus is produced by cell clone M4 (43). These clones were the generous gift of Anthony Shields, Gary Otto, and David Baltimore (Massachusetts Institute of of Technology). Although the same results were obtained with M13 and M13/1, only the M13/1 data are shown below. To be certain that the mutant and wild-type virions used for analysis of viral tRNA maintained their original genotype, in each of the experiments to be described,

fluids from a parallel set of unlabeled cultures were tested for infectivity by the XC plaque assay (39). Reverse transcriptase activity of radioactive viral fluids was measured directly (27, 28) in the course of virus purification (see legend to Fig. 1).

The two classes of mutants differ with respect to their specific defects in the *pol* gene. Clone 23 produces a mutant polymerase protein (17), whereas M13 and M13/1 do not have any detectable enzyme (43). Since the polymerase protein is known to bind tRNA (1–3, 7, 18, 22, 23, 33, 34), it was initially of interest to see whether these classes of mutants would show differences in the relative amount of total free 4S RNA in virions. Viral RNA labeled with $^{32}PO_4^{3-}$ (29) was extracted from mutant and wild-type virions and was then analyzed on composite 1.8% polyacrylamide–0.5% agarose gels (16, 27). The amounts of radioactivity in the 70S and 4S RNA regions of each gel were summed, respectively, and the data were used to calculate the ratio of 70S RNA to 4S RNA. The relative amounts of 4S RNA in clone 23 and clone 32 virions were quite similar (Table 1) and are in agreement with the value obtained for AKR MuLV RNA (29). In contrast, the M13/1 mutant had a twofold deficiency in 4S RNA compared with the M4 wild type. An increase in the 70S/4S RNA ratio has also been reported (40) for the avian sarcoma virus *pol* mutant BH-RSVα (19).

The results described in Table 1 suggested that clone 23 virions may have a normal complement of viral tRNA's, whereas M13/1 virions might be expected to exhibit quantitative and possibly qualitative differences with respect to the tRNA composition of wild-type MuLV. To investigate these possibilities, viral 4S RNA was analyzed in more detail by fractionation in a two-dimensional gel system as previously described (29). Figure 1 illustrates the patterns obtained for clone 23 and the wild-type clone 32. The free 4S RNA patterns for the wild type (Fig. 1a) and mutant (Fig. 1b) were essentially identical. In accord with earlier observations on the free 4S RNA of AKR MuLV and Moloney MuLV (29), three prominent radioactive areas could be distinguished. The central spot, denoted in Fig. 1 by an arrow, has been identified by oligonucleotide fingerprint analysis as the $tRNA^{Pro}$ primer (29, 36). From the autoradiogram it is clear that the mutant and wild type have the same relative amounts of the $tRNA^{Pro}$ species. Analysis of the 4S RNA associated with the genome (70S-associated 4S RNA) also revealed no differences in the gel patterns for clone 23 and clone 32 (data not shown).

Similar analysis was carried out with the mutant M13/1. The free 4S RNA patterns of the M4 wild type and the mutant are shown in Fig.

TABLE 1. *Amounts of 70S RNA and 4S RNA in pol mutants and wild-type MuLV*

Virus	Genotype	70S RNA (cpm)	4S RNA (cpm)	70S RNA/ 4S RNA ratio
Clone 23	Mutant	718	219	3.3
Clone 32	Wild type	1,613	381	4.2
M13/1	Mutant	4,512	552	8.2
M4	Wild type	9,600	2,219	4.3

