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Replication of Avian Leukosis Viruses with Mutations at the Primer Binding Site: Use of Alternative tRNAs as Primers

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We have tested whether avian leukosis viruses (ALVs) can use tRNAs other than $tRNA^{Trp}$ to initiate reverse transcription. The primer binding site (PBS) of a wild-type ALV provirus, which is complementary to the 3′ end of $tRNA^{Trp}$, was replaced with sequences homologous to the 3′ ends of six different chicken tRNAs ($tRNA^{Pro}$, $tRNA^{Lys}$, $tRNA^{Met}$, $tRNA^{Ile}$, $tRNA^{Phe}$, and $tRNA^{Ser}$). Transfection of these proviruses into chicken embryo fibroblasts resulted in the production of infectious viruses, all of which apparently used the tRNA specified by the mutated PBS to replicate. However, growth of these viruses resulted in reversion to the wild-type ($tRNA^{Trp}$) PBS. Some of the viruses revert quite quickly, while others are more stable. The relative stability of a given PBS correlated with the concentration of the corresponding tRNA in the virion. We determined the percentage of viral RNA that had a tRNA bound to the PBS and found that the occupancy rate is lower in the mutants than in the wild-type virus. We conclude that many different tRNAs can be used as primers to initiate reverse transcription in ALV. However, ALVs that use $tRNA^{Trp}$ have a growth advantage over ALVs that use other tRNAs.

A key step in the retroviral life cycle is the copying, by the viral reverse transcriptase (RT), of the single-stranded viral RNA genome into the linear double-stranded DNA form that is subsequently integrated into the host genome (for reviews, see references 43 and 47). RT carries out all of the steps required to produce this linear double-stranded DNA copy of the viral genome through its three known activities: RNA-dependent DNA polymerase, DNA-dependent DNA polymerase, and RNase H. As is true for many DNA polymerases, retroviral RTs require a primer as well as a template. All retroviruses use a cellular tRNA as a primer to initiate reverse transcription; however, different retroviruses use different tRNAs as primers. For example, avian leukosis virus (ALV) uses $tRNA^{Trp}$ (12), murine leukemia virus (MLV) uses $tRNA^{Pro}$ (28), and human immunodeficiency virus (HIV) uses $tRNA_3^{Lys}$ (17, 34, 44). The factors that determine which tRNA is used by a given retrovirus are not well understood. In all retroviruses the 3′ end of the tRNA primer is base paired to the primer binding site (PBS), a complementary region near the 5′ end of the viral genome. Complementarity to the PBS is not the basis for packaging of the specific primer tRNA into the virion. In fact, the selective packaging of tRNAs is not dependent on their association with the viral genomic RNA (22, 29). ALV mutants that do not package viral genomic RNA produce virions with a wild-type distribution of tRNAs. For ALV, there is reasonably good evidence that the Pol region of the Gag-Pol polyprotein plays a role in the selective packaging of tRNAs. ALV mutants in which *pol* is deleted fail to package the normal complement of tRNAs (29, 39). Similar results have been reported for HIV (25). The mature RTs of both ALV (10, 14, 27) and HIV (3, 37), but not that of MLV (26), selectively bind the specific primer tRNA appropriate for that virus. It is not clear whether this in vitro interaction between the tRNA and the mature RT is similar to the interaction of the Gag-Pol polyprotein with tRNAs during viral assembly.

The set of tRNAs packaged into each virion is a nonrandom subset of cellular tRNAs, and different retroviruses package different subsets of cellular tRNAs (46). In ALV, the species used as the primer, $tRNA^{Trp}$, is the predominant tRNA present in the virion, accounting for about 30% of the packaged tRNA but only about 2% of the total cellular tRNA. However, other tRNAs are also present as a relatively high percentage of the packaged tRNAs. While some tRNAs are enriched relative to their concentration in the host cell, others are selectively excluded from the virion. For instance, $tRNA^{Val}$, which is approximately 10% of the tRNA in the host cell, accounts for only about 0.1% of the tRNA found in ALV virions. Each retroviral particle contains approximately 125 tRNA molecules, 10 of which are directly associated with the mature 70S viral RNA dimer (8). Most of these are only loosely associated with the viral RNA and can be removed by mild denaturing conditions. Only the primer tRNA is tightly associated with viral genomic RNA.

To better understand the factors that determine the selection of specific host tRNAs as primers, we investigated the ability of ALV to use primers other than $tRNA^{Trp}$ to initiate reverse transcription. We produced a series of ALV mutants whose RNA genomes contain PBS sequences that are complementary to the 3′ ends of tRNAs other than $tRNA^{Trp}$, specifically, $tRNA^{Pro}$, $tRNA^{Lys}$, $tRNA^{Met}$, $tRNA^{Ile}$, $tRNA^{Phe}$, and $tRNA^{Ser}$. These tRNAs are normally present in ALV virions at different concentrations. We report here on the growth characteristics of these mutant viruses relative to those of wild-type ALV and on their ability to use the alternate tRNA primers to initiate reverse transcription.

MATERIALS AND METHODS

Construction of mutant proviral vectors. We used megaprimer mutagenesis (38) to introduce the various mutant PBS sequences into the RCASBP vector (30), a replication-competent ALV-based vector derived from Rous sarcoma virus. Basically, a specific primer containing the mutation to be introduced is used with two flanking primers in a two-step PCR protocol. The first PCR uses

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the mutant primer and a flanking primer complementary to the opposite strand to generate a 270-bp megaprimer. Following gel purification, the megaprimer is used with the other flanking primer in the second PCR, generating a 610-bp PCR fragment which now contains the sequence originally present in the mutant primer. The PCR was performed as described previously (48) except that 8% dimethyl sulfoxide was used. The PCR products were purified on a 2% low-melting-point Tris acetate-EDTA agarose gel, and the DNA was recovered by using a Qiaex DNA purification kit (Qiagen, Inc., Chatsworth, Calif.).

The 610-bp PCR products were digested with *Mlu*I and *Sac*I restriction enzymes and subcloned into a "collapsed" version of RCASBP [RCASBP(*Aat*II)] that carries only the upstream long terminal repeat (LTR) region and the 5′ end of *gag* (Fig. 1A). The sequence of the entire *Mlu*I-*Sac*I region, which was generated by PCR, was confirmed by DNA sequencing. In the parental RCASBP vector the sequences flanking the upstream and downstream LTRs are identical; therefore, the region flanking the downstream LTR contains an additional copy of the wild-type PBS. For the experiments described here, the downstream PBS was deleted (ΔA/B in Fig. 1A) from both the wild-type and mutant plasmids so that it could not serve as a source of sequence information to facilitate reversion of the mutant PBS sequences. The infectious proviral clones were reconstructed via a three-way ligation as shown in Fig. 1A. All clones were tested for the loss of a *Bst*EII site (present in the $tRNA^{Trp}$ PBS but not in any of the mutants), and the sequence of the PBS was determined.

Cell culture. Primary chicken embryo fibroblasts (CEFs) were cultured from 11-day embryos of line 0 chickens (EV-0). These cells have no endogenous proviruses that are closely related to the RCASBP vectors (2). The CEF cultures were grown in Dulbecco modified Eagle medium (Life Technologies, Inc., Grand Island, N.Y.) supplemented with 10% tryptose-phosphate broth (Life Technologies, Inc.), 5% heat-inactivated fetal bovine serum (Hyclone Laboratories, Inc., Logan, Utah), 5% heat-inactivated newborn calf serum (Life Technologies, Inc.), and 100 U of penicillin per ml and 100 μg of streptomycin per ml (Quality Biological, Inc., Gaithersburg, Md.). The cells were split 1:3 at confluence, usually every 2 days. ALV-6 fibroblasts were kindly provided by Don Salter and were maintained as described above.

Transfections. CEFs were transfected by the $CaPO_4$ precipitate method (49), including a glycerol shock 4 h after the precipitate was added (15). The cells were passaged at confluence. Prior to passage, the culture supernatant was collected, cleared by centrifugation at 1,500 × *g* for 10 min, and stored at −70°C for later analysis.

