Initiation of (–) strand DNA synthesis from tRNA₃^{Lys} on lentiviral RNAs: Implications of specific HIV-1 RNA-tRNA₃^{Lys} interactions inhibiting primer utilization by retroviral reverse transcriptases

(retroviral replication/HIV-1 reverse transcription)

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ABSTRACT Initiation of minus (–) strand DNA synthesis was examined on templates containing R, U5, and primer-binding site regions of the human immunodeficiency virus type 1 (HIV-1), feline immunodeficiency virus (FIV), and equine infectious anemia virus (EIAV) genomic RNA. DNA synthesis was initiated from (i) an oligoribonucleotide complementary to the primer-binding sites, (ii) synthetic tRNA₃^{Lys}, and (iii) natural tRNA₃^{Lys}, by the reverse transcriptases of HIV-1, FIV, EIAV, simian immunodeficiency virus, HIV type 2 (HIV-2), Moloney murine leukemia virus, and avian myeloblastosis virus. All enzymes used an oligonucleotide on wild-type HIV-1 RNA, whereas only a limited number initiated (–) strand DNA synthesis from either tRNA₃^{Lys}. In contrast, all enzymes supported efficient tRNA₃^{Lys}-primed (–) strand DNA synthesis on the genomes of FIV and EIAV. This may be in part attributable to the observation that the U5-inverted repeat stem-loop of the EIAV and FIV genomes lacks an A-rich loop shown with HIV-1 to interact with the U-rich tRNA anticodon loop. Deletion of this loop in HIV-1 RNA, or disrupting a critical loop–loop complex by tRNA₃^{Lys} extended by 9 nt, restored synthesis of HIV-1 (–) strand DNA from primer tRNA₃^{Lys} by all enzymes. Thus, divergent evolution of lentiviruses may have resulted in different mechanisms to use the same host tRNA for initiation of reverse transcription.

All retroviral reverse transcriptases (RTs) initiate proviral DNA synthesis from a host tRNA isoacceptor molecule annealed to the viral RNA (1, 2). This tRNA replication primer has, at its 3' end, 18 nt complementary to the primer-binding site (pbs) found downstream of the 5' unique (U5) sequence in the retroviral RNA genome. During assembly of certain retroviruses, the primer is preferentially packaged into the virion (3–5). The pbs does not appear to play a role in tRNA incorporation, as a reduction or absence of viral genomic RNA does not affect tRNA packaging (3, 4, 6). In the virion, tRNA interacts with the pbs, but may also form higher order structures with other viral RNA sequences (7–11). These interactions do not occur spontaneously at ambient temperatures (12, 13), suggesting they may require additional factors in the virion (e.g., nucleocapsid, RT, *gag-pol*, and *gag* precursor proteins) (6, 12).

tRNA₃^{Lys} is used by all lentiviruses to initiate reverse transcription, but the mechanisms by which it interacts with the viral genome, RT, and other viral factors prior to initiation may differ. For HIV-1, Isel *et al.* (9, 11) and Baudin *et al.* (14) have characterized an interaction between the A-rich loop of the

U5-inverted repeat (IR) stem-loop in genomic RNA and the U-rich anticodon loop of tRNA₃^{Lys}. As predicted by computer folding programs (15), a U5-IR stem-loop exists in the genomes of feline leukemia virus (FIV) and equine infectious anemia virus (EIAV). However, a notable feature in both cases is the absence of an A-rich loop (Fig. 1). Although an interaction between the tRNA anticodon loop and the U5-IR loop of FIV or EIAV is unlikely, it does not rule out the possibility that other tRNA–viral RNA interactions have evolved. For example, the T_ψC loop of tRNA^{Trp} has been shown to pair with the U5-IR stem of the avian genome, whereas interactions between the anticodon loop and the U5-IR loop are less likely (7, 10). Also, preliminary evidence (16) suggests an interaction between the D-loop and sequences 3' of the pbs in the yeast retrotransposon Ty. In an attempt to better define tRNA–viral RNA interactions in HIV we have identified an RT mutant fully competent as an RNA-dependent DNA polymerase, but incapable of initiating tRNA₃^{Lys}-primed DNA synthesis on HIV-1 RNA containing the pbs (17). However, disruption of the U5-IR loop–tRNA anticodon loop complex restored DNA polymerase function to this mutant, suggesting that HIV-1 RT, through specific binding to tRNA₃^{Lys}, may be required to disrupt this loop–loop interaction prior to synthesis of (–) strand DNA.