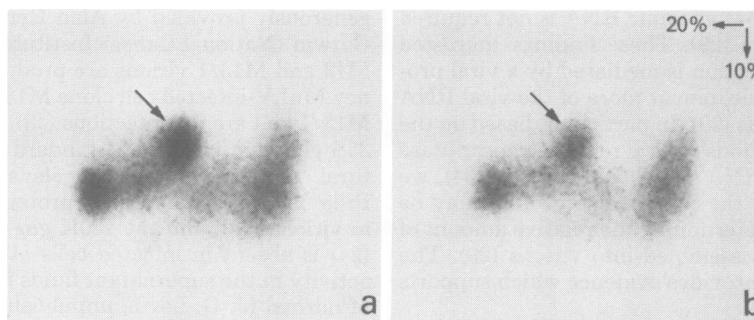

FIG. 1. *Two-dimensional gel patterns of ^{32}P-labeled 4S RNAs from clone 32 wild-type and clone 23 mutant MuLV particles. Cells were labeled with $^{32}PO_4^{3-}$ for 18 h (29). Virions were then harvested at two 4-h intervals after addition of unlabeled McCoy 5A medium containing 10% fetal calf serum and were purified on discontinuous sucrose gradients, sedimented for 90 min at 26,000 rpm in an SW27 rotor (28). Peak fractions were assayed for trichloroacetic acid-insoluble radioactivity and for reverse transcriptase activity (27, 28). Viral RNA was extracted from purified virions (27), and the low-molecular-weight RNAs were then fractionated by two-dimensional gel electrophoresis as previously described (29). The levels of radioactivity detected in these experiments were quite low compared with the amounts observed earlier with AKR and Moloney MuLV (29), and the autoradiograms were exposed at −70°C with the aid of a Kodak Lightning-Plus intensifying screen. The arrows denote the position of the $tRNA^{Pro}$ primer. (a) Clone 32; (b) clone 23.*

2a and b, respectively. These patterns are qualitatively similar and represent typical viral 4S RNA patterns (29). The additional spot in the wild-type pattern (Fig. 2a) is only occasionally resolved in our gels (29), and its presence in one pattern and absence in the other is presumably due to the fact that the runs were performed at different times. The striking result of this experiment is the difference in the relative intensities of the $tRNA^{Pro}$ spots. In the wild type, $tRNA^{Pro}$ is the major constituent of viral free 4S RNA (Fig. 2a), whereas in the mutant, $tRNA^{Pro}$ is the least abundant 4S RNA species (Fig. 2b). Because of the low yields of virus and viral 4S RNA, particularly in the case of M13/1, where there is a deficiency in 4S RNA (Table 1), it was not possible to precisely quantitate the difference in the $tRNA^{Pro}$ content of the mutant and wild type. However, visual inspection of the autoradiograms as well as utilization of an image-processing computer program (26) to estimate differences in intensity suggested that the relative amount of $tRNA^{Pro}$ in the mutant is decreased by a factor of two- to fourfold compared with the wild type. As mentioned above, the same findings were made with mutant M13 virions. These results demonstrate that the *pol* mutation in M13/1 (and M13), which results in the absence of polymerase protein (43), is correlated with a deficiency in the relative amount of $tRNA^{Pro}$ present in free 4S viral RNA.

It was next of interest to determine whether this effect could also be observed with viral 70S-associated 4S RNA from the M13/1 mutant. In this case, however, the same patterns were obtained for the wild type (Fig. 2c) and mutant (Fig. 2d), and the $tRNA^{Pro}$ spots in both patterns were very prominent. Moreover, no differences in the relative intensities of these spots could be detected, indicating that both the mutant and wild type contain the same proportion of $tRNA^{Pro}$ in 70S-associated 4S RNA. Since the deficiency of $tRNA^{Pro}$ in the free 4S RNA of the mutant is not absolute (Fig. 2b), these findings suggest that the tRNA primer which is present in the virion is available for binding to 35S genomic RNA. The implications of this observation will be discussed in more detail below.

The evidence presented in this report indicates that only some mutations in the *pol* gene affect the relative amount of virion-associated tRNA primer. Thus, clone 23, which contains an altered polymerase about 40% smaller than the wild-type enzyme (17), has normal levels of $tRNA^{Pro}$ in free (Fig. 1b) and 70S-associated 4S RNAs. In contrast, in M13/1, where reverse transcriptase is missing (43), the proportion of $tRNA^{Pro}$ in free 4S RNA is significantly reduced (Fig. 2b). Other viral tRNA's are apparently not affected. These results point to an important role of reverse transcriptase in the initial selection of tRNA primer during virus assembly.