RT assays. One milliliter of culture supernatant was centrifuged in a refrigerated Eppendorf tabletop centrifuge for 30 min at the maximum speed. Viral pellets were assayed for RT activity by measuring the incorporation of [α-^{32}P]dGTP into acid-precipitable material, using an oligo(dG) primer and a poly(rC) template as previously reported (30).

Protein preparation and analysis. (i) Virion preparation. One milliliter of culture supernatant was centrifuged in a refrigerated Eppendorf tabletop centrifuge for 30 min at the maximum speed. The supernatant was aspirated, and the viral pellet was dissolved in 30 μl of 1× gel loading buffer (125 mM Tris-Cl [pH 6.8], 1.25% sodium dodecyl sulfate [SDS], 10% glycerol, 0.0125% bromphenol blue, and 568 mM β-mercaptoethanol) and fractionated on an SDS–12% polyacrylamide gel.

(ii) Cell lysates. Plates of transfected cells were washed two times with ice-cold phosphate-buffered saline, and the cells were harvested in 400 μl of 0.5× radioimmunoprecipitation assay buffer (75 mM NaCl, 25 mM Tris-Cl [pH 7.4], 0.5% Triton X-100, 0.5% Na-deoxycholate, 0.05% SDS) and transferred to a 1.5-ml microcentrifuge tube. The cells were subjected to three rounds of freezing and thawing and centrifuged for 15 min at the maximum speed in a refrigerated Eppendorf tabletop centrifuge. Fifty microliters of the cleared supernatant was added to 12.5 μl of 5× gel loading dye (625 mM Tris-Cl [pH 6.8], 6.25% SDS, 50% glycerol, 2.84 M β-mercaptoethanol, 0.0625% bromphenol blue), heated to 100°C, and fractionated on an SDS–10% polyacrylamide gel.

(iii) Western blots (immunoblots). Proteins were transferred to nitrocellulose and probed with a polyclonal anti-MA rabbit serum (generously provided by Volker Vogt, Cornell University) followed by biotinylated goat anti-rabbit antibody and streptavidin-horseradish peroxidase. The complexes were then detected with chemiluminescent substrates (Amersham Corporation, Arlington Heights, Ill.).

RNase protection of virion RNA. Virions were harvested from 1 ml of culture supernatant by centrifugation in a refrigerated Eppendorf tabletop centrifuge for 30 min at the maximum speed. The supernatant was aspirated, and RNase protection was performed with a Lysate Ribonuclease Protection Kit (United States Biochemical Corp., Cleveland, Ohio).

Determination of viral PBS sequence. CEFs were infected with virus at various times after transfection. One milliliter of cleared culture supernatant was added to fresh CEFs at the time of passage. After the cells had attached to the plates, the medium was changed, and 24 h later the cells were washed twice with phosphate-buffered saline, scraped into 1 ml of ice-cold phosphate-buffered saline, and transferred to a 1.5-ml microcentrifuge tube. The cells were collected by centrifugation at 1,500 × *g* for 10 min, the supernatant was removed by aspiration, and the cell pellet was frozen for later analysis.

PCR was performed on crude cell lysates. Two microliters of the loose cell pellet was added to 8 μl of a lysis buffer (1× PCR buffer with 0.1% Triton X-100). To disrupt the cells, the suspension was overlaid with 200 μl of paraffin oil and sonicated with a VibraCell sonicator (Sonics and Materials Inc., Danbury, Conn.) equipped with a microprobe at level 3 for 15 s. The emulsion was then centrifuged at the maximum speed in a refrigerated Eppendorf tabletop centrifuge for 10 min to separate the phases, and 1.5 μl of the aqueous phase was used in the PCR. The PCR used primers in the U3 region (5′ TGGACGAACCACT GAATTCC 3′) and *gag* (5′ CCTTAATGACGGCTTCCATGC 3′) to generate a 464-bp product that was gel purified as described above and sequenced.

Cycle sequencing was performed by using a two-temperature thermal-cycle method in which the incorporation of radiolabel and termination reactions occurred simultaneously. A reaction mixture containing 50 mM Tris-Cl (pH 8.3), 50 mM KCl, 7 mM $MgCl_2$, 100 mM β-mercaptoethanol, 170 μg of bovine serum albumin per ml, 0.25 pmol of primer, 2.5 μM deoxynucleoside triphosphates (dNTPs), 0.5 μCi of [α-^{32}P]dATP (800 Ci/mmol), 1 U of *Taq* polymerase, and 8% dimethyl sulfoxide was prepared. The reaction mixture was divided into four separate tubes, each containing 1 μl of the appropriate ddNTP (ddA at 2.5 mM, ddC at 2.5 mM, ddG at 0.25 mM, and ddT at 5.0 mM). The reaction mixtures were overlaid with paraffin oil, placed in an Uno Thermocycler (Biometra Inc., Tampa, Fla.), and heated at 95°C for 5 min. The mixtures were then subjected to 25 cycles of 90°C for 40 s and 62°C for 80 s. Fifty percent formamide loading dye was added to the reaction mixtures, and the samples were fractionated on a 6% polyacrylamide–urea sequencing gel. The gel was autoradiographed overnight, and the X-ray film was developed with a Kodak RP X-OMAT processor.

Viral RNA purification. Supernatants were collected from CEFs stably transfected either with the wild-type plasmid RCASBP or with one of the RCASBP plasmids containing a mutated PBS. Thirty milliliters of cell culture supernatant was centrifuged at 3,000 rpm for 10 min at 4°C in a Sorvall RC-3 centrifuge and then filtered through cellulose-polyvinyl chloride (0.45-μm pore size) to remove cellular debris. Virions were pelleted through a 15% sucrose cushion by centrifugation at 25,000 rpm for 1 h at 4°C with an SW28 rotor in a Beckman L8 M ultracentrifuge. The viral pellets were lysed in 0.5 ml of lysis buffer (100 mM NaCl, 50 mM Tris-Cl [pH 7.4], 10 mM EDTA, 1% SDS, 100 μg of proteinase K per ml) at 37°C for 30 min. The crude lysates were extracted once with an equal volume of phenol-chloroform (1:1). The viral RNA was precipitated overnight in 2.5 volumes of ethanol at −20°C and collected by centrifugation at the maximum speed in an Eppendorf microcentrifuge for 15 min at 4°C. The RNA pellet was dried under vacuum, resuspended in 30 μl of TEN (10 mM Tris-Cl [pH 7.5], 1 mM EDTA, 50 mM NaCl), and stored at −80°C.

In vitro primer tagging. The tRNA primer attached to the viral RNA genome was labeled by using exogenous avian myeloblastosis virus (AMV) RT or MLV RT (Boehringer-Mannheim, Indianapolis, Ind.) and [α-^{32}P]dATP (800 Ci/mmol). The reaction was carried out at 37°C for 1 h in 10 μl of the RT buffer specified by the manufacturer. The sample was extracted once with an equal volume of phenol-chloroform (1:1). The nucleic acids were precipitated overnight in 2.5 volumes of ethanol at −20°C, collected by centrifugation at the maximum speed in a refrigerated Eppendorf microcentrifuge for 15 min at 4°C, and dried under vacuum. Samples were resuspended in buffer containing 50% formamide, boiled for 5 min, and fractionated in a 10% denaturing polyacrylamide–urea gel in Tris-borate-EDTA (TBE) buffer at 1,500 V for approximately 4 h. The gel was autoradiographed overnight, and the X-ray film was developed with a Kodak RP X-OMAT processor.

Primer extension. (i) End labeling of primer. A single-strand DNA oligonucleotide primer with the sequence 5′ CTCCACCAGGGTCATCGAACTCGC CTC 3′ was synthesized and designated no. 14743. This primer is complementary to a 27-base segment of the RNA genome of RCASBP that is located 67 bases downstream of the PBS. Oligonucleotide 14743 was labeled at the 5′ end by using polynucleotide kinase (New England Biolabs, Beverly, Mass.) and [γ-^{32}P]ATP (6,000 Ci/mmol) as described by the supplier. Unincorporated nucleotides were removed by using a Sephadex G-25 column prepared in a Millipore Ultrafree MC 0.22-μm-pore-size filter unit (Millipore, Bedford, Mass.). The radioactivity in 1 μl of the labeled oligonucleotide was counted, and the remainder was stored at −80°C overnight.