Although the DNA polymerase and ribonuclease H activities of human immunodeficiency virus type-2 (HIV-2), simian immunodeficiency virus (SIV), FIV, and EIAV RTs have been extensively studied and compared with those of the HIV-1 enzyme (18–22), the manner in which related lentiviruses initiate (–) strand DNA synthesis is poorly characterized. In this study, we evaluated the efficiency with which lentiviral (HIV-1, FIV, EIAV, HIV-2, and SIV) and retroviral [murine leukemia virus (MLV) and avian myeloblastosis (AMV)] RTs initiate DNA synthesis from tRNA₃^{Lys} annealed to pbs-containing RNA templates of HIV-1, FIV, and EIAV. To test involvement of the anticodon loop–U5-IR–loop interaction in initiation, an 85-nt tRNA₃^{Lys}-DNA chimera and tRNA₃^{Lys} were employed as primers on wild-type HIV-1 pbs RNA, in addition to a mutant HIV-1 template lacking the -A-A-A-A- sequence in the U5-IR loop. Using these materials, it was possible to test the contribution of specific lentiviral RT/tRNA₃^{Lys}/lentiviral

Abbreviations: AMV, avian myeloblastosis virus; EIAV, equine infectious anemia virus; FIV, feline immunodeficiency virus; MLV, murine leukemia virus; RT, reverse transcriptase; SIV, simian immunodeficiency virus; pbs, primer-binding site; IR, inverted repeat.

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FIG. 1. Predicted secondary structures of the HIV-1_{HXB2}, EIAV, and FIV genomes immediately adjacent to the pbs. For each structure, pbs nucleotides complementary to tRNA_{3^{lys}} (the cognate primer of HIV, EIAV, and FIV) are in boldface type, whereas the first nucleotide of (–) strand DNA is indicated +1. For each RNA genome, a U5-IR loop 5' to the pbs is indicated.

RNA interactions toward efficient initiation and synthesis of (–) strand strong-stop DNA.

MATERIALS AND METHODS

Enzymes. Highly purified lentiviral and retroviral RTs were used in these studies. p66/p51 and p66/p66 HIV-1 RT (19), p66/p66 EIAV RT (20), and p66/p66 SIV RT were purified by a combination of metal chelate and ion exchange NTA-Sepharose chromatography. Heterodimeric HIV-2 RT was prepared according to Müller *et al.* (21), p66/p66 FIV RT according to North *et al.* (22), and p80 MLV RT as described in Wöhrle *et al.* (23). AMV RT ($\alpha 63/\beta 95$) was purchased from Boehringer Mannheim. All enzymes were >90% homogeneous and free of contaminating nucleases.

Plasmids and RNA Templates. RNA expression plasmids, containing long terminal repeat sequences from several lentiviral genomes, were linearized by restriction endonuclease digestion and transcribed *in vitro*. All RNA transcripts were fractionated by high-voltage electrophoresis on 6% denaturing polyacrylamide gels, excised, and eluted as described (17).

HIV-1 pbs RNA. Transcription of *AccI*-digested pHIV-pbs_{HXB2} yielded a 497-nt RNA, designated HIV-1 pbs RNA (24). The -A-A-A-A- sequence of the U5-IR loop (Fig. 1) was deleted by site-directed mutagenesis from pHIV-pbs_{HXB2} to create pHIV-pbs Δ A (X.L. and M.A.W., unpublished data). A 493-nt RNA template (HIV-1 pbs Δ A RNA) was derived from this plasmid template as described above.

