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Placement of tRNA Primer on the Primer-Binding Site Requires *pol* Gene Expression in Avian but Not Murine Retroviruses

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In an early step in the retroviral infectious process, reverse transcriptase copies the genomic RNA of the virus into complementary minus-strand DNA. The primer for this synthetic event is a molecule of cellular tRNA, which is annealed by its 3′ 18 nucleotides to a region of the genomic RNA termed the primer-binding site (PBS); the sequence of the PBS and hence the identity of the tRNA depend upon the retrovirus species. In addition to the primer tRNA, retrovirus particles contain a substantial number of other tRNA molecules. The latter tRNA population is enriched for the tRNA species which serves as primer for the virus. While there is considerable evidence that the enrichment for the primer species can be attributed to the *pol* gene product, nothing is known regarding mechanisms of annealing the primer to the PBS. We have analyzed *pol*$^-$ mutants of avian leukosis virus (ALV) and murine leukemia virus (MuLV) for the presence of primer at the PBS in virion genomic RNA. Remarkably, the results were different for the two viruses: the PBS was substantially occupied by primer in MuLV but not in ALV. Previous data indicates that the Pol-dependent enrichment of the primer within the virion is much greater in ALV than in MuLV. We therefore propose that the absence of primer at the PBS in *pol*$^-$ ALV is due to the deficiency of the primer species within the particle. The results suggest that, at least in MuLV, the tRNA is unwound by either the Gag protein or a cellular protein for annealing to the PBS. Further, the C-terminal 17 amino acids of Gag are unnecessary for this function in MuLV.

In all retroviruses, the primer for minus-strand DNA synthesis is a cellular tRNA (for reviews, see references 1 and 18). The viral genomic RNA contains a region, termed the primer-binding site (PBS), that is complementary to 18 bases at the 3′ end of the tRNA species used as the primer. The particular tRNA species serving as the primer is different in different retroviruses; thus, human immunodeficiency virus type 1 (HIV-1) and its close relatives use $tRNA_3^{Lys}$, avian leukosis viruses (ALVs) use $tRNA^{Trp}$, and murine leukemia viruses (MuLVs) use $tRNA^{Pro}$ (for a review, see reference 18). In a mature infectious retrovirus particle, the primer tRNA is base-paired to each molecule of genomic RNA along the entire 18-nucleotide length of the PBS. The mechanisms by which the primer tRNA is incorporated into the particle during virus assembly and hybridized to the PBS are not well understood.

Several studies (3, 26) have shown that mutant particles lacking viral protease (PR) activity contain primer tRNA bound to the PBS; therefore, if viral proteins participate in tRNA incorporation into the particle and hybridization to the PBS, they are presumably the Gag or Gag-Pol polyproteins rather than the cleavage products found in the mature infectious virion.

In addition to the primer tRNA, retrovirus particles contain a large number of molecules of small cellular RNAs, including other tRNA molecules. These "free" tRNA molecules are enriched for the tRNA species that serves as the primer for reverse transcription (for a review, see reference 31). While the mechanism of this enrichment during virus assembly is also not fully understood, studies with mutants have shown that the enrichment depends upon the presence of the *pol* gene product in several retrovirus systems (14, 21, 24). In ALV, the mature reverse transcriptase (RT) molecule has a specific affinity for $tRNA^{Trp}$ (20), while in HIV-1, deletions in the RT coding region prevent the enrichment for $tRNA_3^{Lys}$ among the free tRNAs in the particle (17). These observations suggest that it is the RT domain of the Gag-Pol polyprotein that is responsible for the enrichment of the primer species during virus assembly.

In contrast to these observations on free tRNA in virions, the presence of primer tRNA at the PBS has not been extensively studied in *pol*-negative mutant particles. The apparent role of the RT domain of Gag-Pol in incorporation of the primer species into the virion might suggest that occupancy of the PBS by the primer would be quite deficient in *pol* mutant particles. We have tested this prediction in both ALV and MuLV. Interestingly, the results were different in the two cases: the prediction was confirmed in the former case but not in the latter case.

MATERIALS AND METHODS

Cells and viruses. Experiments with ALV used the replication-competent molecular clone, RCAS, described by Hughes et al. (13). ALV was grown in primary cultures of chicken embryo fibroblasts prepared from line 0 (EV-0) chickens as described previously (32).

Wild-type Moloney MuLV (Mo-MuLV) was derived from the molecular clone pRR88 (7). MuLV was produced in 293T cells containing simian virus 40 large T antigen (6).