(ii) Hybridization and primer extension. Purified viral RNA was either left untreated or heated to 100°C for 5 min to melt off the endogenous tRNA primer. One nanogram of ^{32}P-labeled oligonucleotide 14743 was annealed to approximately 1 ng of viral genomic RNA at 42°C for 5 min in a buffer containing dNTPs (1 mM each), 50 mM Tris-Cl (pH 8.3), 20 mM dithiothreitol, 12 mM $MgCl_2$, 60 mM NaCl, 0.1% Nonidet P-40, and 50 μg of actinomycin D per ml. Twenty-five units of AMV RT (Boehringer Mannheim) was added to initiate the reaction (see Fig. 5). Primer extension took place at 42°C for 1 h, after which the reaction mixtures were passed through a Sephadex G-50 column and the samples were dried under vacuum. Samples were resuspended in buffer containing 50% formamide, boiled for 5 min, and fractionated in a 5% denaturing polyacrylamide–urea gel in Tris-borate-EDTA buffer at 1,500 V for 4 h. The gel was autoradiographed overnight, and the X-ray film was developed with a Kodak RP X-OMAT processor. The results of the experiment were analyzed with a Molecular Dynamics PhosphorImager SF. Gel bands were quantitated by integration of volumes and subtraction of local background levels.

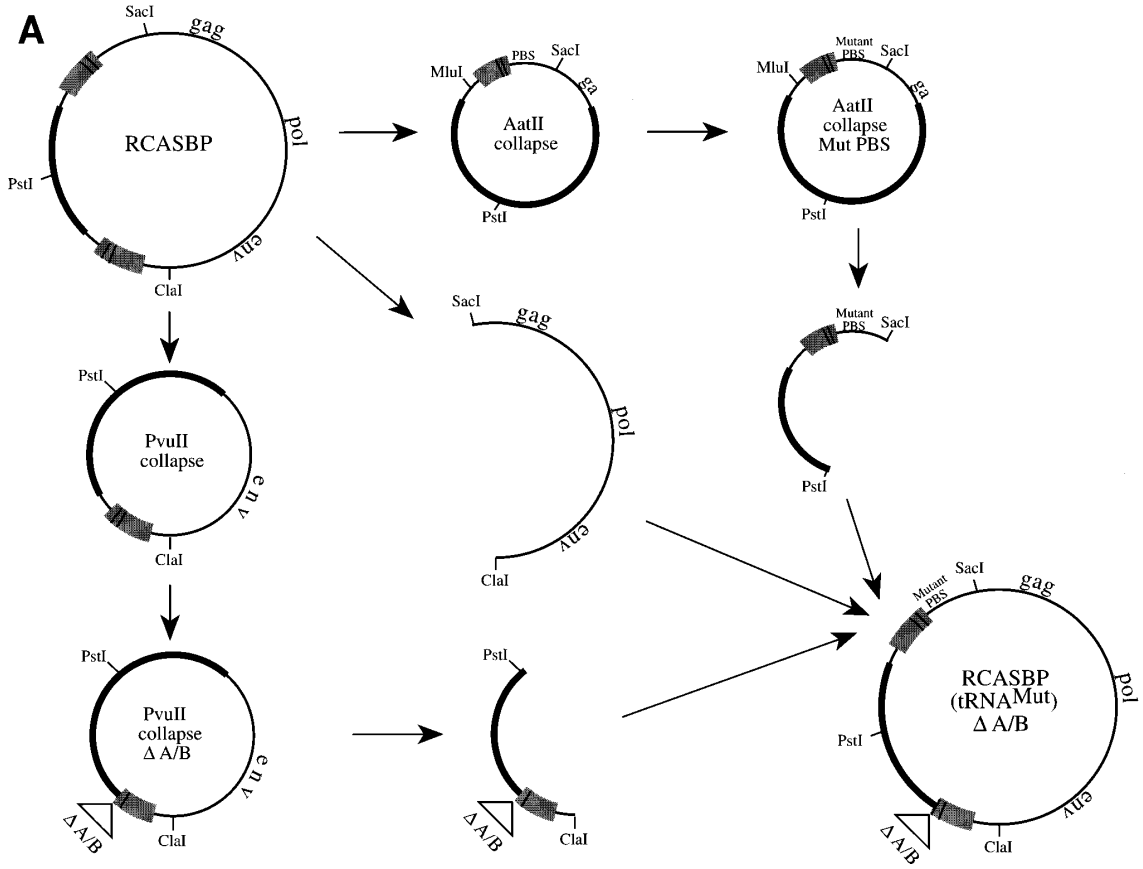


FIG. 1. (A) Schematic diagram depicting the construction of proviral clones containing mutated PBSs. The *Aat*II and *Pvu*II collapse plasmids were generated by digestion with the appropriate enzyme (*Aat*II or *Pvu*II, respectively) and religation in dilute conditions. These two plasmids simplified the manipulation of the upstream and downstream LTRs. For the wild-type and mutant PBS proviral plasmids described here, the downstream LTR was modified by deletion of the U5 and flanking sequences that have homology with the 5′ end of the provirus. The *Aat*II collapse plasmid was used to subclone the PBS mutations generated by megaprimer mutagenesis (see Materials and Methods). The infectious proviral clones containing the mutated PBS sequences were reconstructed by a three-way ligation (*Cla*I-*Pst*I, *Pst*I-*Sac*I, *Sac*I-*Cla*I). All of the viral coding sequences are present on the *Sac*I-*Cla*I fragment, which decreased the risk of introducing mutations into the coding sequences while manipulating the LTR and PBS sequences. (B) Sequences of the wild-type (tRNA^{Trp}) PBS with the 3′ end of a tRNA^{Trp} annealed to it and of the mutant PBS sequences introduced into the proviral plasmids. The white letters in the black boxes highlight the differences between the wild-type and mutant PBSs. The PBS complementary to tRNA^{Ser} has one extra base when aligned with the other PBSs. The limits of the tRNA^{Ser} PBS were selected on the basis of the overall alignment of tRNA^{Ser} with other tRNAs and specifically taking into consideration the position of the first modified base in the tRNA. The tRNAs specified are of the following anticodon specificities: tRNA$^{Met(CAU)}$, tRNA$^{Pro(AGG)}$, tRNA$^{Lys(CUU)}$, tRNA$^{Phe(GAA)}$, tRNA$^{Ile(AAU)}$, and tRNA$^{Ser(UCA)}$.

RESULTS

Construction of viral vectors with mutated PBSs. Six alternative PBS sequences (41) were introduced into RCASBP in place of the wild-type PBS, which is complementary to the 3′ end of $tRNA^{Trp}$ (Fig. 1B). The mutated viral vectors were tested by restriction enzyme digestion and then subjected to DNA sequencing to show that the desired mutations had been introduced. The alternative PBSs are complementary to the 3′ ends of chicken cellular tRNAs that are present in the ALV virion at different relative abundances (46).

