

# Synthetic tRNA<sup>Lys,3</sup> as the replication primer for the HIV-1<sub>HXB2</sub> and HIV-1<sub>Mal</sub> genomes

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## ABSTRACT

In order to determine the contribution of modified bases on the efficiency with which tRNA<sup>Lys,3</sup> is used *in vitro* as the HIV-1 replication primer, the properties of synthetic derivatives prepared by three independent methods were compared to the natural, i.e. fully modified, tRNA. When prepared directly by *in vitro* run-off transcription, we show here that the predominant tRNA species is 77 nt, representing a non-templated addition of a single nucleotide. As a consequence, this aberrant tRNA inefficiently primes (–) strand strong stop DNA synthesis from the primer binding site (PBS) on the HIV-1 viral RNA genome to which it must hybridize. In contrast, correctly sized tRNA<sup>Lys,3</sup> can be prepared by (i) total chemical synthesis and ligation of ‘half’ tRNAs, (ii) transcription of a cassette whose DNA template contained strategically placed 2'-O-Methyl-containing ribonucleotides and (iii) processing from a larger precursor by means of targeted cleavage with *Escherichia coli* RNase H. When each of these 76 nt tRNAs was supplemented into a (–) strand strong stop DNA synthesis reaction utilizing the HXB2 strain of HIV-1, the amount of product obtained was comparable to that from the fully modified counterpart. Parallel assays monitoring early events in (–) strand strong stop DNA synthesis using either the HXB2 or Mal strain of HIV-1 RNA as the template indicated little difference in the pattern or total product amount when primed with either natural or synthetic tRNA<sup>Lys,3</sup>. In addition, nuclease mapping of PBS-bound tRNA suggests inter-molecular base pairing between bases of the tRNA anticodon domain and the U-rich U5-IR loop of the viral 5' leader region is less stable on the HIV-1<sub>HXB2</sub> genome than the HIV-1<sub>Mal</sub> isolate.

## INTRODUCTION

Minus-strand DNA synthesis in retroviruses and many long terminal repeat (LTR)-retrotransposons is initiated from the 3' terminus of a host-coded tRNA particular to each element [for review see (1)]. Examples of this include tRNA<sup>Lys,3</sup> in the

type 1 and type 2 human immunodeficiency viruses (HIV-1, HIV-2) (2,3), tRNA<sup>Pro</sup> in murine leukemia virus (4), tRNA<sup>Trp</sup> in avian myeloblastosis virus (5) and tRNA<sup>iMet</sup> in the *Saccharomyces cerevisiae* LTR-retrotransposons Ty1 and Ty3 (6). In retroviruses, complementarity between a region of 18 contiguous nucleotides near the 5' terminus of the RNA genome, defined as the primer binding site (PBS), and the 3' end of the cognate tRNA primer establishes the site of initiation (7). Ty1 and Ty3 exhibit a variation of this theme inasmuch as nucleotides defining the PBS are non-contiguous (8–10). However, despite these differences, a wealth of genetic and biochemical data indicates that long-range interactions between the tRNA primer and viral RNA genome contribute to the efficiency with which minus-strand DNA synthesis is initiated (10–25). Alternate regions of complementarity between the tRNA primer and viral RNA genome include (i) nucleotides at the tRNA 5' terminus in feline immunodeficiency virus (20), (ii) TΨC loop nucleotides in Rous sarcoma virus (12), (iii) D-loop nucleotides in Ty1 (14) and Ty3 (6), and (iv) TΨC and anticodon loop nucleotides in HIV-1 and HIV-2 (11,13,15,16,18,19,22–30). Taken together, these studies illustrate initiation of reverse transcription as a multi-step and highly regulated event involving considerable cross talk between the retroviral polymerase and tRNA/viral RNA duplex (31).

A more detailed kinetic analysis using the HIV-1<sub>Mal</sub> isolate with fully modified tRNA<sup>Lys,3</sup> has indicated that initiation of minus-strand DNA synthesis is biphasic (32). Slow and distributive synthesis occurs during the first few cycles of polymerization, after which the rate of polymerization increases by three orders of magnitude. However, when the same event is studied using tRNA<sup>Lys,3</sup> derived by *in vitro* transcription, RT immediately moves into the elongation mode, while the overall level of minus-strand strong stop DNA product is substantially diminished (33). Based on studies with viral RNA from this HIV-1 isolate, these differences between natural and *in vitro*-transcribed tRNA<sup>Lys,3</sup> were attributed to the stabilizing effect exerted by hypermodified bases in the U-rich anticodon loop of the tRNA primer, which was shown by a combination of chemical and enzymatic footprinting to interact with the A-rich U5-IR loop of the viral genome (16,17,32–35). Experiments of this nature therefore implied that, although substantial amounts of tRNA<sup>Lys,3</sup> could be prepared by *in vitro* transcription, the lack of base modifications made this unsuitable for enzymatic or biophysical studies. However, our work with the HIV-1<sub>HXB2</sub> isolate suggested that differences in

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minus-strand DNA synthesis catalyzed from natural and synthetic tRNA<sup>Lys,3</sup> were less severe, implying that tRNA base modifications may not be as critical as the overall structure and stability of the initiation complex established on other HIV-1 genomes (26). At the same time, we considered a purely practical reason for the reduced efficiency of minus-strand DNA synthesis from *in vitro*-transcribed tRNA, namely heterogeneity at the 3' terminus of the transcript. Since RNA polymerase will catalyze non-templated base additions (36,37), it was conceivable that a portion of the tRNA transcript was of an inappropriate length. tRNAs containing additional 3' nucleotides, while appropriate for use in gel mobility shift and enzymatic footprinting studies, would therefore be inefficient primers, since initiation would require DNA synthesis from an unpaired base.