Construction of ALV and MuLV mutants. Four deletion mutants and one mutant with an inversion in the ALV genome were constructed from RCAS by

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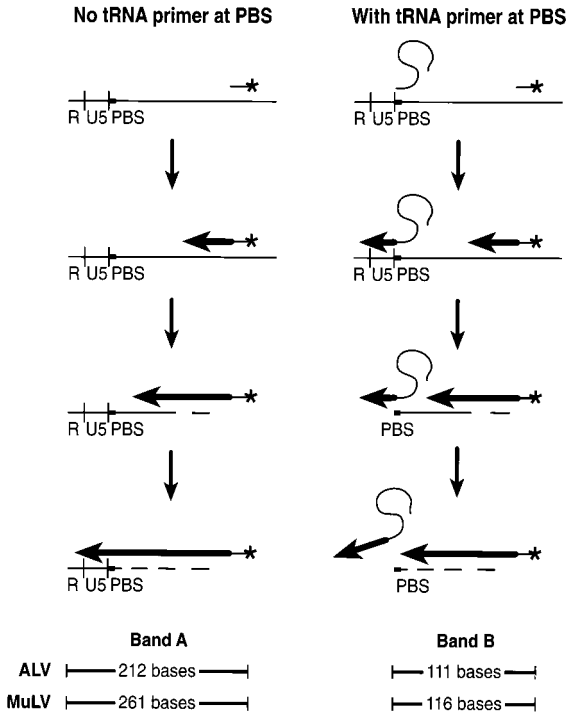

FIG. 1. Schematic depicting the primer extension assay used to determine the occupancy of PBS in mutant and wild-type genomic RNA. A ^{32}P-labeled oligodeoxynucleotide primer (—*) is extended by AMV RT in the presence of deoxynucleoside triphosphates to map the termini of the viral RNAs present. If no tRNA is present at the PBS, the labeled primer will be extended to the 5′ end of the viral RNA, forming a product of 212 bases in ALV and 261 bases in MuLV. In contrast, if the PBS is occupied by a tRNA molecule, the tRNA and the labeled primer will both be extended. The RNase H activity in RT will digest the genomic RNA template on the 5′ side of the PBS; in this case, the labeled primer will be extended only 111 bases (ALV) or 116 bases (MuLV) before reaching the end of the template.

excising restriction fragments by standard techniques (23). In all cases except RCAS-AvrIIinvert, the digested DNAs were rendered blunt ended with T4 DNA polymerase before religation. Each deletion was confirmed by restriction mapping.

A mutant of Mo-MuLV with a point mutation, R44ter, has been described previously (22); in this mutant, codon 44 of the nucleocapsid protein has been changed from arginine to a termination codon.

Transfection, virus preparation, and RNA purification. Transient transfections were performed by the $CaPO_4$ technique (8). In the case of ALV, the cells were exposed to a glycerol shock 4 h after addition of the DNA-$CaPO_4$ precipitate (12). Virus was collected 24 and 48 h later and was pelleted out of clarified cell culture supernatants, and the RNA was isolated as described previously (32).

In the case of MuLV, successive 24-hour harvests of virus were taken for 4 days following transfection. Virus was isolated and the RNA was purified as described by Fu and Rein (5). Levels of MuLV genomic RNA were analyzed in nondenaturing Northern blots as described previously (5) followed by quantitation of hybridized radioactivity with a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, Calif.).

Primer extension assay for PBS occupancy in viral RNA. The assay for the level of PBS occupancy in genomic RNA, detailed below and depicted in Fig. 1, was described previously (32).

(i) End labeling of primer. A single-stranded DNA oligonucleotide primer was labeled at the 5′ end with [γ-^{32}P]ATP (6,000 Ci/mmol) and polynucleotide kinase (New England Biolabs, Beverly, Mass.). A Sephadex G-25 column (Boehringer Mannheim Corp., Indianapolis, Ind.) was used to remove unincorporated nucleotides.

In studies of ALV, the primer had the sequence 5′CTCCACCAGGGTC ATCGAACTCGCCTC3′. This 27-base sequence is complementary to nucleotides 214 to 188 of the genomic RNA of RCAS (taking the 5′ end of the genomic RNA as nucleotide 1); nucleotide 188 is 67 bases downstream of the 3′ end of the PBS. In the experiments with MuLV, the primer was 5′TCAGTCATAGA CACTAGACAATCGG3′, complementing nucleotides 261 to 237 of the Mo-MuLV sequence (25). The Mo-MuLV PBS is at nucleotides 146 to 163 (25).