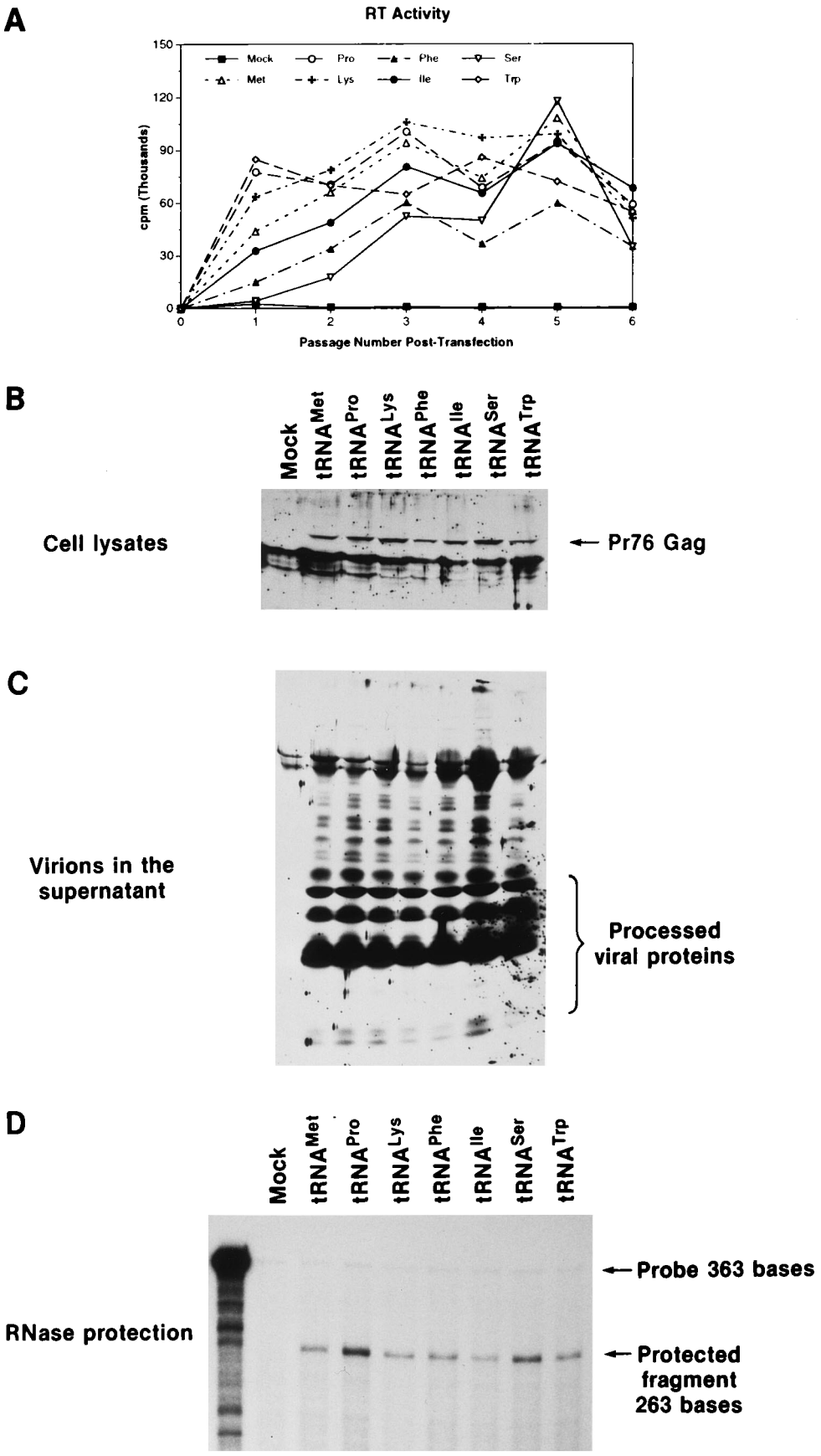
Proviruses carrying mutant PBSs give rise to replication-competent virus. The provirus-containing plasmids were transfected into CEFs, and the culture supernatants were collected at specific times after transfection and analyzed for the production of viral particles by measurement of RT activity or by Western transfer analysis. RT assays showed that all of the mutant proviruses gave rise to infectious virus (Fig. 2A); however, there was a slight delay in the appearance of some of the mutant viruses. In transient-transfection assays, all of the plasmids gave rise to equivalent amounts of viral particles as determined by Western analysis (Fig. 2B and C). These assays were done with fibroblasts derived from ALV-6 chickens. These cells are resistant to infection by subgroup A ALV because they produce the ALV(A) envelope. Therefore, the growth lag of the mutant viruses appears to be due to a slower spread of virus through the culture and not to any deficiency in transcription, translation, or assembly of the viral mutants.

The mutant viruses package normal levels of viral RNA. Viral pellets prepared in parallel to those used for the Western blots shown in Fig. 2C were used in RNase protection experiments employing a probe specific for the viral RNA. Figure 2D shows that all of the mutant PBS viruses packaged approximately equal amounts of RNA per virion (compare the protected RNA fragment in Fig. 2D with the amount of virion protein present in Fig. 2C), suggesting that the mutations that we introduced into the PBS do not significantly affect the packaging of genomic RNA.

Sequence analysis of the PBSs in infectious viruses. The sequences of the PBSs in the viruses were determined, as described in Materials and Methods, at various times following transfection of CEFs with the plasmids carrying the mutant proviruses. Cell-free supernatants from the transfected cultures were used to infect fresh CEFs, and cell lysates were prepared 24 h after infection. PCR was performed to amplify a region that included the PBS. These lysates contain the newly reverse-transcribed viral DNA but may also contain some viral RNA. *Taq* polymerase has weak RT activity (18, 42), so it is theoretically possible that viral RNA could give rise to a product of the desired size through reverse transcription of the viral RNA sequences and a crossover event during PCR. To favor the specific amplification of sequences from viral DNA, the two PCR primers were chosen to be homologous to sequences in U3 (there is no upstream U3 in viral RNA) and in *gag*, respectively, and only 25 cycles of PCR were done. Infection of fresh CEFs served two purposes. First, the transfected DNA used to initiate the infection was not available as a substrate for PCR. Second, during reverse transcription, the PBS sequences are copied from two different sources: the minus strand is copied from the viral RNA, and the plus strand is copied from the tRNA primer used to initiate reverse transcription (for reviews, see references 43 and 47). Thus, the two strands of the PBS in the DNA produced following infection contain sequences both from the PBS of the viral RNA and from the tRNA primer used to initiate reverse transcription. Therefore, it is possible that the PBSs of the two strands could differ in sequence. Any mismatches would be expected to be resolved by additional rounds of replication; however, both sequences would be represented in the results of the PCR-cycle sequencing.

At short times after infection, all of the mutant viruses contained only mutant PBS sequences, indicating that the viruses with the mutated PBSs are replication competent and suggesting that the viruses can use the tRNA primers specified by the mutated PBSs to initiate reverse transcription. Figure 3 shows the sequences of the PBSs present in the viruses after six cell passages. Most of the mutant viruses still had only mutant sequences at their PBSs; however, the mutant containing a PBS homologous to $tRNA^{Ser}$ had already reverted, and the virus in the culture contained the wild-type $tRNA^{Trp}$ PBS. If the mutant viruses were using $tRNA^{Trp}$ to initiate reverse transcription, we would have expected to have seen some $tRNA^{Trp}$ sequences present at the PBS in the DNA produced following infection. The viruses were also analyzed following a series of virus passages onto fresh CEFs. In these experiments, CEFs were maintained for six passages following transfection, and then 1 ml of supernatant from passage 6 was used to infect fresh CEFs. These infected CEFs were grown for three passages, and the virus was again transferred to fresh CEFs. Each transfer of virus to fresh CEFs allowed replication of the virus and provided an opportunity for reversion of the PBS to occur and for any revertant virus to compete with the mutant virus and overgrow the culture. With repeated passage of the virus in culture, all of the mutant-PBS viruses reverted to contain wild-type $tRNA^{Trp}$ PBS sequences, but different mutant viruses reverted at different rates. The exact kinetics of reversion in any given experiment depends on a number of factors, such as the efficiency of transfection and the doubling time of the CEFs, etc., that can vary from experiment to experiment. However, the relative reversion times were entirely consistent between experiments. Figure 4 summarizes the reversion data from a single experiment. In this experiment, for the least stable of the mutant viruses, RCASBP($tRNA^{Ser}$), reversion was evident during the first six cell passages posttransfection. RCASBP($tRNA^{Phe}$) reverted after one virus transfer. Reversion of RCASBP($tRNA^{Ile}$) was observed after two virus transfers, that of RCASBP($tRNA^{Met}$) was observed after three virus transfers, and those of RCASBP($tRNA^{Lys}$) and RCASBP ($tRNA^{Pro}$) were observed only after four virus transfers.