In this paper, we addressed the issue of tRNA<sup>Lys,3</sup> heterogeneity by directly comparing minus-strand DNA synthesis from natural, fully modified tRNA with derivatives prepared by (i) *in vitro* transcription, (ii) total chemical synthesis, (iii) *in vitro* transcription of a cassette whose template contains modified nucleotides and (iv) RNase H-mediated processing of a 'precursor' tRNA. Analysis of tRNAs indicates that those prepared by methods (ii)–(iv) are, in fact, 1 nt shorter than tRNA<sup>Lys,3</sup> prepared by conventional *in vitro* transcription. The issue is also complicated by the observation that fully modified tRNA<sup>Lys,3</sup> and the major transcript prepared by method (i) migrate on high-resolution polyacrylamide gels as a 77 nt species. Thus, as speculated, a significant proportion of tRNA<sup>Lys,3</sup> prepared by *in vitro* transcription under standard conditions is likely to contain an additional 3' nucleotide. Subsequent comparison of minus-strand strong stop DNA synthesis with tRNA prepared by methods (ii)–(iv) to the same event initiated from fully modified tRNA indicated minimal difference in efficiency, regardless of whether the template was the HXB2 or Mal strain. Based on these observations, we propose that, provided the appropriate conditions are established for *in vitro* synthesis, the 76 nt version of tRNA<sup>Lys,3</sup> is a suitable primer for studies on initiation of HIV-1 DNA synthesis. However, enzymatic probing of tRNA<sup>Lys,3</sup> hybridized to HIV-1<sub>HXB2</sub> versus HIV-1<sub>Mal</sub> viral RNA demonstrated a difference in the sensitivity of the anticodon loop region to a single-strand-specific nuclease. This loop remained predominantly unpaired when complexed with the HIV-1<sub>HXB2</sub> isolate, while it base paired when hybridized to the HIV-1<sub>Mal</sub> genome. Taken together, the results reported here suggest that, under our assay conditions, modified nucleotides present in tRNA<sup>Lys,3</sup> do not affect the efficiency of (–) strand strong stop synthesis, yet the binary complexes of HIV-1<sub>HXB2</sub> and HIV-1<sub>Mal</sub> are different in structure.

## MATERIALS AND METHODS

### Materials

Restriction enzymes, DNA/RNA-modifying enzymes, dNTPs, rNTPs and glycogen were purchased from Roche (Indianapolis, IN). <sup>32</sup>P-labeled nucleotides were the products of Amersham Pharmacia Biotech (Piscataway, NJ). T7 Megashortscript<sup>TM</sup> kits were from Ambion (Austin, TX). All other reagents were of the highest purity and were purchased from Fisher, Sigma, Invitrogen or Bio-Rad.

### Enzymes and nucleic acids

(His)<sub>6</sub>-tagged p66/p51 HIV-1 RT was prepared as described previously (38). pShortPBS, a plasmid containing a 99 nt region surrounding the HIV-1<sub>HXB2</sub> PBS cloned downstream of the T7 promoter, was a gift from Dr Karin Musier-Forsyth (39). HIV-1<sub>HXB2</sub> PBS RNA was prepared by *in vitro* transcription of RNA nucleotides 125–223 with the trinucleotide sequences GGG and CCC added at the 5' and 3' ends, respectively, using an Ambion Megashortscript<sup>TM</sup> kit. The HIV-1<sub>Mal</sub> PBS DNA template was created by a PCR reaction containing two sense and two antisense oligonucleotides, which when annealed, overlap to facilitate amplification of the T7 promoter immediately followed by nucleotides 123–217 of the Mal PBS region. The HIV-1<sub>Mal</sub> PBS RNA was created as above. Fully modified tRNA<sup>Lys,3</sup> was provided by B. Ehresmann (Strasbourg, France) and was prepared from bovine liver as described previously (35). Chemically synthesized tRNA<sup>Lys,3</sup> (Figure 1, Scheme A) was prepared by Dharmacon RNA technologies (Lafayette, CO) by ligation of the two RNAs below using T4 RNA ligase.

5'-GCCCGGAUAGCUCAGUCGGUAGAGCAUCAGACUUUU-3'

5'-P-AAUCUGAGGGUCCAGGGUUCAAGUCCCUGU-UCGGGCGCCA-3'

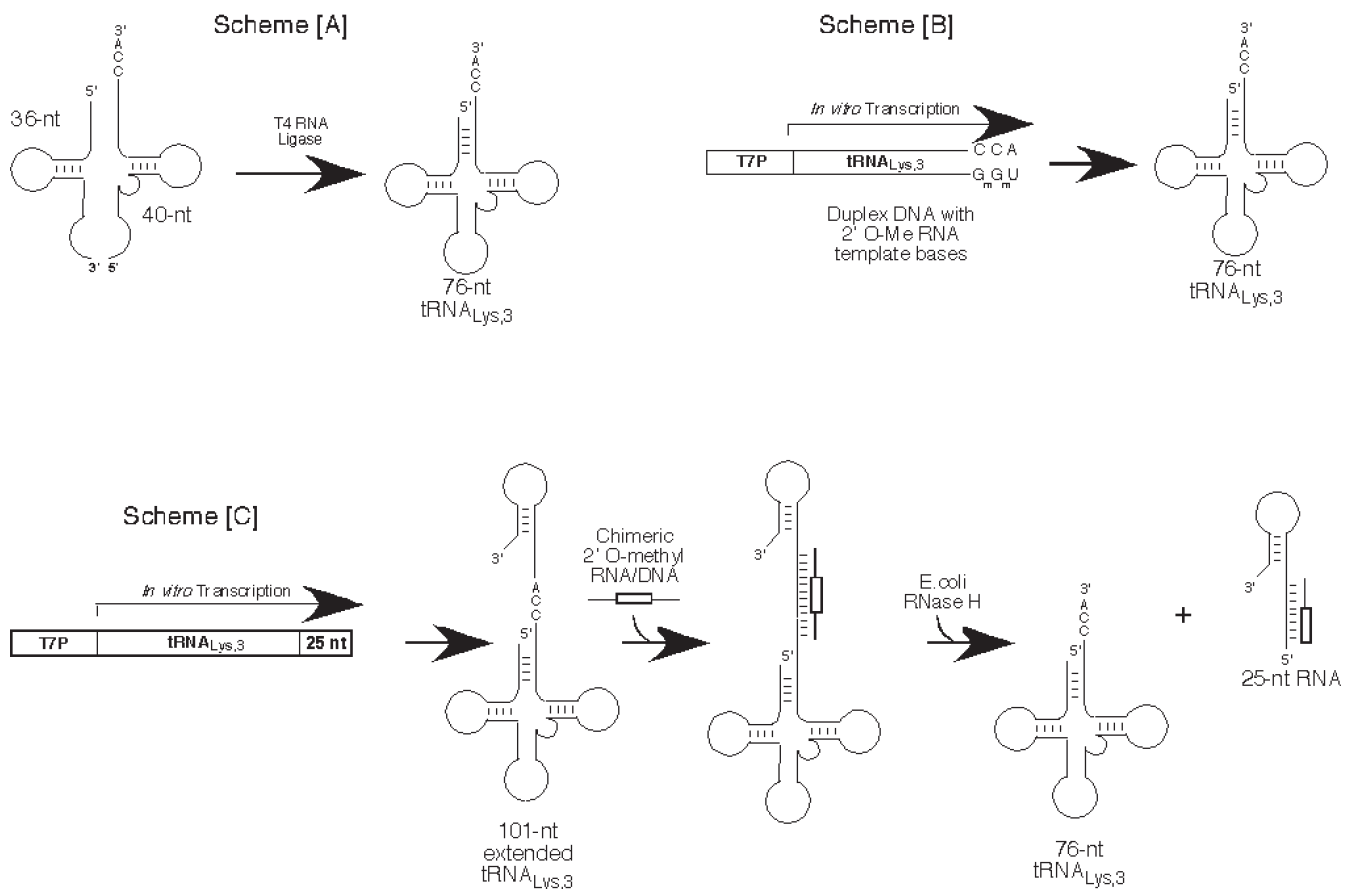
### tRNA preparation by *in vitro* transcription