The idea that reverse transcriptase is responsible for the selection of the tRNA primer was originally suggested by Panet et al. (34) and is supported by the present study as well as by data from the avian system. It has been shown by Sawyer and Hanafusa (40) and more recently by Peters and Hu (37) that both the free and 70S-associated 4S RNAs in avian sarcoma virus *pol* mutants are severely deficient in $tRNA^{Trp}$, the tRNA primer for the avian sarcoma and leukosis virus group (14, 21, 48). In addition, the free 4S RNA of the mutants appears to contain a random selection of host tRNA's, suggesting that the avian reverse transcriptase also controls the selection of nonprimer tRNA's (37, 40). With the exception of $tRNA^{Trp}$, however, normal amounts of the other virion tRNA's are present in the 70S RNA complex (37, 40). This finding, taken together with our results showing a normal viral tRNA pattern for the 70S-associated 4S RNA of M13/1 (Fig. 2d), suggests that *pol* mutations do not affect tRNA binding interactions with genomic RNA.

It is also of interest to compare the present observations on clone 23 (Fig. 1b) with those of Peters and Hu (37) on the avian *pol* mutant SE52d (30). Both mutants produce abnormally small polymerase proteins with little or no functional activity. However, clone 23 has the tRNA composition of wild-type virus (Fig. 1b), whereas the free 4S RNA of SE52d shows no selective incorporation of host tRNA's (37).

Although our findings and the studies on the avian system indicate that reverse transcriptase is a major determinant in the selection of tRNA primer, the fact that the amount of primer in free 4S RNA is dramatically reduced in avian mutants and only partially decreased in M13/1 may be significant. It is interesting that the results obtained with the avian and murine mutants parallel known differences between wild-type avian and murine viruses. Thus, free $tRNA^{Trp}$ in avian myeloblastosis virus is enriched 30-fold with respect to its proportion in total cell tRNA, whereas $tRNA^{Pro}$ is only enriched three- to fourfold in MuLV (47). Moreover, using a high-affinity binding assay, it was shown that the avian virus reverse transcriptase forms specific complexes with $tRNA^{Trp}$, $tRNA^{Pro}$, and, to a lesser extent, with $tRNA_4^{Met}$, but not with other tRNA's (22, 34). In contrast, although nonspecific binding of tRNA's to the murine enzyme has been demonstrated (33), specific complex formation with $tRNA^{Pro}$ has not been detected by currently available assays (22). The present data showing that a deficiency in