Which tRNA is used as the primer to initiate reverse transcription in the mutant viruses? To determine whether viruses with mutant PBSs were using tRNAs specified by their PBSs to initiate reverse transcription or whether they were using $tRNA^{Trp}$ even in the absence of complete sequence homology at the PBS, primer tagging was used to label the tRNAs annealed to the PBSs on viral genomic RNAs. The first two bases added to a tRNA primer annealed to the PBS in ALV are dA (Fig. 1B); thus, in a primer-tagging experiment using AMV RT, the $tRNA^{Trp}$ was labeled by the addition of either one or two ^{32}P-dA residues (Fig. 5). Under these conditions, AMV RT could label tRNA primers from several of the mutant viruses (Fig. 5). Approximately equal amounts of virus, as determined by RT activity, were used in each assay. All of the alternate tRNAs were labeled less efficiently than the wild-type $tRNA^{Trp}$. The tRNAs labeled by using RNAs isolated from the mutant viruses showed electrophoretic mobilities different from that of $tRNA^{Trp}$, suggesting that the mutant viruses are indeed replicating by using tRNAs other than $tRNA^{Trp}$ to initiate reverse transcription. While it is possible that there are smaller amounts of the alternative tRNAs bound to the PBSs of genomic RNAs in the mutant viruses, it is also possible that the non-Trp tRNAs are poorly extended by the AMV RT. To test this hypothesis, we tried to label each of the alternative


A
RT Activity
Mock
Pro
Phe
Ser
Met
Lys
Ile
Trp
cpm (Thousands)
150
120
90
60
30
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0
1
2
3
4
5
6
Passage Number Post-Transfection
B
Mock
tRNAMet
tRNAPro
tRNALys
tRNAPhe
tRNAIle
tRNASer
tRNATrp
Cell lysates
Pr76 Gag
C
Virions in the supernatant
Processed viral proteins
D
Mock
tRNAMet
tRNAPro
tRNALys
tRNAPhe
tRNAIle
tRNASer
tRNATrp
RNase protection
Probe 363 bases
Protected fragment 263 bases

FIG. 2. (A) Reverse transcriptase assay to show the spread of infectious ALV following transfection of CEFs with wild-type or mutant-PBS proviral plasmid DNAs. Assays were done as described in Materials and Methods with 1-ml samples of culture supernatants harvested at the indicated cell passage following transfection. (B, C, and D) Transient transfection of ALV-6 CEFs with wild-type or mutant-PBS proviral plasmid DNAs. ALV-6 CEFs contain a defective ALV provirus. They produce subgroup A Env protein, but no other viral proteins, and are resistant to infection by subgroup A ALV. Parallel 1-ml samples were analyzed. (B) Western blot analysis of intracellular Gag protein 24 h posttransfection; (C) Western blot analysis of anti-MA reactive viral proteins in the supernatant 24 h posttransfection; (D) RNase protection of viral particle-associated RNA from wild-type and mutant-PBS ALVs 24 h posttransfection.

tRNA primers with a different enzyme, MLV RT. As seen in Fig. 5, there are both quantitative and qualitative differences in the labeling of the tRNAs by the MLV RT. All of the alternate tRNAs could be labeled by either AMV or MLV RT.

To further investigate the tRNAs bound to the PBSs of the viral RNAs, we designed the primer extension assay described in Fig. 6. In this assay we monitor extension of an exogenously added ^{32}P-labeled DNA primer annealed to the viral RNA downstream of the PBS. Several possible outcomes in this experiment are diagrammed in Fig. 6. If there is no tRNA primer annealed to the viral genomic RNA (Fig. 6A), the ^{32}P-labeled oligonucleotide can be extended to the end of the viral RNA (i.e., through the PBS, U5, and R). In the presence of an extendable tRNA primer annealed to the PBS (Fig. 6B), RT can extend the tRNA primer, and RNase H can then cleave the RNA template strand at or near the PBS-U5 boundary. In this case, the labeled oligonucleotide can be extended to either the 3′ end of the PBS, where it encounters an annealed tRNA, or, if RT can displace the tRNA, to the 5′ end of the PBS as shown in Fig. 6B. If the tRNA primer cannot be extended by the RT, there are once again two possible outcomes: the oligonucleotide could be extended to the 3′ end of the PBS or to the 5′ end of the viral RNA, depending on whether or not the unextended tRNA could be displaced. We compared the products derived from templates that were either native viral RNA or RNA that had been heated to 100°C to melt off any tRNA primers.

Figure 7 shows the results of one such experiment. The band at 212 bases (band A) is generated by extension of the exogenous primer to the end of the viral RNA genome. The band at 111 bases (band B) corresponds to primer that has been extended to the 5′ end of the PBS. Band B presumably represents the fraction of viral RNA that had extendable tRNA primers annealed at the PBS that were subsequently displaced by RT. The series of bands present below band B are of the sizes expected if the extended primer was unable to displace the tRNA from the PBS irrespective of whether the tRNA was extended. The different sizes may reflect a partial displacement of the tRNA-PBS complex by RT. In the wild-type virus ($tRNA^{Trp}$), the majority of the product in the unheated lane is in band B, or in the bands below band B, indicating a high percentage of viral RNA with tRNA annealed at the PBS. Following heating, the majority of the primer extension product is in band A, indicating that most of the tRNA has been melted off. The residual band B that is seen in the heated sample may be due either to incomplete removal of the tRNA

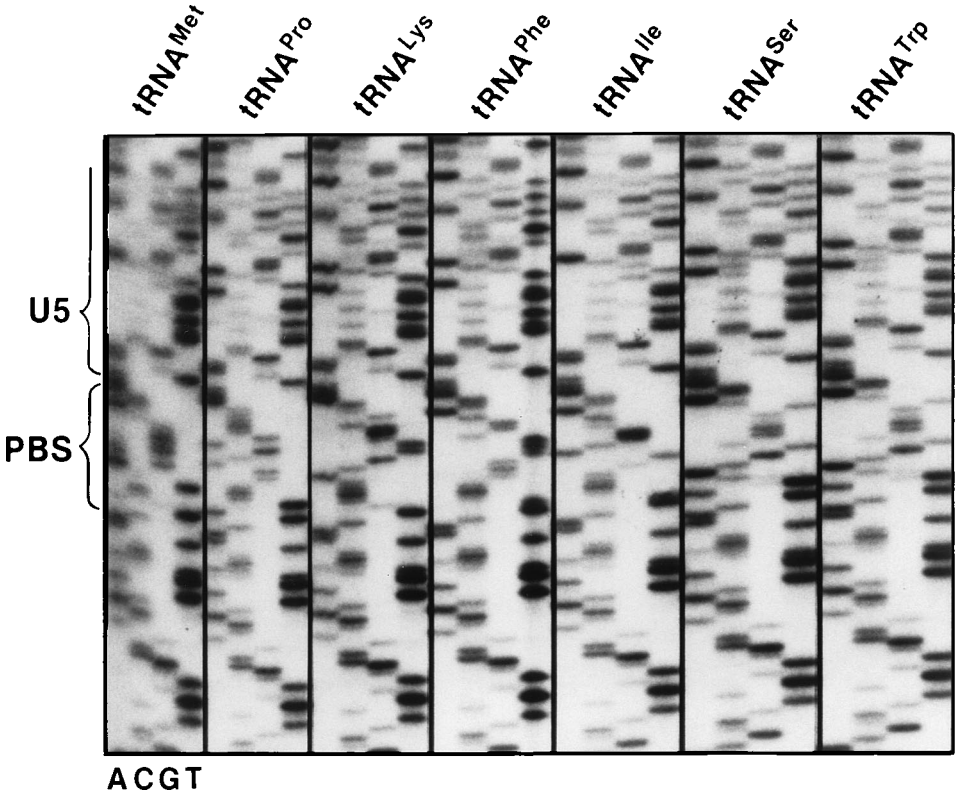

FIG. 3. Sequences of the PBSs of viruses present six cell passages posttransfection. Analysis of sequences was performed as described in Materials and Methods. At this time, most of the infectious viruses that originally encoded mutant PBS sequences still contained mutant PBS sequences. However, the RCASBP($tRNA^{Ser}$) culture had already reverted and contained the wild-type $tRNA^{Trp}$ PBS sequence. At earlier time points (up to passage 3), this virus had the mutant $tRNA^{Ser}$ PBS sequence.