FIV pbs RNA. A 1093-bp *HindIII* fragment (nt 148–1241), containing U5, R, and pbs sequences, and a portion of U3 was excised from the FIV molecular clone 34TFIO (25) and ligated into *HindIII*-linearized pSP72 (Promega) to create plasmid

pFIV-pbs. This plasmid was linearized with *MnII* and transcribed to generate a 454-nt FIV pbs RNA.

EIAV pbs RNA. A *MunI*-*PvuII* fragment (nt 203–618) was excised from the EIAV proviral clone pSPEIAV19 (a generous gift of S. L. Payne, Case Western Reserve University) and ligated into *EcoRI*-*PvuII*-digested pSP72 to create plasmid pEIAV-pbs. pEIAV-pbs was linearized with *PvuII* and transcribed to yield a 444-nt EIAV pbs RNA (26).

Primers. DNA synthesis from lentiviral RNA templates was initiated from the following primer variants: (i) synthetic 76-nt tRNA_{3^{lys}} prepared by *in vitro* transcription; (ii) an 85-nt tRNA–DNA chimera (see below); (iii) natural, i.e., fully modified 76-nt tRNA_{3^{lys}}; and (iv) an 18-nt oligoribonucleotide complementary to the pbs. Primers (i) and (ii) were internally labeled with ³²P during *in vitro* transcription, whereas (iii) and (iv) were 5' labeled with ³²P and polynucleotide kinase.

RNA-Dependent DNA Synthesis of (–) Strand Strong-Stop DNA. For (–) strand DNA synthesis experiments, annealed template–primer (0.2–0.4 pmol) was added to a reaction mixture (total volume 10 μ l) containing 50 mM KCl, 10 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, and RT. This mixture was incubated 2 min at 37°C prior to addition of dNTPs (100 μ M). After a 30-min incubation, (–) strand DNA synthesis products were fractionated by denaturing high-voltage electrophoresis, visualized by autoradiography, and quantified via phosphorimaging (Molecular Dynamics PhosphorImager, using IMAGEQUANT software provided by the supplier).

tRNA–DNA Chimera Synthesis. Using the large fragment of *Escherichia coli* DNA polymerase I and a pbs-containing 37-nt DNA template (i.e., 18 pbs nucleotides flanked 5' and 3' by 9 and 10 nt, respectively), synthetic tRNA_{3^{lys}} was extended at its 3' terminus by 9 deoxynucleotides as described (17). This 85-nt chimera was subsequently hybridized to the pbs-containing HIV-1 viral RNA genome and assayed for its ability to support RT-catalyzed (–) strand synthesis as described above.

RESULTS

RNA-Dependent DNA Polymerase Activities of Heterologous RTs. The DNA polymerase activity of each enzyme was first evaluated by its ability to support (–) strong-stop synthesis from an 18-nt RNA primer annealed to HIV-1 pbs RNA. The amount of each enzyme used was previously standardized in an assay limited to a single round of DNA synthesis (17). The RNA/viral RNA template–primer combination and predicted (–) strand strong-stop DNA product are shown schematically in Fig. 2A and the reaction products in Fig. 2B. As determined by phosphorimaging analysis of Fig. 2B, all enzymes used the same amount of RNA primer for the synthesis of (–) strand DNA; a similar observation was made in the presence of the competitor heparin, which limits each enzyme to a single round of synthesis (not shown). Although a low-enzyme:template–primer ratio (2:1) was employed, the product was primarily full-length (–) strand strong-stop DNA. With all RTs, pausing was most apparent within the first 5–6 nt of DNA synthesis on the HIV-1 RNA template. Other pause products were present but varied in intensity with each RT, FIV RT being the least processive.