Synthetic tRNA<sup>Lys,3</sup> was prepared by the following methods of *in vitro* transcription.

- (i) An unmodified, duplex template containing a T7 promoter, and specifying the 76 nt tRNA, was transcribed by standard procedures using an Ambion Megashortscript<sup>TM</sup> kit.
- (ii) A modified DNA duplex containing 2'-methoxyribose (2'-O-Methyl) groups as the two final template nucleotides was prepared by PCR, according to the method described previously (40), and transcribed as in (i). This is depicted diagrammatically in Figure 1, Scheme B.
- (iii) A longer 101 nt 'precursor' tRNA was created by targeted RNase H cleavage (Figure 1, Scheme C). A transcription cassette containing the tRNA sequence followed by 25 non-specific nucleotides was prepared by PCR. Following 5' end labeling and purification by high-resolution denaturing gel electrophoresis, the 'pre-tRNA' was annealed to a synthetic 2'-O-Methyl RNA–DNA chimera of the following sequence:

5'-mUmAmGmCmAmGdTdGdGdCmGmCmCmCmGm-  
AmA-3'

where the DNA component is underlined and in boldface and m represents the 2'-methoxyribose group of the RNA component. After annealing, the duplex was incubated in 20 mM HEPES/KOH, 50 mM KCl, 10 mM MgCl<sub>2</sub>, 1 mM DTT with 2 U *Escherichia coli* RNase H (Sigma) at 37°C for 1 h to facilitate site-specific cleavage of the RNA after the canonical –C–C–A– trinucleotide at the tRNA<sup>Lys,3</sup> 3' terminus. This was followed by phenol:CHCl<sub>3</sub>:isoamyl alcohol (25:24:1) extraction and precipitation with glycogen (final 0.4 mg/ml), sodium acetate, pH 5.2, and ethanol. After precipitation,



**Figure 1.** Methods of preparation of synthetic tRNA<sup>Lys,3</sup>. Details for preparation of each version are provided in Materials and Methods. T7P, T7 promoter.

the pellet was washed with 70% EtOH, dried and resuspended in double-distilled water.

All tRNAs were dephosphorylated in 10 mM Tris, pH 8, with 150 U bacterial alkaline phosphatase at 65°C for 1 h, extracted with an equal volume of phenol:CHCl<sub>3</sub>:isoamyl alcohol (25:24:1) and precipitated with glycogen (final concentration 0.4 mg/ml), sodium acetate, pH 5.2, and ethanol. After precipitation, the pellets were washed with 70% EtOH, dried and resuspended in double-distilled water. Subsequently, 5'-<sup>32</sup>P-labeled primers were prepared by phosphorylation utilizing T4 polynucleotide kinase and [γ-<sup>32</sup>P]ATP (3000 Ci/mmol) following standard protocols. 3'-<sup>32</sup>P-labeled primer was prepared by ligation of cytidine 3',5'-bis [α-<sup>32</sup>P] phosphate to tRNA<sup>Lys,3</sup> (Scheme B, see above) using T4 RNA ligase and following standard procedures. After labeling, each primer was fractionated through a denaturing polyacrylamide gel and the band corresponding to full-length tRNA was excised and eluted in 0.3 M NaAc, 0.1% SDS and 1 mM EDTA at 37°C overnight. Following elution, nucleic acid was precipitated, washed with 70% ethanol, dried and resuspended in double-distilled water.

### RNA-dependent DNA synthesis reactions

tRNA-viral-RNA duplexes were prepared by mixing template with primer at a 2-fold molar excess of the former in 10 mM Tris-HCl, pH 7.6, 25 mM KCl, heating to 90°C for 2 min

followed by cooling at the rate of 1°C/min to 4°C. The concentration of primer was normalized across the samples by adding a mixture of radiolabeled and cold tRNA. For analysis of tRNA-primed (-) strand DNA synthesis, the tRNA-viral-RNA duplex was added at a final concentration of 370 nM to a reaction buffer containing 50 mM Tris-HCl, pH 7.6, 6 mM MgCl<sub>2</sub>, 60 mM KCl, 1 mM DTT. p66/p51 HIV-1 reverse transcriptase was added to a final concentration of 733 nM and complex formation was allowed to proceed for 2 min at 37°C. All four dNTPs were added to a final concentration of 200 μM and aliquots removed for analysis at various time points were extracted with an equal volume of phenol:CHCl<sub>3</sub>:isoamyl alcohol (25:24:1) and precipitated with glycogen (final 0.4 mg/ml), sodium acetate, pH 5.2, and ethanol. After precipitation, the pellets were washed with 70% EtOH, dried, resuspended in 89 mM Tris-borate, pH 8.3, 2 mM EDTA, 7 M urea, 0.1% xylene cyanole, 0.1% bromophenol blue and subjected to high-voltage denaturing PAGE. Percentage extension of each primer species was determined using a BioRad FX phosphorimager, quantified by Quantity One™ software (Bio-Rad), and plotted with Delta Graph software.