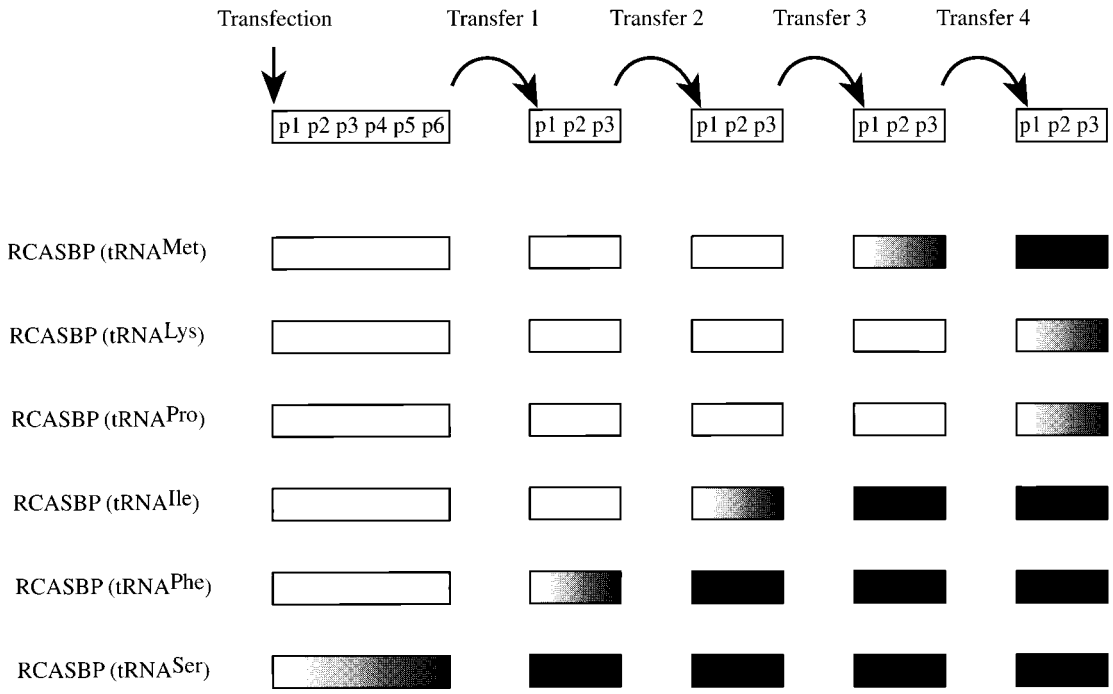

FIG. 4. Summary of reversion data for the viruses carrying mutated PBSs. Each arrow represents a separate viral transfer onto fresh CEFs followed by three passages (p) of CEFs between each viral transfer. The first viral transfer took place at six cell passages posttransfection. The sequences of the PBSs present in the viruses were determined by PCR sequencing as described in Materials and Methods. The white boxes indicate that the virus present had mutant PBS sequences, and the black boxes represent cultures in which the viruses had reverted to predominantly wild-type PBS sequences.

from the viral RNA or to reannealing of the tRNA to the viral PBS under these conditions. The lanes labeled $tRNA^{Ser}$ are from a culture in which the majority of the virus has reverted to the wild-type $tRNA^{Trp}$ PBS (Fig. 3); therefore, the pattern seen is essentially wild type. For the other mutants there is considerably less label present in band B and the lower bands than there is for the wild type. For all of the viral RNAs there are some bands that appear to correspond to incompletely displaced or nondisplaced primer tRNA. All of the mutant viruses give rise to some band B, suggesting that some extendable tRNA is present on all of the viral PBSs. On the basis of the primer-tagging data shown in Fig. 5, it is probable that the degree to which the various primers are extended (and displaced) is to some extent AMV RT specific, and we would not be surprised to obtain different results with different RTs. However, AMV RT is the same as the RT present in the virion in vivo, and therefore these results should be relevant to what is occurring during replication of the mutant viruses. The numbers in Fig. 7B were generated by PhosphorImager analysis of the gel shown in Fig. 7A. On the basis of the ratio of the primer

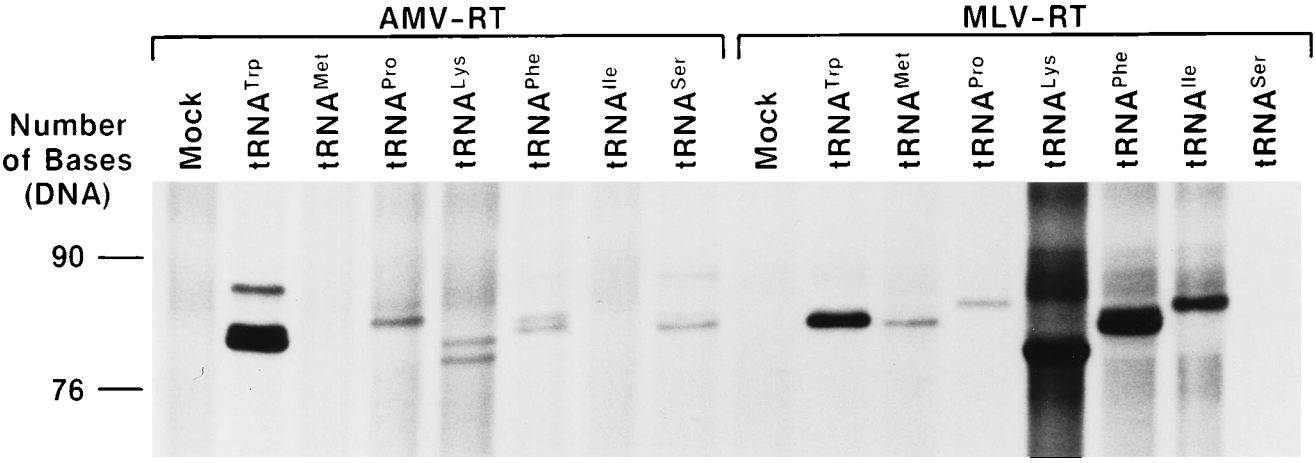

FIG. 5. Autoradiograph of a 10% polyacrylamide–urea gel showing primer tagging of tRNAs present on the wild-type and mutant viral RNAs. Purified viral RNA was incubated in the presence of [α-^{32}P]dATP and either AMV RT or MLV RT under conditions that resulted in the addition of one or two dA bases onto any tRNA primers that were bound to the PBS. The $tRNA^{Trp}$ primer on the wild-type RCASBP RNA was easily labeled under these conditions. We were able to label tRNAs from all of the mutants with AMV RT and/or MLV RT. In all cases, the labeled tRNAs from the mutant viruses show electrophoretic mobilities different from that of the wild-type $tRNA^{Trp}$.