(ii) Hybridization and primer extension. Purified viral genomic RNA was either left untreated or heated to 100°C for 5 min to remove the endogenous tRNA primer. A 1-ng portion of ^{32}P-labeled oligonucleotide primer was annealed to approximately 1 ng of viral RNA at 42°C for 5 min in 50 mM Tris-Cl (pH 8.3)–60 mM NaCl–12 mM $MgCl_2$–20 mM dithiothreitol–1 mM each deoxynucleoside triphosphate–0.1% Nonidet P-40–50 μg of actinomycin D per ml. Primer extension was initiated by the addition of 25 U of avian myeloblastosis virus RT (Boehringer Mannheim) and allowed to proceed at 42°C for 1.5 h. The reaction products were passed through Sephadex G-50 columns, dried under vacuum, and resuspended in gel loading buffer containing 50% formamide. The samples were boiled for 5 min prior to fractionation in a 5% denaturing polyacrylamide–urea gel in Tris-borate-EDTA (TBE) buffer at 1,500 V for 2.5 h. The dried gel was autoradiographed, and the X-ray film was developed with a Kodak RP X-OMAT processor. Gel bands were quantitated by integration of volume and subtraction of local background levels with a PhosphorImager.

Primer-tagging assay on MuLV. MuLV genomic RNA was also assayed for the presence of primer at the PBS by a primer-tagging assay, performed as described previously (32), except that the RT was from Mo-MuLV (Life Technologies, Inc., Gaithersburg, Md.) rather than AMV. Purified viral RNA was incubated with MuLV RT and [α-^{32}P]dATP in 10 μl of reverse transcription buffer (supplied by the manufacturer) for 1 h at 37°C. The samples were extracted with phenol-chloroform and precipitated with ethanol. The RNA was resuspended in gel-loading buffer containing 50% formamide, boiled for 5 min, and analyzed by electrophoresis in a 10% polyacrylamide–urea gel in TBE followed by autoradiography.

Analysis of MuLV proteins. MuLV particles were analyzed by immunoblotting following sodium dodecyl sulfate-polyacrylamide gel electrophoresis with goat antiserum against $p30^{CA}$ (27) and rabbit antiserum made against a recombinant protein containing RT and a portion of IN (11). The RT activity in the MuLV particles was assayed as described previously (6).

RESULTS

***pol*⁻ mutants of ALV.** Four ALV mutants with the *pol* coding region disrupted were generated for the present study; they are depicted in Fig. 2. In one of these mutants, termed RCAS-AvrIIinvert, the disruption also extends into the PR (p15) region of Gag and alters the last 16 residues of PR. The PR defect in this mutant was confirmed by immunoblotting assays, which demonstrated the presence of unprocessed Gag polyprotein in virions (data not shown). Two other *pol* mutants, RCAS-ΔHpa/Cla and RCAS-ΔHpa/Kpn, have a deletion that removes all but the first 77 amino acids of RT. These mutants differ in that RCAS-ΔHpa/Cla lacks the IN coding region and the entire *env* gene while in RCAS-ΔHpa/Kpn the *env* gene is intact. The fourth *pol* mutant, RCAS-ΔKpn/Cla, has a deletion that removes the 3′ end of the IN coding region (eliminating the C-terminal 63 amino acids of IN) and the entire *env* gene. As a control, a fifth mutant, RCAS-ΔSal/Cla, was also made; this mutant lacks the 3′ half of the *env* gene, but its *gag* and *pol* genes are intact. All of these mutants generated virus particles at levels similar to that of the wild type, as determined by immunoblotting with antibody against matrix protein (data not shown).

PBS occupancy in *pol*⁻ mutants of ALV. To determine whether the PBS is occupied by primer tRNA in *pol* mutants of ALV, we transfected molecular clones of the mutant and wild-type viral genomes into chicken cells. Supernatants were harvested from these cultures 24 and 48 h after transfection; virus was pelleted, and its RNA was analyzed for PBS occupancy by a primer extension assay as described in Materials and Methods (Fig. 1). Under the conditions of this assay, a genomic RNA molecule whose PBS is occupied by a tRNA primer will give rise to a 111-nucleotide radioactive band (band B in Fig. 1) whereas a genomic RNA molecule lacking tRNA will yield a 212-nucleotide radioactive band (band A; this band is the same as strong-stop DNA). Thus, the ratio of radioactivity in the 111-base product to that in the sum of the 111- and 212-base products was taken as a measure of the occupancy of the PBS by the primer. As an additional control, an aliquot of the genomic RNA was heated to 100°C to remove the tRNA from