Ability of Heterologous RTs to Support (–) Strand DNA Synthesis on a Wild-Type HIV-1 RNA/tRNA_{3^{lys}} Duplex. Synthesis of (–) strand DNA from synthetic and natural tRNA_{3^{lys}} on the wild-type HIV-1 genome is described schematically in Fig. 2A. Although each RT efficiently extended an 18-nt RNA primer (Fig. 2B) and all lentiviruses of the current study employ tRNA_{3^{lys}} as primer *in vivo*, only HIV-1 p66/p51 (lane 1) and AMV RT (lane 8) initiated (–) strand DNA synthesis from the synthetic tRNA primer (Fig. 2C). When the natural tRNA primer was employed, these two were again active, in addition to the homodimeric SIV enzyme (Fig. 2D). With the exception of pause products within 5–6 nt of the primer terminus, a paucity of paused intermediates suggested the inability of certain enzymes to sup-

port tRNA-primed synthesis of (–) strand strong-stop DNA was not the result of inefficient extension.

To accurately determine the efficiency of tRNA-primed DNA synthesis by these enzymes, we calculated, by phosphorimaging analysis, the amount of (–) strand DNA synthesis initiated by tRNA₃^{Lys} relative to that initiated by an 18-mer RNA primer was determined (Table 1). For example, values from Table 1 reveal that, even though FIV RT had reduced polymerase activity from both RNA and tRNA primers, the product ratio of these primed events were similar to that of other RTs that failed to initiate from tRNA₃^{Lys}. The relative amount of tRNA-primed synthesis by HIV-1 RT was 5- to 10-fold greater than that catalyzed by HIV-1 p66/p66, HIV-2 p66/p51, FIV p66/p66, EIAV p66/p66, or MLV RTs (Table 1).

Variants of the HIV-1 RNA Template or tRNA₃^{Lys} Primer Restore (–) Strand Synthesis Capacity to Heterologous RTs. Two systems were evaluated to test the significance of an interaction between the tRNA anticodon loop and the viral RNA U5-IR loop in initiation of (–) strand DNA synthesis. First, the A-rich loop in HIV-1 pbs RNA was deleted from the parental RNA by site-directed mutagenesis, creating the HIV-1 pbsΔA RNA template. Nuclease footprinting confirmed that the RNA secondary structure in the vicinity of the pbs was conserved in the absence of the -A-A-A-A- sequence (E.J.A., unpublished work). tRNA₃^{Lys} was then annealed to HIV-1 pbsΔA RNA and offered as primer for the heterologous enzymes (Fig. 3A). Second, an 85-nt tRNA-DNA chimera (tRNA₃^{Lys} extended by nine deoxynucleotides) was annealed to the wild-type HIV-1 pbs RNA to ensure disruption of the loop-loop complex, as well as a proposed interaction between the tRNA anticodon stem and the U5-IR stem (11) (Fig. 3C).

When HIV-1 pbsΔA RNA is substituted as template, tRNA-primed synthesis of (–) strand DNA is restored to all enzymes (Fig. 3B). However, the ratio of extended to unextended primer on HIV-1 pbsΔA RNA did not differ to that ratio on

HIV-1 pbs RNA. Similar levels of (–) strand DNA synthesis by the heterologous RTs were also observed when the 85-nt tRNA-DNA chimera was employed as primer on wild-type HIV-1 RNA template (Fig. 3D). Thus, two approaches capable of disrupting an interaction of tRNA with the U5-IR loop of HIV-1 RNA allow all enzymes to initiate HIV-1 (–) strand DNA synthesis from tRNA₃^{Lys}. With those enzymes inefficient at initiating (–) strand DNA synthesis from tRNA₃^{Lys} hybridized to HIV-1 pbs RNA, there is a 5- to 10-fold increase in (–) strand DNA production from the chimeric primer or from tRNA₃^{Lys} on the HIV-1 pbsΔA RNA template (Table 1). The results of Fig. 3B and D also suggest that the inability of several enzymes to use tRNA₃^{Lys} on wild-type HIV-1 RNA (Fig. 2C and D) is not due to steric inhibition by tRNA₃^{Lys} or by the inability of these RTs to bind free tRNA₃^{Lys}. On all HIV-1 template/tRNA primer duplexes, there was more (–) strand DNA synthesis catalyzed by HIV-1 RT than by any heterologous RT (Table 1). Thus, even in the absence of the proposed anticodon loop-U5-IR-loop complex, increased affinity of HIV-1 RT for tRNA₃^{Lys} most likely resulted in augmented usage of primer tRNA₃^{Lys} (Fig. 3B and D).