### Cleavage of tRNA<sup>Lys,3</sup> by S1 nuclease

The annealing reaction was composed of 3'-<sup>32</sup>P-labeled synthetic tRNA<sup>Lys,3</sup> (Scheme B, see above) with a specific activity of  $3.7 \times 10^3$  c.p.m./pmol combined with a 2-fold excess of

viral RNA in a buffer of 100 mM Tris, pH 7.6, and 25 mM KCl. The mixture was heated to 90°C for 2 min and then slowly cooled at 1°C/min to 4°C. Subsequently, 5 mM MgCl<sub>2</sub> was added and the mixture was incubated for 15 min at room temperature. Cleavage reactions were performed as described previously [Miller *et al.* (20)] and the products were visualized by high-voltage denaturing PAGE.

## RESULTS

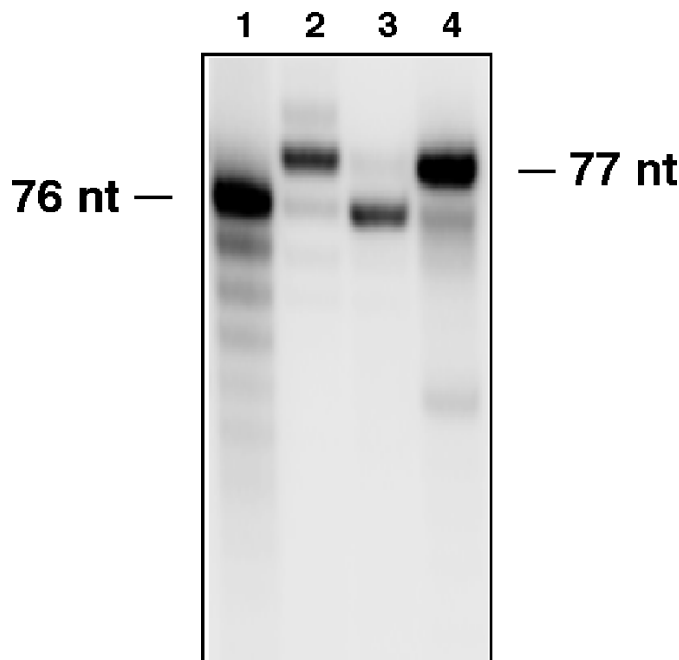
### Heterogeneity of synthetic tRNA<sup>Lys,3</sup> variants

Because we were concerned with heterogeneity following conventional *in vitro* transcription from a DNA cassette or endonucleolytically cleaved plasmid, several methods were employed to create homogeneous preparations of tRNA<sup>Lys,3</sup>. Figure 1 depicts these strategies schematically. While total chemical synthesis of the 76 nt tRNA would be most desirable in terms of yield for physical characterization of the HIV-1 initiation complex, Scheme A requires an additional ligation step, which can reduce recovery efficiency of the intact tRNA. Likewise, *E. coli* RNase H-mediated release of tRNA<sup>Lys,3</sup> from a precursor (Scheme C) has an additional processing step, the specificity of which is dependent on the source of *E. coli* RNase H (41). Thus, the most practical approach appeared to be *in vitro* transcription of a tRNA-expressing cassette derived by PCR with a primer set, one of which is modified to introduce 2'-*O*-Methyl ribonucleotides (Scheme B). This strategy was used previously to cause specific arrest of the T7 RNA polymerase leading to a decrease in non-templated addition at the 3' end of an *in vitro*-transcribed RNA (40). Nevertheless, each strategy was tested and compared with respect to tRNA length and suitability as primer in minus-strand strong stop RNA-dependent DNA synthesis reactions.

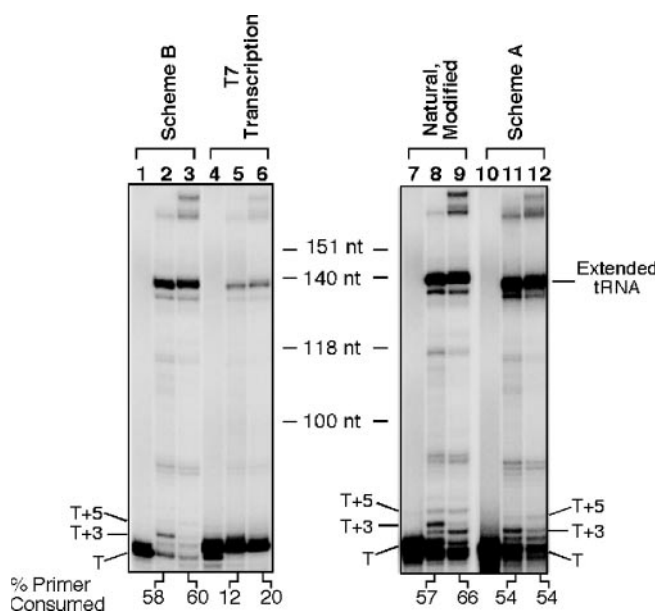
Figure 2 illustrates tRNA<sup>Lys,3</sup> heterogeneity following preparation via Schemes A and B, comparing this with either the natural, i.e. fully modified counterpart, and the version derived from transcription of an unmodified DNA template. Lane 1 shows the migration of the 76 nt tRNA created by ligation of two chemically synthesized oligoribonucleotides. Based on this 'reference' tRNA, it is clear that most of the transcript derived from an unmodified DNA cassette is heterogeneous (lane 2) with the major species migrating at a position corresponding to 77 nt. The inclusion of natural tRNA<sup>Lys,3</sup> (lane 4) indicates that, as a consequence of base modifications, this also migrates corresponding to a 77 nt RNA fragment. Although a 77 nt species is evident following transcription of a cassette containing 2'-*O*-Methyl RNA bases (lane 3), this is a minor product. Thus, while tRNA<sup>Lys,3</sup> 3' heterogeneity might not be expected to influence its use in footprinting and gel-mobility shift experiments with HIV-1 RT, such heterogeneity would have serious consequences on the efficiency with which it is used as the minus-strand primer for reverse transcription, since this would require initiation from an unpaired base. This aspect is addressed in the following section.