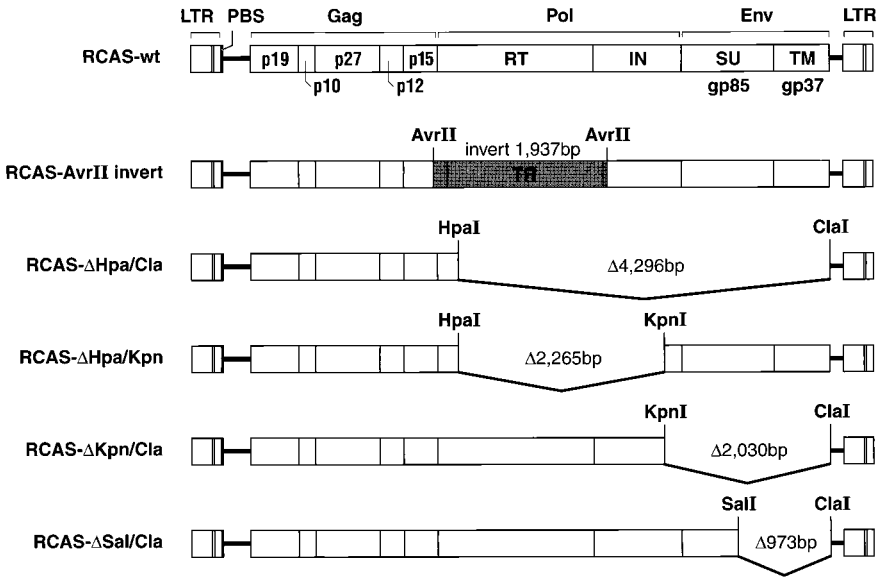

FIG. 2. Schematic depicting mutants of RCAS used in the present study. The mutants included RCAS-AvrIIinvert, with an inversion of the sequence between bases 2435 and 4372, disrupting PR, RT, and IN; RCAS-ΔHpa/Cla, with a deletion of bases 2734 to 7030, disrupting RT, IN, and Env; RCAS-ΔHpa/Kpn, with a deletion of bases 2734 to 4999, disrupting RT and IN; RCAS-ΔKpn/Cla, with a deletion of bases 5000 to 7030, disrupting IN and Env; and RCAS-ΔSal/Cla, with a deletion of bases 6057 to 7030, disrupting Env. Nucleotide positions are given with the 5′ end of genomic RNA taken as position 1.

the PBS; as noted previously (32), the tRNA primer was evidently only partially removed by this procedure.

Results of a typical experiment are shown in Fig. 3 and summarized in Table 1. In the wild-type sample, the radioactivity in the 111-nucleotide band was slightly greater than that in the 212-nucleotide band; this indicates that the PBS is occupied by primer tRNA in at least half of the molecules of genomic RNA. In contrast, in each of the four *pol*$^-$ mutants, very little radioactivity was detected in the 111-nucleotide region but a prominent band was seen at 212 nucleotides. These results show that PBS occupancy in the *pol*$^-$ mutants is 1/10 or less of that in the wild-type control (Fig. 3; Table 1). This