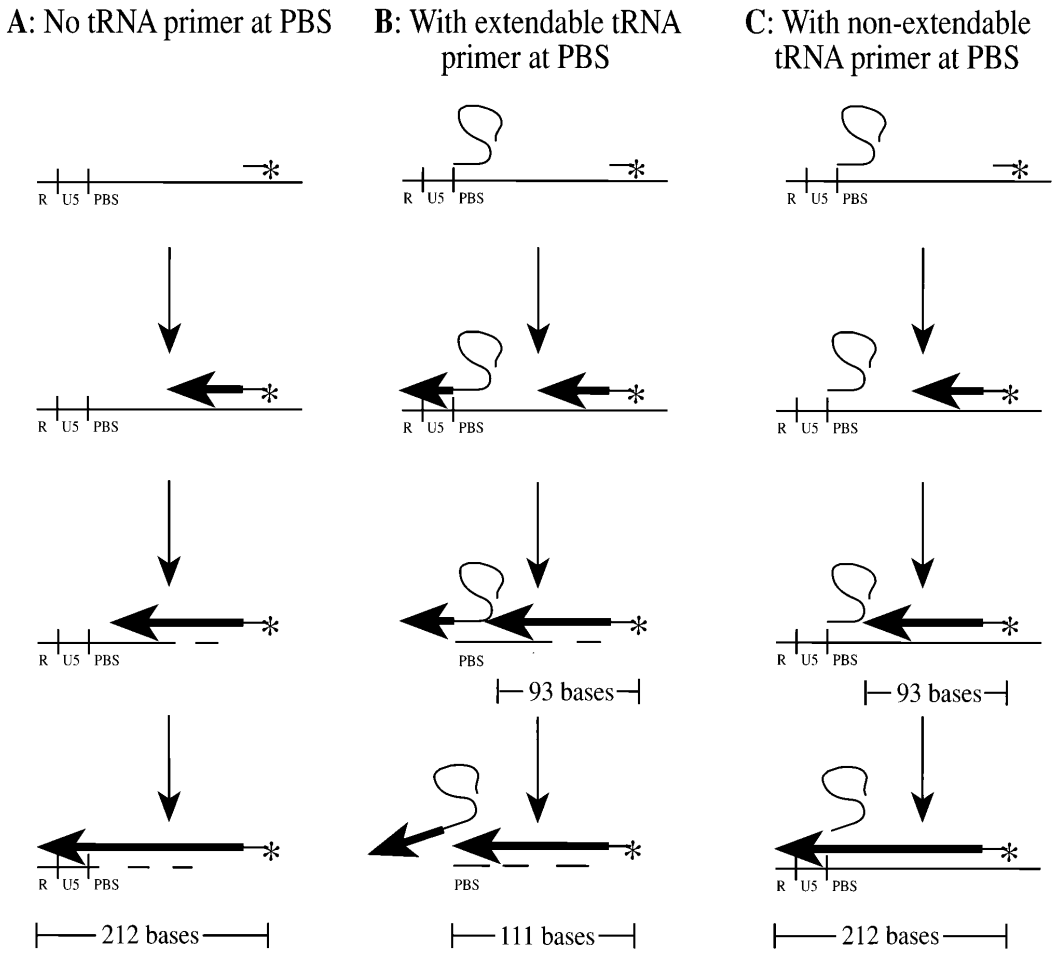

FIG. 6. Schematic depicting the primer extension assay used to determine tRNA occupancy in the wild-type and mutant-PBS viruses. In this experiment, a ^{32}P-labeled DNA oligonucleotide primer (—*) was extended by AMV RT in the presence of dNTPs to map the termini of the viral RNAs present. The different panels show the different results expected depending on whether or not a tRNA is annealed to the PBS and is extendable by AMV RT (see Results). Because each viral RNA sample is a mixture of RNAs that do or do not have tRNA annealed, both bands can be generated from each sample. The ratio between the radioactivity present in the tRNA-specific band and the total radioactivity present in both bands is a measure of the percentage of viral genomic RNA that has a tRNA primer annealed at the PBS.

extension products, about 70% of wild-type ALV RNA has a tRNA primer bound to the PBS which can be extended by AMV RT. The extension ratios for all of the mutant tRNAs are lower. This suggests that a limiting step in the replication of these viruses may be the annealing and/or subsequent extension of the appropriate tRNA primer. These results also show that AMV RT can displace the tRNA from the PBS, although not with 100% efficiency, in the absence of any other viral or cellular factors.

DISCUSSION

The initiation of reverse transcription is a key step in the retroviral life cycle and requires specific interactions between a cellular tRNA primer, the viral RNA genome, and viral proteins. We have produced a series of ALV mutants containing alternate PBSs in order to investigate the role of the PBS sequences in the choice of the tRNA primer used to initiate reverse transcription. These studies show that, in addition to $tRNA^{Trp}$, other tRNAs can function as primers for the initiation of reverse transcription in ALV, both in vitro and in vivo. However, replication of these mutant-PBS viruses results in reversion of the PBS sequences to sequences that are complementary to the wild-type primer $tRNA^{Trp}$.

Why do the mutant-PBS viruses revert? Our data show that although several different tRNAs can serve as primers, ALV does show a clear preference for $tRNA^{Trp}$, and the viruses that use $tRNA^{Trp}$ have a growth advantage over those that use other tRNAs as primers. This is suggested both by the fact that the viral cultures revert to wild type with passage and by the slower initial spread of the mutant-PBS viruses through the culture following transfection of the viral DNAs. Thus, the rates of reversion that we have observed reflect both the frequency of reversion and the ability of a mutant virus to compete with reverted wild-type virus during spread through the culture.

Why do the viruses with mutant PBSs grow more slowly? One possible explanation for the slower growth of the mutant PBS viruses is that the tRNA primer does not anneal to the mutant viral RNA genome with the same efficiency and/or affinity as that with which $tRNA^{Trp}$ anneals to wild-type viral RNA. This could be either because there are smaller amounts of the cognate tRNA in the virion or because the alternate tRNA is unable to properly interact either with viral proteins

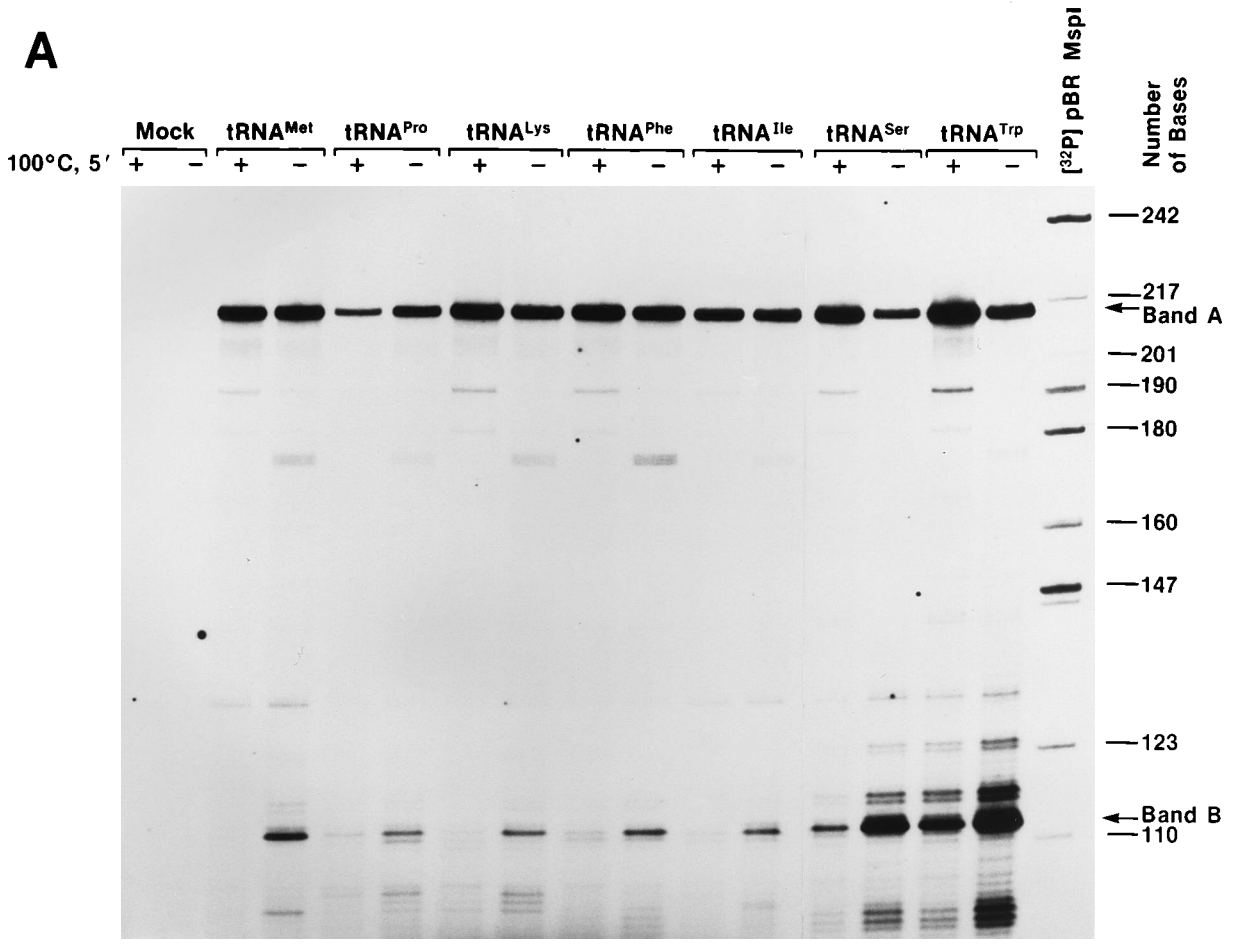

FIG. 7. (A) Autoradiograph of 5% polyacrylamide gel showing results of the primer extension assay. Samples were (+) or were not (−) heated to 100°C for 5 min to remove the tRNA primer before the primer extension assay was done. The sources of the different bands are described in Results. (B) Quantitation of the primer extension products present in bands A and B. The ratio [B/(A + B)] is a measure of the percentage of viral RNAs with an extendable tRNA primer annealed at the PBS.

that are involved in annealing the tRNA to the genome or with viral RNA sequences outside the PBS.