Heterologous RTs Support (–) Strand DNA Synthesis from tRNA₃^{Lys} Hybridized to EIAV and FIV RNA Templates. The inability of several related RTs to use the cognate primer hybridized to the HIV-1 RNA genome may indicate a selection of retroviral RTs for their cognate primer and genomic RNA template. To test this hypothesis, RNA containing R, U5, and pbs sequences of the FIV and EIAV proviral genomes was prepared by *in vitro* transcription. Synthetic tRNA₃^{Lys} was annealed to each to assess (–) strand DNA synthesis as described schematically in Fig. 4A. In contrast to our findings with the wild-type HIV-1 genome (Fig. 2), heterologous RTs use tRNA₃^{Lys} as primer on the FIV and EIAV templates for productive synthesis of (–) strand strong-stop DNA (Fig. 4B and C). With those enzymes catalyzing poor levels of (–) strand DNA synthesis from tRNA₃^{Lys} hybridized to HIV-1 pbs

FIG. 2. Heterologous RTs exhibit differential (–) strand DNA synthesis activity on the HIV-1_{HXB2} genome from oligonucleotide and tRNA₃^{Lys} primers. (A) Schematic representation of RNA-dependent DNA synthesis system. Hybridization of an 18-nt RNA primer to the pbs of the 497-nt HIV-1 RNA leads to synthesis of a 192-nt (–) strand product. Replacing oligoribonucleotide primer with tRNA₃^{Lys} results in synthesis of a 250-nt (–) strand strong-stop DNA. (B) Oligoribonucleotide-primed (–) strand synthesis activity. Lanes: 1, HIV-1 p66/p51 RT; 2, HIV-1 p66/p66 RT; 3, p66/p51 HIV-2 RT; 4, p66/p66 SIV RT; 5, p66/p66 FIV RT; 6, p66/p66 EIAV RT; 7, MLV RT; 8, AMV RT. The migration positions of the 18-nt RNA primer and full-length 192-nt (–) strand strong-stop product are indicated. (C) (–) strand strong-stop DNA synthesis from *in vitro*-synthesized, i.e., unmodified, tRNA₃^{Lys}. Lane notations are as in B. Migration positions of the 76-nt tRNA primer and 250-nt (–) strand strong-stop product are indicated. Synthetic tRNA₃^{Lys} was internally radiolabeled during *in vitro* transcription with ³²P. (D) (–) strand strong-stop DNA synthesis from *natural*, i.e., fully modified bovine tRNA₃^{Lys}. Lane notations are as in B. Lane "RT" refers to a tRNA₃^{Lys} - HIV-1 pbs RNA complex in the absence of enzyme.

Table 1. Relative efficiencies of (–) strand strong-stop DNA synthesis initiated from tRNA^{Lys} (over that initiated from an 18-mer RNA primer on various lentiviral RNA templates)

Enzymes	Relative synthesis of (–) strand strong-stop DNA from tRNA ^{Lys} / synthesis of (–) strand strong-stop DNA from an 18-mer RNA primer					
	HIV-1 pbs RNA (s)tRNA/RNA	HIV-1 pbs RNA (n)tRNA/RNA	HIV-1 pbsΔA RNA (s)tRNA/RNA	HIV-1 pbs RNA (c)tRNA/RNA	FIV pbs RNA (s)tRNA/RNA	EIAV pbs RNA (s)tRNA/RNA
HIV-1 p66/p51	1	1	1	1	1	1
HIV-1 p66/p66	0.07	0.05	0.72	0.69	0.41	0.79
HIV-2 p66/p51	0.03	0.04	0.78	0.62	0.64	0.84
SIV p66/p66	0.11	0.32	0.49	0.71	0.66	0.72
FIV p66/p66	0.16	0.02	0.61	1.2	0.74	0.61
EIAV p66/p66	0.09	0.08	0.69	0.52	0.41	0.72
MLV RT	0.04	0.10	0.40	0.63	0.63	0.79
AMV RT	0.31	0.94	0.65	0.69	0.53	0.61