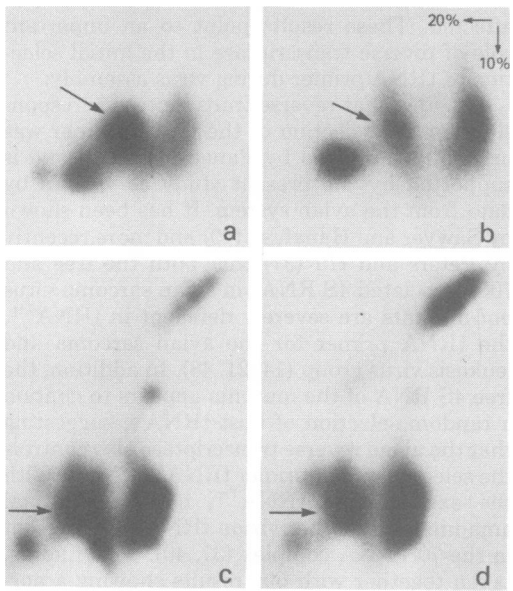

FIG. 2. *Two-dimensional gel patterns of ^{32}P-labeled free and 70S-associated 4S RNAs from M4 wild-type and M13/1 mutant MuLV particles. ^{32}P-labeled virions were prepared as described in the legend to Fig. 1 except that Dulbecco medium containing 10% fetal calf serum was used for harvesting the particles. Analysis of the free 4S RNA present in total viral RNA by two-dimensional polyacrylamide gel electrophoresis has been described previously (29) (see the legend to Fig. 1). Identical results were obtained with free 4S RNA which was isolated by fractionating total viral RNA in sucrose density gradients (data not shown). The 70S RNA was prepared as follows. The peak virus-containing fractions from the discontinuous sucrose gradients were pooled and centrifuged at 26,000 rpm for 90 min in an SW27.1 rotor. Viral pellets were resuspended in 0.3 ml of buffer containing 0.01 M Tris, pH 7.4, and 0.001 M EDTA and were incubated with 1% sodium dodecyl sulfate in the presence of proteinase K (400 μg/ml) at room temperature for 20 to 30 min. The disrupted virions were sedimented in 11.5 ml of linear 15 to 30% (wt/vol) sucrose gradients in buffer containing 0.01 M Tris, pH 7.4, 0.1 M NaCl, and 0.001 M EDTA (TNE buffer) and 0.2% sodium dodecyl sulfate for 210 min at 35,000 rpm in an SW41 rotor. Peak fractions corresponding to 70S RNA were pooled, 50 μg of carrier E. coli tRNA were added, and the RNA was then precipitated with 2 volumes of absolute ethanol and left at −20°C overnight. The 70S RNA was recovered by centrifugation and reprecipitated one time in ethanol. Each 70S RNA preparation was characterized by analyzing a portion of the RNA on a composite 1.8% polyacrylamide–0.5% agarose gel (16, 27) together with marker AKR viral [^{3}H]RNA. To separate small RNAs from 35S genomic RNA, 70S RNA was heated at 100°C for 1.75 min and then plunged into ice. The resulting mixture consisting of 35S RNA, 70S-associated 4S RNA, and other low-molecular-weight RNAs (4, 8, 12, 13, 47) was subjected to two-dimensional polyacrylamide gel electrophoresis (29) (see the legend to Fig. 1); 35S RNA remains at the origin of the 10% gel (29, 36). The arrows denote the position of the tRNA^{Pro} primer. Free 4S RNA: a, M4; b, M13/1. 70S-associated RNA: c, M4; d, M13/1.*

pol gene products affects the level of tRNA^{Pro} in MuLV suggest that such binding does take place in vivo. Differences in the tRNA binding properties of the avian and murine reverse transcriptase enzymes, however, may affect the degree to which tRNA primer is enriched in virions and could reflect differences in the protein structure of these enzymes. For example, the MuLV enzyme contains a single subunit (31, 45), whereas the avian polymerase is composed of two subunits (25). In addition to these considerations, it is also possible that qualitative differences between the mutations in the avian and murine mutants could result in a quantitative effect on the amount of tRNA primer encapsidated into mutant virions. It should be emphasized, however, that despite these quantitative differences, it is clear that polymerase has a major effect on selection of the tRNA primer used by avian and murine retroviruses. Further work will be needed to assess the possible role of other viral RNA binding proteins in this process, particularly in the case of MuLV.

In addition to interacting with polymerase, tRNA primer molecules bind to 35S genomic RNA in a highly specific reaction involving hybridization of 16- to 19-nucleotide base pairs near the 3′ terminus of the tRNA (6, 10, 35). Since this region of native tRNA is already intramolecularly base paired (6, 7, 20, 21, 35), disruption of most or all of these hydrogen bonds would be necessary to facilitate binding to genomic RNA (6). It has been postulated (1, 6, 7, 20) that tRNA primer binding to reverse transcriptase results in the unfolding of the native tRNA conformation and thereby promotes the subsequent interaction with 35S RNA. However, this idea is not consistent with the M13/1 data which show that normal levels of tRNA^{Pro} are associated with 35S RNA (Fig. 2d) in virions which lack polymerase (43). Thus, it would appear that polymerase is not required for tRNA primer binding to 35S RNA.

Although polymerase itself may not unfold the tRNA primer, it seems reasonable to assume that it is a viral protein which has this function. Schulein et al. (42), in a study on the nucleocapsid protein p10 (9), showed that the nucleic acid-binding properties of p10 are consistent with it being a nucleic acid-unwinding protein. In addition, Fresco, Miller, and Long (personal com-

munication) have found that the Rauscher MuLV p10 protein has potent unwinding activity in vitro and is able to unwind helical regions of tRNA. These observations suggest the possibility that p10 itself or possibly a viral precursor protein such as $Pr65^{gag}$ which contains p10 (32) may fulfill the function originally assigned to the polymerase. Studies with other well-defined viral mutants, such as those which might be generated by recombinant DNA techniques, should prove useful in further elucidating the interaction between viral proteins and tRNA during virus assembly and viral RNA-directed DNA synthesis.

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