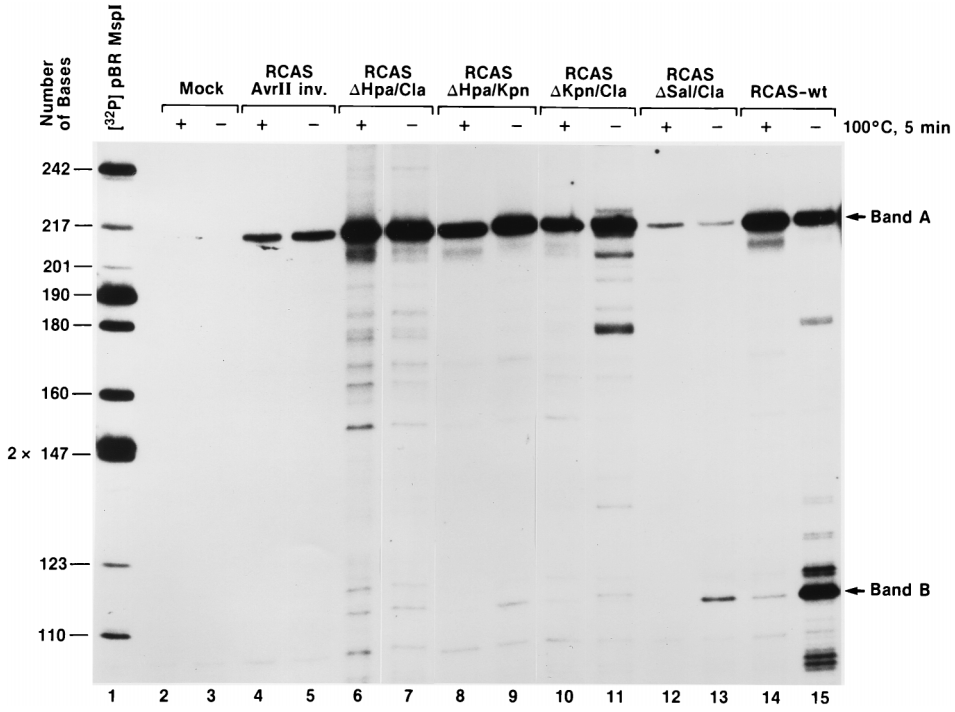

FIG. 3. Autoradiograph of a gel showing results of the primer extension assay in ALV. Samples of genomic RNA were (+) or were not (−) heated to 100°C for 5 min to remove the tRNA primer before the primer extension assay was performed. Band A, 212-base primer extension product; Band B, 111-base primer extension product. The mock-transfected cells received no DNA during the transfection procedure.

TABLE 1. Percentage of ALV genomic RNA with tRNA primer bound at the PBS

Lane(s) in Fig. 3	DNA construct	Viral proteins affected	% PBS occupancy[a]	
			Unheated	Heated[b]
1	[^{32}P] pBR-MspI	NA[c]	NA	NA
2, 3	Mock transfection	NA	NA	NA
4, 5	RCAS-AvrII invert	RT, IN, PR	5.4	8.1
6, 7	RCAS-ΔHpa/Cla	RT, IN, SU, TM	3.2	3.1
8, 9	RCAS-ΔHpa/Kpn	RT, IN	3.2	2.6
10, 11	RCAS-ΔKpn/Cla	IN, SU, TM	4.6	6.5
12, 13	RCAS-ΔSal/Cla	SU, TM	54.0	13.0
14, 15	RCAS-wt	None	52.0	4.5

[a] Percent PBS occupancy = [(PhosphorImager units in 111-nt band)/(PhosphorImager units in 111- plus 212-nt band)] × 100.
[b] Purified ALV genomic RNA was heated to 100°C for 5 min to remove the tRNA primer prior to primer extension experiment.
[c] NA, not applicable.

deficit in occupancy is specifically attributable to the disruption of the Pol coding region in these mutants, rather than to their inability to replicate, since the mutant with a deletion entirely in *env* (i.e., RCAS ΔSal/Cla) shows the same level of occupancy as the wild type (Table 1). (There is also a significant level of radioactivity at 180 nucleotides in the wild-type and ΔKpn/Cla samples. The nature of this band is under investigation.)

***pol*⁻ mutant of MuLV.** The results presented above indicate that *pol* gene expression is required for efficient placement of tRNA on the PBS in ALV. To determine whether this is generally true for retroviruses, we also investigated the presence of the tRNA primer on the PBS in a *pol* mutant of MuLV. This mutant, R44ter (22), contains a premature termination codon which eliminates the 17 C-terminal amino acids of the Gag polyprotein. While this change has little or no effect on virion production or RNA encapsidation (22, the mutation would be expected to prevent translation of the *pol* coding region. This is because *pol* expression in wild-type MuLV is accomplished by translational suppression of the *gag* termination codon. A complex, bipartite signal in the viral mRNA is required in *cis* for this suppression event (4, 33; reviewed in reference 10); this signal is located immediately 3′ of the *gag* termination codon. It seemed extremely unlikely that the context of the premature termination codon in R44ter would induce efficient readthrough of this codon.

We examined R44ter particles for the presence of *pol* gene products in several ways. First, the particles were tested by immunoblotting with anti-p30^{CA} antiserum. As shown in Fig. 4A, this antiserum reacted with Pr65^{gag} in these particles, rather than with p30^{CA} as in the wild-type control. The fact that Pr65^{gag} is not cleaved shows that R44ter lacks a functional PR; this is expected since PR is encoded in the *pol* gene, on the 3′ side of the normal *gag* termination codon, in MuLV.

We also tested for the other *pol*-encoded proteins, i.e., RT and IN, by direct immunoblotting of virus particle preparations with an antiserum capable of reacting with both of these proteins. As demonstrated in Fig. 4B, neither of these proteins was seen in R44ter particles, although both were detected in the wild-type control.

In addition, we tested R44ter particles for RT activity. This should be a sensitive assay for the expression of RT sequences, since the Gag-Pol polyprotein present in PR⁻ MuLV particles possesses a high level of RT enzymatic activity (3). To determine the level of sensitivity of our assay, we also measured the RT activity in a series of dilutions of the wild-type particle preparation. The results are presented graphically in Fig. 5 and show that the wild-type sample gave a practically linear dose-response over a 1,000-fold range. In contrast, incorporation of [^{3}H]TTP with R44ter was indistinguishable from the background level of incorporation seen with supernatants of cells transfected with the plasmid vector alone. Hence, in all of these tests, we found no evidence for expression of the *pol* gene in the R44ter mutant.