The values reported represent the synthesis of (–) strand strong-stop DNA from tRNA^{Lys} relative to that of (–) strand strong-stop DNA from an 18-mer RNA primer (second line in each heading) on each respective template (first line in each heading). The ratio obtained for each enzyme was standardized to the ratio obtained with HIV-1 p66/p51 (given value of 1). (s)tRNA = 76 nt synthetic tRNA^{Lys}; (n)tRNA = natural 76 nt tRNA^{Lys}; (c)tRNA = chimera of synthetic tRNA^{Lys} and 9 deoxynucleotides.

RNA, there was at least a 4-fold increase in (–) strand DNA synthesis in reactions employing tRNA^{Lys} as primer on the FIV or EIAV pbs RNA (Table 1). In addition, there was no selective advantage of FIV or EIAV RTs for their respective RNA genomes, i.e. the level of (–) strand DNA synthesis initiated from tRNA^{Lys} on their respective templates by the feline and equine RTs was equal to (or in some cases less than) that determined for all other enzymes (Table 1).

FIG. 3. Heterologous RTs productively extend a tRNA^{Lys}-DNA chimera hybridized to HIV-1 pbs RNA or tRNA^{Lys} hybridized to a modified HIV-1 RNA. (A) Schematic representation of the 5' end of the pbsΔA HIV-1 genome. The pbs (thin solid line) is looped out and annealed to the 3' end of tRNA^{Lys} (shaded line), whereas the -A-A-A-A- sequence has been removed from the U5-IR loop. (B) tRNA^{Lys} primed (–) strand strong-stop DNA on the HIV-1 pbsΔA RNA template by heterologous RTs. Lane notations are as in Fig. 2B. (C) Extended complementarity between the tRNA-DNA chimera at the 3' end of the primer and the HIV_{HXB2} genome. Dotted line represents the 3' end of tRNA^{Lys} annealed to PBS of viral RNA (thin solid line). The thick solid line represents a 9-nt deoxynucleotide extension of tRNA^{Lys}. Complementarity between the chimeric tRNA primer and the wild-type viral genome is thereby extended from 18 to 27 nt. (D) Synthesis of (–) strand strong-stop DNA from the 85-nt tRNA-DNA chimera by heterologous RTs. Lane 1, 85-nt tRNA-DNA chimera in the absence of RT; other lane notations are as in Fig. 2B.

Unlike the HIV-1 genome, computer-generated folding of 500 nt adjacent to the pbs of FIV and EIAV genomes predicted no alternative RNA structures containing an A-rich loop. Although tRNA^{Lys} may interact with other FIV and EIAV RNA sequences, these interactions are unlikely to represent barriers to initiation or elongation of (–) strand DNA. Interestingly, EIAV pbs RNA, when compared with FIV pbs RNA, supported more (–) strand DNA synthesis from tRNA^{Lys}. The diminution of DNA synthesis from tRNA^{Lys} on FIV pbs RNA appears attributable to decreased efficiency of tRNA^{Lys} annealing to the RNA genome, whereas augmented synthesis by the HIV-1 heterodimer may be explained by its higher affinity for free tRNA (E.J.A., unpublished work).