### tRNA<sup>Lys,3</sup>-primed minus-strand strong stop DNA synthesis

Figure 3 indicates the efficiency with which different preparations of synthetic tRNA<sup>Lys,3</sup> support HIV-1 minus-strand DNA



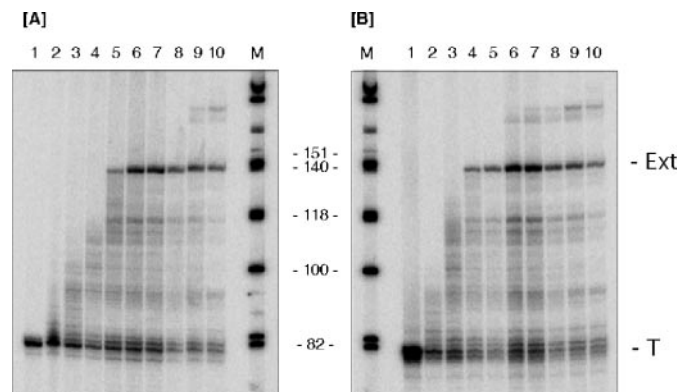
**Figure 2.** Evaluation of tRNA<sup>Lys,3</sup> variants by high-resolution denaturing gel electrophoresis. Lane 1, ligation of chemically synthesized oligoribonucleotides; lane 2, *in vitro* transcription of an unmodified transcription cassette; lane 3, *in vitro* transcription of a cassette whose template contains two 2'-*O*-Methyl ribonucleotides at its 5' terminus; lane 4, 5' end-labeled, natural tRNA<sup>Lys,3</sup>. Note that all tRNA species are 5' end-labeled, thus excluding any effects due to 5' heterogeneity.



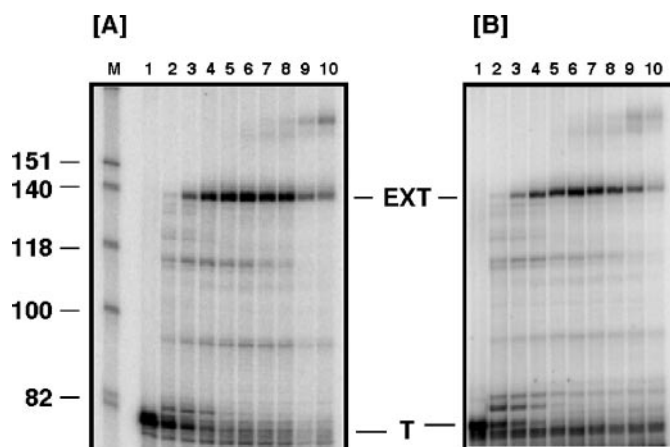
**Figure 3.** tRNA-primed (-) strand strong stop DNA synthesis on the HIV-1<sub>HXB2</sub> viral RNA template. Lanes 1–3, tRNA prepared from a transcription cassette containing 2'-*O*-Methyl ribonucleotides on its template; lanes 4–6, tRNA prepared from an unmodified transcription cassette; lanes 7–9, fully modified, natural tRNA; lanes 10–12, chemically synthesized tRNA. Lanes 1, 4, 7 and 10: tRNA alone; lanes 2, 5, 8 and 11: 5 min (-) strand DNA synthesis; lanes 3, 6, 9 and 12: 60 min (-) strand DNA synthesis. The amount of tRNA primer consumed after 5 and 60 min is indicated under each lane. In addition to the fully extended (-) strand strong stop product, early initiation products (T+3 and T+5) are highlighted. T, full-length tRNA primer.

synthesis. The RNA template for these experiments was derived from the HIV-1<sub>HXB2</sub> isolate, differing in sequence from the HIV-1<sub>Mal</sub> isolate used in some earlier initiation and nucleic acid probing studies (16,32,33). However, the sequence in the vicinity of the PBS in our clone is identical to the HIV-1<sub>Lai</sub> strain, for which alternate interactions between the viral RNA and the tRNA primer have been proposed (30). From our analysis, it is clear that tRNA prepared either by total chemical RNA synthesis (lanes 10–12) or via transcription of a 2'-O-Methyl RNA/DNA template (lanes 1–3) is vastly superior in priming efficiency to that derived by transcription of an unmodified template (lanes 4–6). Quantitation of the data indicated that, after 5 min, ~60% of the tRNA could be extended into full-length product, after which there was no further increase. To our surprise, this was very close to the level of minus-strand strong stop DNA synthesis achieved using fully modified tRNA<sup>Lys,3</sup> as the minus-strand primer (lanes 7–9). The data of Figure 3 thus suggest that modifications to tRNA<sup>Lys,3</sup>, in particular hypermodified bases of the anticodon loop, do not contribute to the efficiency with which it is extended by HIV-1 RT on the HIV-1<sub>HXB2</sub> RNA template. A second important feature of Figure 3 concerns the short reverse transcription products diagnostic of the transition from initiation to elongation of minus-strand synthesis, namely the T + 3 and T + 5 products (defining T as the full-length tRNA primer). These are both visible during minus-strand synthesis from natural tRNA<sup>Lys,3</sup>, with the T + 3 product predominating after 5 min (Figure 3, lanes 8 and 9). Equivalent products are also visible when minus-strand synthesis is catalyzed from a chemically synthesized tRNA<sup>Lys,3</sup> (Figure 3, lanes 11 and 12). Although these appear 1 nt smaller, this is corrected by the altered migration properties of natural tRNA (Figure 2). Similar products are evident, albeit at a slightly lower level, using tRNA derived from a 2'-O-Methyl RNA-modified transcription cassette (Figure 3, lanes 2 and 3).