PBS occupancy in R44ter MuLV. The occupancy of the PBS in R44ter virion RNA was initially tested by the primer-tagging assay described in Materials and Methods. Genomic RNA was extracted from wild-type and R44ter particles and quantitated following nondenaturing Northern analysis as described previously (5). In the experiment shown in Fig. 6, the yield of viral RNA from the R44ter particles was approximately 1.4 times that from the wild-type control (Fig. 6A; Table 2). The same

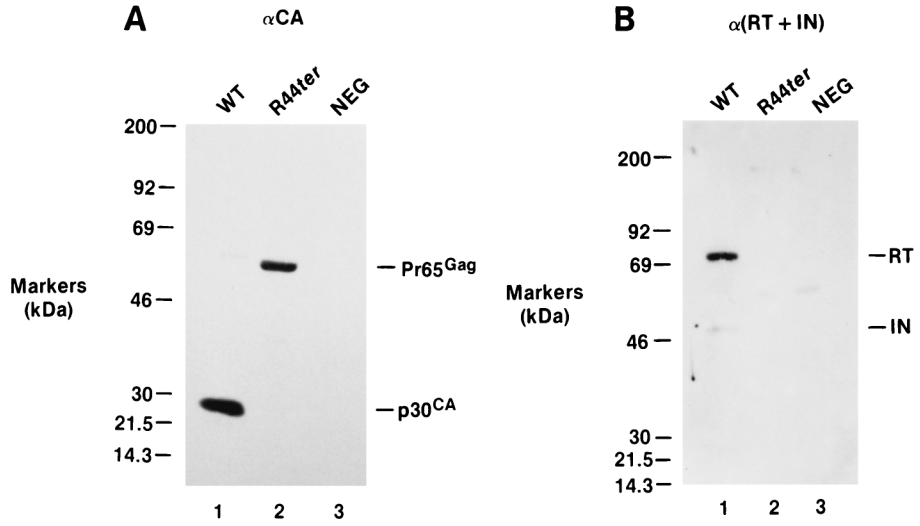

FIG. 4. Comparison of the protein composition of wild-type and R44ter Mo-MuLV particles by immunoblotting. (A) Analysis with anti-p30^{CA} antiserum (αCA). (B) Analysis with anti-(RT + IN) antiserum [α(RT + IN)]. NEG, analysis of supernatant from 293T cells transfected with the pGCcos3neo plasmid vector, lacking a proviral genome; WT, wild type.

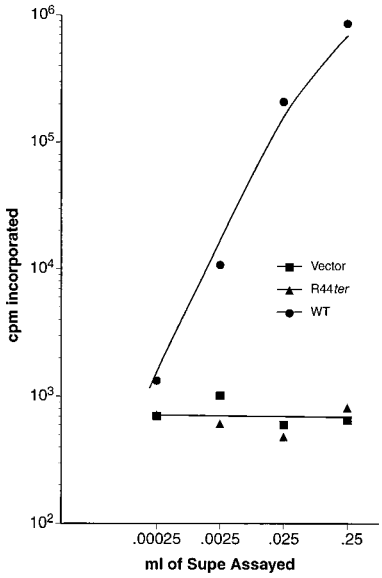

FIG. 5. Absence of detectable RT activity in R44ter particles. Particles were disrupted and assayed at several dilutions as described previously (6). Vector, supernatant (Supe) from 293T cells transfected with the pGCcos3neo plasmid vector.

RNA preparations were then incubated with MuLV RT and [^{32}P]dATP; under these conditions, primer tRNA annealed to the PBS would be extended by one or two dAMP residues, generating a radioactive band 76 or 77 nucleotides in length. The RNAs were then reextracted and analyzed by electrophoresis on polyacrylamide gels followed by autoradiography. As shown (Fig. 6B; Table 2), the radioactivity in the primer tRNA band in the R44ter assay was about 1.5 times that in the wild-type control; thus (Table 2), the extent of PBS occupancy in R44ter genomic RNA, as measured by primer tagging, is virtually identical to that in the wild-type control. It should also be noted (Fig. 6B) that the radioactively tagged primer in R44ter had the same electrophoretic mobility as that in the wild type. Since different primers exhibit different mobilities under these conditions (32), this observation strongly suggests