It has been suggested that the binding of $tRNA^{Trp}$ to the viral RNA may involve sequences outside the PBS (1, 16). This would imply that the interactions with other tRNAs may be suboptimal. ALV RT may also show preferences for $tRNA^{Trp}$ in the initiation complex. The finding that MLV RT can label several of the non-Trp tRNAs more efficiently than AMV RT suggests that the inefficient extension of the non-Trp tRNAs may also play a role in the decreased growth of the mutant viruses. However, in vitro ALV RT uses many different primer-template combinations with considerable facility. During replication of the viral genome, RT efficiently extends several other RNA primers, and ALV RT can efficiently extend a murine $tRNA^{Pro}$ annealed to 70S MLV RNA (9a, 13).

Waters and Mullin (46) have measured the concentrations of various tRNAs in AMV virions. The most abundant tRNA present is $tRNA^{Trp}$, which accounts for approximately 30% of the total packaged tRNA. $tRNA^{Lys}$ and $tRNA^{Pro}$ each account for about 12%, and $tRNA^{Met}$ accounts for about 10%, of the total tRNA packaged. $tRNA^{Phe}$, $tRNA^{Ile}$, and $tRNA^{Ser}$ are present at very low levels (1 to 3%) in the virion. We observed a fairly good correlation between the reported concentrations of various tRNAs in the virion and the relative stabilities of

various mutant-PBS viruses. It should be noted, however, that the tRNA concentrations reported by Waters and Mullins do not differentiate between different isoacceptor species of tRNA, which prevents us from making any strong statements about this correlation.

It is also possible that sequences in the PBS may be important for functions other than primer binding and that these sequences may in some way favor the use of $tRNA^{Trp}$. Several essential viral functions, such as RNA dimerization (4, 31), RNA packaging (35, 40), and viral RNA splicing, have been mapped to the 5′ end of the genome. Although a great deal of effort has been expended in attempting to delineate the precise boundaries of these signals, in most cases the data have been difficult to interpret. Viral genomic RNA is highly structured, and the signals are probably not contiguous in the genome. It is likely, in fact, that these signals are determined by both structure and sequence components, which could overlap in a complicated fashion and may constrain the sequence within or immediately adjacent to the PBS.

Our data suggest that for ALVs with mutant PBSs, the alternate tRNAs are extended by AMV RT less efficiently than is $tRNA^{Trp}$. This decreased amount of extendable tRNA primer annealed at the PBS would certainly limit the initiation of reverse transcription, and we believe that this may be the primary determinant of slower growth in the mutant-PBS viruses.

There are reports of other types of retroviruses that can use alternate tRNAs as primers. Wild-type MLV uses $tRNA^{Pro}$ to initiate reverse transcription; however, there are several endogenous murine retroviruses that use $tRNA_1^{Gln}$ (9). Colicelli and Goff (9) isolated a recombinant MLV that had acquired a PBS complementary to the 3′ end of $tRNA_1^{Gln}$, presumably through a recombination event involving one of the endogenous viruses. The recombinant virus, which used $tRNA_1^{Gln}$ as a primer to initiate reverse transcription, was reported to replicate in a manner indistinguishable from that of the wild type. Furthermore, there are endogenous murine retroviruses that have PBSs complementary to $tRNA_3^{Lys}$, $tRNA^{Phe}$, and $tRNA^{Gly}$ (9). Lund et al. (24) have reported that a replication-defective vector derived from MLV can use other cellular tRNAs, specifically, $tRNA_1^{Gln}$ or $tRNA_3^{Lys}$, as efficiently as the wild-type primer $tRNA^{Pro}$. In these experiments, the alternative PBS sequences were introduced into a defective vector in which replication of the viruses was limited to a single round of infection, so no clear conclusions could be drawn about the subtle differences in the tRNA primer preferences or in the stabilities of these alternative PBSs in MLV. A recent report by Li et al. (23) described the effects of mutations that changed the PBS of HIV type 1 so that it was homologous to the 3′ end of $tRNA_{1,2}^{Lys}$ or $tRNA^{Phe}$ instead of the wild-type $tRNA_3^{Lys}$. When these mutations were present in infectious clones of HIV type 1, the viruses were capable of replication using the alternate tRNA primers, but the mutant PBS reverted to the wild-type $tRNA_3^{Lys}$ PBS during viral replication.

How do the mutant PBSs revert to the wild-type sequence? The vast majority of avian retroviruses described use $tRNA^{Trp}$ as a primer. This raises the possibility that mutant PBSs could revert by recombination with an endogenous avian retrovirus. To avoid this type of reversion in these experiments, we used EV-0 chicken cells, which contain no endogenous viruses closely related to ALV (2). The reversion events that we observed always restored the PBS and the flanking regions to sequences that were identical to wild-type ALV sequences. A recombination event with an endogenous virus would be expected to involve flanking sequences, and the resulting recombinant viruses would have had changes in the flanking sequences (6, 7). For these reasons, we believe that the reversion events that we observed did not involve recombination with endogenous retroviruses.

We favor a model for reversion of the PBS sequences in which sequence information is captured from a $tRNA^{Trp}$ that, on rare occasions, is used to initiate reverse transcription even though there is only limited homology between the mutant PBS and the 3′ end of $tRNA^{Trp}$. If a majority of the mutant PBSs do not have a cognate tRNA bound, these unoccupied PBSs might favor the "inappropriate" binding of $tRNA^{Trp}$. Additional interactions between $tRNA^{Trp}$ and the viral genome outside the PBS or with viral proteins, such as nucleocapsid (11, 21), might stabilize the binding of a $tRNA^{Trp}$ at a mutant PBS. In the standard model of reverse transcription (43, 47), the first 18 bases of the 3′ end of the tRNA are copied to generate the positive DNA strand of the PBS, whereas the negative-strand PBS sequences are copied from the viral RNA. The second jump in reverse transcription is believed to involve homology between the positive- and negative-strand PBS sequences (43, 47). As a consequence, a virus with a mutant PBS sequence that used a $tRNA^{Trp}$ to initiate reverse transcription would generate a double-stranded DNA with mismatches at the PBS (one strand would contain mutant sequence, and the other strand would contain wild-type sequence). There is compelling evidence that the capture of information from a mismatched tRNA is the mechanism for the reversion of HIV type 1 mutants with partial deletions of the PBS (36, 45). There is additional evidence that internal mismatches in the PBS can exist and are tolerated in MLV (5, 20). Pulsinelli and Temin (33) have found that mutations at the 3′ end of the PBS in a spleen necrosis virus-based vector had little or no effect on the ability of a viral vector to complete a single round of replication. In this system, RT could extend mismatches of as many as 3 bases at the 3′ end of the PBS following the second jump. This supports the idea that the mismatches generated by the mutant-PBS ALVs using a $tRNA^{Trp}$ to initiate reverse transcription would be tolerated during the replication of ALV and could allow reversion to the wild-type PBS sequences.

Our data indicate that ALVs can replicate by using tRNA primers other than $tRNA^{Trp}$, if they contain a PBS complementary to an alternative chicken tRNA. The ALVs with alternative PBSs show no measurable defects in virion production or packaging efficiency; however, all of the mutant viruses revert with passage in culture. The mutant viruses all replicate less efficiently than ALV with a wild-type $tRNA^{Trp}$ PBS. A major determinant of the replication efficiency of the mutant viruses is probably occupancy of the PBS by a primer tRNA that can be efficiently extended by the avian RT. PBS occupancy in the mutant viruses is correlated with and may be dependent on the concentration of a given tRNA in the virion.

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