Because the DNA synthesis reaction had reached completion when the initial time point was taken, we performed a more intensive study to analyze events earlier in initiation of (–) strand synthesis. This study would elucidate any difference in the kinetics of initiation or early pause patterns when using the natural tRNA primer versus one that was created synthetically. We elected here to focus on fully modified tRNA<sup>Lys,3</sup> and its counterpart created by Scheme B. Additionally, we chose to compare both the HIV-1<sub>HXB2</sub> (Figure 4) and the HIV-1<sub>Mal</sub> (Figure 5) PBS RNA templates with each primer. Figures 4 and 5 demonstrate that the profiles utilizing either primer tRNA species are similar over the entire time course regardless of the template strain, ruling out the possibility that natural tRNA<sup>Lys,3</sup> may be utilized more efficiently at early time points in the initiation reaction. It is worth noting that despite the fact that the hypermodified nucleotides present in the primer do not seem to affect the total amount of (–) strand strong stop species, pause patterns early in the reaction are different depending on the strain of the HIV-1 template RNA. The T + 3 and T + 5 products are much more pronounced in the reaction containing the HIV-1<sub>Mal</sub> template RNA. Taken together, the data of Figures 4 and 5 suggest that base modifications present in the primer tRNA<sup>Lys,3</sup> do not affect the accumulation of (–) strand strong stop DNA.



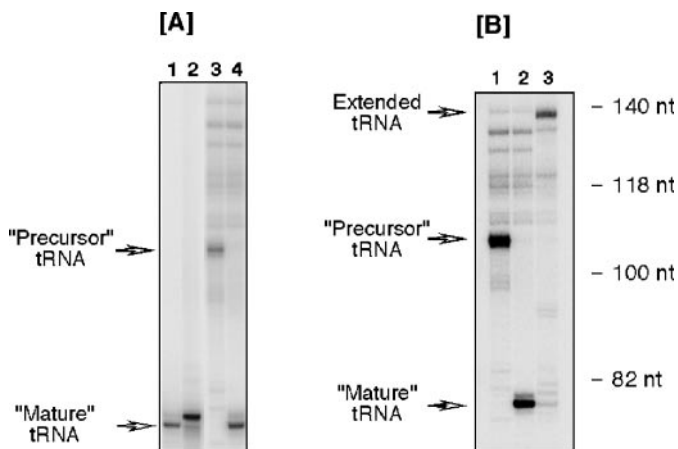
**Figure 4.** Expanded primer extension analysis comparing either natural, fully modified tRNA<sup>Lys,3</sup> or synthetic tRNA<sup>Lys,3</sup> annealed to HIV-1<sub>HXB2</sub> PBS RNA. (A) Extension of synthetic tRNA<sup>Lys,3</sup> prepared by Scheme B; (B) extension of natural, fully modified tRNA<sup>Lys,3</sup>. Lanes 1, unextended primer; lanes 2, 15 s; lanes 3, 30 s; lanes 4, 1 min; lanes 5, 2 minutes; lanes 6, 3 min; lanes 7, 4 min; lanes 8, 5 min; lanes 9, 15 min; lanes 10, 30 min. M, marker ΦX174DNA HinfI digest. Unextended primer is indicated by T, and the fully extended DNA synthesis product by the notation EXT.



**Figure 5.** Expanded primer extension analysis comparing either natural, fully modified tRNA<sup>Lys,3</sup> or synthetic tRNA<sup>Lys,3</sup> annealed to HIV-1<sub>Mal</sub> PBS RNA. (A) Extension of synthetic tRNA<sup>Lys,3</sup> prepared by Scheme B; (B) extension of natural, fully modified tRNA<sup>Lys,3</sup>. Lanes 1, unextended primer; lanes 2, 15 s; lanes 3, 30 s; lanes 4, 1 min; lanes 5, 2 min; lanes 6, 3 min; lanes 7, 4 min; lanes 8, 5 min; lanes 9, 10 min; lanes 10, 30 min. M, marker ΦX174DNA HinfI digest. Unextended primer is indicated by T, and the fully extended DNA synthesis product by the notation EXT.

### Preparation of tRNA<sup>Lys,3</sup> via RNase H-mediated processing

The final method of tRNA<sup>Lys,3</sup> preparation involved its release from a 101 nt precursor, to which an RNA–DNA chimera was hybridized, via targeted hydrolysis with *E. coli* RNase H. The efficiency and accuracy of processing is illustrated in Figure 6A, indicating that at least 80% of the RNase H hydrolysis product is correctly sized tRNA, and a small proportion 1 nt larger (compare lanes 3 and 4). Figure 6B, lane 3 indicates that this tRNA species also supports efficient (–) strand strong stop DNA synthesis. Furthermore, the presence of T + 3 and T + 5 products also suggests that the replication machinery progresses through initiation and into productive elongation as (–) strand DNA synthesis occurs.



**Figure 6.** (A) Preparation of tRNA<sup>Lys,3</sup> via RNase H-mediated hydrolysis of a 101 nt precursor. Lane 1, 76 nt, chemically synthesized; lane 2, 76 nt natural tRNA<sup>Lys,3</sup>; lane 3, 101 nt precursor-tRNA transcript; lane 4, 76 nt tRNA product of RNase H-mediated processing of the 101 nt precursor. (B) (-) strand strong stop DNA synthesis primed by the 76 nt tRNA<sup>Lys,3</sup> maturation product. Lane 1, 101 nt tRNA precursor; lane 2, 76 nt 'mature' tRNA product; lane 3, (-) strand synthesis supported by tRNA<sup>Lys,3</sup> maturation product.