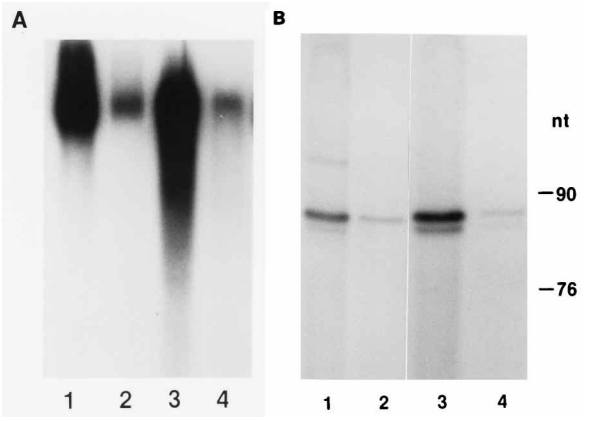

FIG. 6. Assay by primer tagging of PBS occupancy in genomic RNA from wild-type and R44ter Mo-MuLV particles. (A) Level of genomic RNA in wild-type and R44ter samples, as assayed by nondenaturing Northern blotting at two dilutions. (B) Level of primer at the PBS, assayed by primer tagging at two dilutions of genomic RNA. Lanes: 1, wild type; 2, wild type, 1:10; 3, R44ter; 4, R44ter, 1:10.

TABLE 2. Occupancy of Mo-MuLV PBS measured by primer tagging

Sample	Relative PhosporImager units		% PBS occupancy[c]
	Total RNA[a]	Primer tagging[b]	
Wild-type Mo-MuLV	100.0	100.0	100.0
Wild-type 1:10	13.5	9.8	73.0
R44ter Mo-MuLV	143.0	153.0	107.0
R44ter 1:10	10.6	8.3	78.0

[a] As measured by radioactivity associated with genomic RNA in a nondenaturing Northern blot, normalizing to undiluted wild type = 100.0%.
[b] Incorporation of [α-^{32}P]-dATP to the primer band, normalizing to undiluted wild type = 100.0%.
[c] (PhosphorImager units in primer band)/(PhosporImager units in genomic RNA band) $\times$ 100.

that the primer on the PBS in R44ter particles, like that in wild-type Mo-MuLV, is tRNA^{Pro}.

The results in Fig. 6 and Table 2 strongly suggested that MuLV differs from ALV in the requirement for *pol* gene products for PBS occupancy. To ensure that this difference was not due to the difference in the assay methods used with the two viruses, we also measured PBS occupancy in wild-type and R44ter MuLV RNA by primer extension, using the same procedures as with ALV (Fig. 3; Table 1). The results of a typical experiment are shown in Fig. 7 and summarized in Table 3. Quantitation of the 261- and 116-nucleotide bands in Fig. 7 showed that the level of occupancy in R44ter RNA is approximately 60% of that seen with RNA from wild-type virus. Thus, these experiments confirm that primer placement is efficient in the absence of the *pol* gene product in MuLV. In addition, these results (Tables 1 and 3) demonstrate that in both ALV and MuLV, the PBS of wild-type genomic RNA is at least 50% occupied.

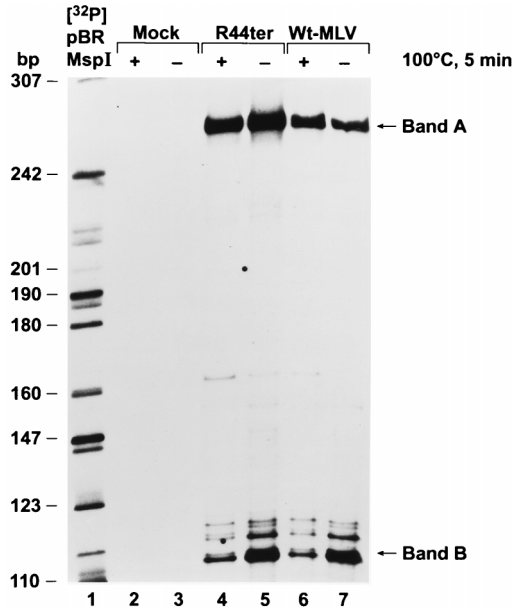

FIG. 7. Assay by primer extension of PBS occupancy in genomic RNA from wild-type and R44ter Mo-MuLV particles. Samples of genomic RNA were (+) or were not (−) heated to 100°C for 5 min to remove the tRNA primer before the primer extension assay was performed. Band A, 261-base primer extension product; Band B, 116-base primer extension product. Mock-transfected cells received salmon sperm DNA during the transfection procedure.