### Comparative analysis of the tRNA<sup>Lys,3</sup>/HIV-1<sub>HXB2</sub> versus HIV-1<sub>Mal</sub> RNA complex

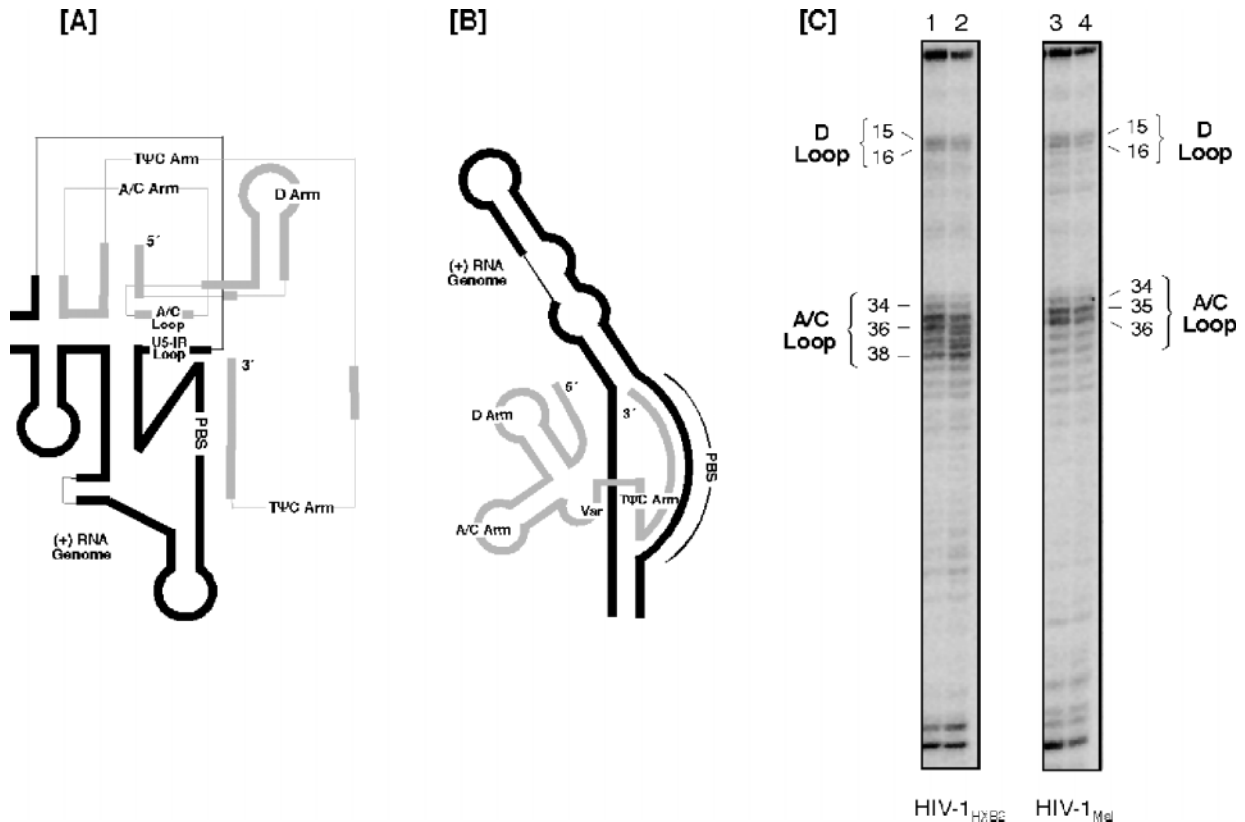
As indicated earlier, models of the binary complex between HIV-1 viral RNA and its tRNA<sup>Lys,3</sup> primer differ with the strain under examination (16,30,32,42). We hypothesized that our strain, HIV-1<sub>HXB2</sub>, due to its absolute homology with HIV-1<sub>Lai</sub> in the vicinity of the PBS, might form a complex similar to that proposed by Beehrens and Berkhout (30) (Figure 7A) and differing from the structure proposed by Ehresmann and coworkers (16) (Figure 7B). In addition, the difference in the pattern of pause products, possibly the result of the enzyme encountering RNA structure, when comparing Figures 4 and 5 supports this hypothesis. To this end, we performed limited S1 nuclease digestion of a synthetic tRNA<sup>Lys,3</sup>, annealed to either HIV-1<sub>HXB2</sub> or HIV-1<sub>Mal</sub> PBS RNA. The results of Figure 7C clearly show that these two RNAs interact differently with the tRNA in the anticodon loop region. HIV-1<sub>Mal</sub> shows limited susceptibility in this region, supporting previous data showing that this region is involved in intermolecular pairing with the viral U5-IR loop (Figure 7C, lanes 3 and 4) (35). The small amount of cleavage seen here may be due to the fact that the tRNA lacks base modifications and therefore this interaction is not as stable as that formed by the previous study. However, significantly enhanced cleavage of the tRNA<sup>Lys,3</sup> anticodon domain is evident when in a complex with HIV-1<sub>HXB2</sub> RNA, indicating this loop is not present in a base paired configuration (Figure 7C, lanes 1 and 2). These results suggest that the binary complex formed by tRNA<sup>Lys,3</sup> and HIV-1<sub>HXB2</sub> RNA likely does not form the complex structure involving the anticodon domain of the primer RNA proposed by Isel *et al.* (35) but instead is present in an altered conformation.

## DISCUSSION

Reports in the literature to this point have been disparate with regard to the requirement for hypermodified bases in

the anticodon loop of the tRNA primer for efficient reverse transcription. Isel *et al.* (35) suggest that modified residues in the anticodon loop of tRNA<sup>Lys,3</sup> are critical for stabilizing its base pairing interaction with the A-rich HIV-1 viral U5-IR loop. On the other hand, base modifications were suggested to destabilize interactions between tRNA<sup>Pro</sup> and the Moloney murine leukemia virus genome (43). Many other groups have analyzed reverse transcription in a variety of retroviruses and retroelements *in vitro*, but with no consensus on the source of the primer tRNA used in the studies (12,14,20,26,28,30,39). This discrepancy likely exists because, while the primers for reverse transcription *in vivo* contain hypermodified bases, it is technically challenging to obtain enough natural tRNA for work *in vitro*. Nevertheless, all studies agree that the priming efficiency of hypermodified tRNA is greater than the tRNAs created *in vitro* by transcription with T7 RNA polymerase. We hypothesized that an alternative explanation for the inefficient use of the *in vitro*-transcribed tRNA may be the presence of an additional non-templated base at its 3' end. Therefore, we elected to compare several methods for creating a homogeneous, 76 nt primer *in vitro*. Using the strategies outlined in Figure 1, our results show that the procedure by which the tRNA is created has a profound effect on its ability to be utilized as a primer for reverse transcription by HIV-1 RT *in vitro*. Production of a 76 nt tRNA<sup>Lys,3</sup> molecule by ligation of two synthetic RNA oligonucleotides or RNase H-directed cleavage of a 'precursor' RNA was successful but time consuming. However, transcription with T7 RNA polymerase from a template containing two 2'-methoxyribonucleotides at the 5' terminus of the template strand produced a product that was 95% homogeneous, compared to transcription with a conventional template, the latter producing almost exclusively the *n* + 1 species. Transcription with T7 RNA polymerase also has the advantage of producing large amounts of RNA with little cost or labor. In our assay, the tRNAs created by Schemes A, B and C (Figure 1) were extended by HIV-1 RT to an amount comparable to that of the natural tRNA<sup>Lys,3</sup> containing base modifications. Comparison of the HIV-1 strains Mal and HXB2 as the template RNA for tRNA primer extension indicated no dependence on base modifications. In addition, we found that HIV-1<sub>HXB2</sub> RNA is not base paired to the anticodon loop of its cognate primer tRNA. This supports the hypothesis that while varying strains of HIV-1 use the primer tRNA<sup>Lys,3</sup> as a primer for DNA synthesis, the specific binary complex recognized by the replication machinery in each case can vary with regard to the structure formed between the viral RNA and this primer (16,30,32,42).

Analysis of several viral replicases in addition to HIV-1 have shown that heterogeneity at the 3' end of viral RNAs may influence replication. Brome mosaic virus RNA-dependent RNA polymerase has been shown to possess an initiation defect when the template contains 3' extensions (44). Paramyxovirus simian virus 5 RNA demonstrated reduced infectivity when nucleic acid preparations were found to contain 3' end heterogeneity (45). In order to circumvent this obstacle, the presence of 2'-methoxyribonucleotides in the DNA transcription template has proven useful in allowing creation of an RNA of defined length in our system and other systems (40,44,45). One can envisage that analysis of RNA structure may be complicated when multiple nucleic acid species are present. In analyses employing X-ray crystallography



**Figure 7.** Schematic diagram of the proposed binary complex of tRNA<sup>Lys,3</sup> with HIV-1<sub>Lai</sub> RNA (A) or with HIV-1<sub>Mal</sub> RNA (B). Viral RNA bases are indicated in thick black lines, tRNA<sup>Lys,3</sup> bases are in thick gray lines. Thin lines indicate connectivity but do not represent RNA sequence. (C) S1 nuclease digestion of tRNA<sup>Lys,3</sup> in binary complex with either HIV-1<sub>HXB2</sub> RNA (lanes 1 and 2) or HIV-1<sub>Mal</sub> RNA (lanes 3 and 4). Lanes 1 and 3, 5 min; lanes 2 and 4, 10 min. Migration positions of the cleavage products are indicated, as well as the corresponding location on the tRNA.

or NMR, a heterogeneous RNA population can lead to aberrant signals that make data interpretation difficult. Indeed, Wu and Tinoco (46) demonstrated by NMR gel-purified RNAs created by T7 transcription using a standard DNA template and with a 2'-O-Methyl RNA-DNA chimeric template produced different spectra in the region representing the last three nucleotides. These data indicated that the extra nucleotide can influence the resonance of bases in the immediate vicinity and therefore lead to errors in data interpretation (46). Ribozyme cleavage has also been used to create a homogeneous RNA population in assays as diverse as virus replication and crystallographic analyses of plant and animal virus replicases (44,45,47). Pata *et al.* (47) performed crystallization of HIV-1 RT with its cognate template/primer, and created a tRNA<sup>Lys,3</sup> of defined length by cleavage with a hammerhead ribozyme. In this case, however, due to the sequence requirement of the ribozyme, the technique was limited by the necessity for a mutation at the 3' end of the tRNA. Synthesis of the tRNA utilizing a 2'-O-Methyl RNA-DNA chimera would circumvent the need for mutation, and, in addition, the total yield of RNA would likely be increased, an obvious advantage for crystallography. Transcription utilizing a template modified with 2'-methoxyribonucleotides should become the standard procedure when an RNA species with a defined 3' end is required.

The varied structures of different HIV-1 isolates demonstrated *in vitro* (16,30,31) lead to the obvious question of the

native conformation of the tRNA<sup>Lys,3</sup>/viral RNA binary complex in the virion. In this case, the situation becomes complicated by the fact that the viral RNA is much longer than that used by the *in vitro* studies and also dimeric, which might provide additional opportunities for inter- and intra-strand base pairing. In addition, proteins, both viral and cellular, are present in the dense core of the virion where the initiation complex is thought to form. The retroviral nucleocapsid protein (NC) has been shown to facilitate annealing of the tRNA primer onto the viral RNA *in vitro* in many systems (8,28,39,48–51). However, comparison between the heat-annealed primer-template complex versus that annealed by NC demonstrated no marked differences in the binary complexes formed, further supporting the use of *in vitro* studies to dissect the initiation complex (48). In the cell, Mak *et al.* (52) have shown that the viral polyprotein pr160<sup>gag-pol</sup> is necessary for selective packaging of the primer tRNA into virions. Later work by this group proposes a complex interaction between pr55<sup>gag</sup>, pr160<sup>gag-pol</sup>, tRNA<sup>Lys,3</sup> and human lysyl tRNA synthetase (53–56). In summary, the reverse transcription initiation complex may be more intricate and could involve not only nucleic acids but also multiple protein components as well to facilitate efficient replication. Analyses that allow examination of the nucleic acid conformation in virion cores are ongoing in this laboratory, as well as footprinting studies to map areas that interact with any protein component present.

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