TABLE 3. Occupancy of Mo-MuLV PBS measured by primer extension

Lane(s) in Fig. 7	DNA construct	% PBS occupancy[a]	
		Unheated	Heated[b]
1	[^{32}P]pBR-MspI	NA[c]	NA
2, 3	Mock transfection	NA	NA
4, 5	R44ter	27.1	15.5
6, 7	Mo-MuLV wt	52.8	18.2

[a] Percent PBS occupancy is (PhosphorImager units in 116-nucleotide band)/(PhosphorImager units in 116- plus 261-nucleotide band) × 100.
[b] Purified Mo-MuLV genomic RNA was heated to 100°C for 5 min to remove the tRNA primer prior to primer extension.
[c] NA, not applicable.

DISCUSSION

In this study, we analyzed a series of *pol*⁻ mutants of ALV and MuLV with respect to the presence of the primer tRNA on the PBS of the encapsidated genomic RNAs. The results show that efficient occupancy of the PBS requires expression of the *pol* coding region in ALV but not in MuLV.

Why might *pol*⁻ mutants of ALV and MuLV differ with respect to the occupancy of the PBS? A number of reports have shown that expression of the *pol* gene enriches for the primer tRNA species among the free tRNAs in the virion (14, 17, 21, 24). Since these studies have analyzed ALV, MuLV, and HIV-1, it seems likely that this is a general characteristic of retroviruses. However, the magnitude of this enrichment is quite different for different viruses. Thus, $tRNA^{Trp}$, the primer for ALV, makes up only 1.4% of cellular tRNA but constitutes ~32% of the tRNA present in the MuLV virion (31). In contrast, $tRNA^{Pro}$, the MuLV primer species, is enriched only three- to fourfold in the virion by *pol* expression (14) and even after enrichment is not the most abundant tRNA species in the particle (31). It seems plausible that this difference accounts for the difference between primer occupancy in the ALV mutants (Fig. 3; Table 1) and in the MuLV mutant (Fig. 6 and 7; Tables 2 and 3). We suggest that the low level of $tRNA^{Trp}$ in *pol*⁻ ALV particles is insufficient for significant occupancy of the PBS while the amount of $tRNA^{Pro}$ in *pol*⁻ MuLV virions is evidently large enough for nearly complete occupancy.

The experiments presented here have analyzed the effect of a specific type of *trans*-acting factor, i.e., the *pol* gene product, on the placement of the primer tRNA on the PBS. An alternative approach to analysis of the mechanism of primer placement has been to replace the normal PBS with sequences complementary to the 3′ ends of other cellular tRNA species (15, 16, 28–30, 32). A spontaneous recombinant MuLV with an altered PBS has also been described (2). Briefly, these experiments show that retroviruses can use a number of tRNAs as primers. (While viruses using some alternative tRNA primers replicate more efficiently than others, none of the mutants with altered PBS sequences replicates as well as the corresponding wild-type virus.) These results, taken together with those in this report, strongly suggest that mechanisms exist for the selection and placement of virtually any tRNA primer on a PBS with a complementary sequence, provided that the primer species is present at a sufficient level in the particle. Our results do not shed light on these mechanisms, except to show that they do not require any *pol* products in MuLV. This conclusion is in agreement with the results of an early analysis of a natural *pol*⁻ mutant of MuLV (14) and extends previous reports that PR activity is unnecessary for primer placement in both MuLV and ALV (3, 26). Our results also show that the C-terminal 17 residues of Gag are unnecessary in MuLV. If a viral protein participates in the unwinding of secondary and tertiary structures in the tRNA and genomic RNA, it would presumably be the Gag polyprotein precursor; again, the C-terminal 17 amino acids of this protein are evidently not necessary for this function in MuLV.

While the viral protein that is responsible for enrichment of the primer species in wild-type virions is apparently the Gag-Pol precursor, in vitro studies have previously measured the affinity of mature RT, a cleavage product of Gag-Pol, for the respective primer tRNA species. Interestingly, it was found that mature ALV RT can specifically bind $tRNA^{Trp}$, while no corresponding specificity for $tRNA^{Pro}$ was detected in MuLV RT (9, 19).

It is also interesting that RCAS-ΔKpn/Cla, which has an intact RT coding region but a disrupted IN gene, was defective with respect to PBS occupancy (Fig. 2 and 3; Table 1). This might mean that the IN, as well as the RT, region of Gag-Pol participates in bringing the primer tRNA into the particle and/or placing it on the PBS. However, an alternative explanation is that only the RT region is directly involved but the IN and RT regions of the polyprotein interact with each other; in this case, mutations in IN might alter the conformation of the relevant regions of the RT domain within the Gag-Pol precursor. Indeed, the active RT in an ALV particle, unlike that of MuLV or HIV, includes IN as part of a larger RT subunit; therefore, interactions between the two regions are quite likely.

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W.F. and B.A.O.C. contributed equally to this work.

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