Pausing During Early (–) Strand DNA Synthesis Events on Different Lentiviral RNA Templates. In previous studies, prematurely terminated products were observed 1–6 nt from the primer terminus when HIV-1 RT was used in combination with tRNA^{Lys} and HIV-1 RNA (27, 28). To accurately determine the amount and position of pause products on heterologous lentiviral RNA templates, (–) strand DNA synthesized in conditions of low RT:primer/template ratio (1:2) was fractionated by high-resolution PAGE alongside products of dideoxy-sequencing reactions (Fig. 5A). With the exception of early pause products, synthesis of (–) strand strong-stop DNA under these conditions was as efficient as observed in Figs. 2–4 (data not shown). Using HIV-1 p66/p51 RT, pause products were observed 1–6 nt from the primer tRNA^{Lys} annealed to HIV-1 pbs RNA or to HIV-1 pbsΔA RNA (Fig. 5A). Deletion of the A-rich sequence disrupts the tRNA^{Lys} anticodon loop-U5-IR loop interaction but appears to have little effect on the U5-IR stem structure determined by nuclease footprinting (E.J.A., unpublished work). A similar observation was made in experiments where the A-rich sequence of the HIV-1_{mal} template was deleted and partially substituted with an unrelated sequence (28). Pausing near the primer terminus may be induced by the U5-IR stem in HIV-1 RNA (Fig. 5B) because disruption of this stem with the 85 nt tRNA^{Lys}-DNA chimera eliminated pause products near the primer terminus during synthesis of (–) strand DNA (Fig. 3D).

Data of Figs. 5B and 3D and of previous work (27, 28) suggest that pausing during initiation of (–) strand DNA synthesis may be enzyme specific rather than dependent on primer/template interactions. To test this hypothesis, pausing following initiation of (–) strand DNA was studied in related lentiviral systems, i.e., using FIV and EIAV RTs with their respective templates. The use of primer tRNA^{Lys} annealed to the FIV pbs RNA template induced stalling of both HIV-1 p66/p51 RT and FIV p66/p66 RT 11–12 nt from the primer terminus (Fig. 5D). With EIAV pbs RNA as template, pause products were present 1, 3, 4, 7, and 9 nt from the tRNA^{Lys}

FIG. 4. tRNA^{Lys}-primed synthesis of (-) strand strong-stop DNA on EIAV and FIV genomes by heterologous RTs. (A) Schematic representation of pbs-containing portions of the EIAV and FIV genomes. The pbs (boldface type) and surrounding sequences of the EIAV (Upper) and FIV genomes (Lower) are presented, whereas those immediately preceding the R region are vector-derived. tRNA^{Lys}-primed (-) strand strong-stop products from the each genome are indicated. (B) tRNA^{Lys}-primed (-) strand strong-stop DNA on the EIAV genome by heterologous RTs. Lane notations are as in Fig. 2B. (C) tRNA^{Lys}-primed (-) strand strong-stop DNA on the FIV genome. Lane notations are as in Fig. 2B.

terminus when DNA synthesis was catalyzed by HIV-1 p66/p51 RT and primarily 1, 3, and 4 nt from the primer terminus with EIAV p66/p66 RT. Analysis of RNA secondary structures by Zucker algorithms (15) predict that the translocating enzymes encounter a G-C-rich region of secondary structure on the RNA genome when tRNA^{Lys} is extended by 4 nt on EIAV pbs RNA (Fig. 5C), and when tRNA^{Lys} is extended by 12 nt on FIV pbs RNA (Fig. 5F). Short stem-loops encountered by RT prior to the first RNase H cleavage of the template (i.e., after synthesis of 18 nt) has also been shown to induce pausing (26). Thus, it appears that pausing by HIV-1, FIV and EIAV RTs was induced by RNA secondary structures rather than properties specific to these enzymes.

DISCUSSION

To study initiation of reverse transcription, we have evaluated the efficiency with which several lentiviral and retroviral RTs support (-) strand DNA synthesis from tRNA^{Lys} hybridized to pbs-containing RNA templates of HIV-1, FIV, and EIAV. Although the lentiviral enzymes studied here share a common tRNA replication primer, our data suggest that efficient initiation of (-) strand DNA synthesis may be defined by retrovirus-specific interactions of both RT with tRNA^{Lys} and tRNA^{Lys} with the viral RNA genome. First, all enzymes supported (-) strand DNA synthesis from tRNA^{Lys} on EIAV and FIV pbs-containing RNAs, whereas only HIV-1, AMV, and SIV RT productively extended tRNA^{Lys} hybridized to HIV-1 RNA. Second, only HIV-1 p66/p51 RT showed high affinity for free tRNA^{Lys}. Finally, tRNA^{Lys} has been shown to interact with HIV-1 genomic RNA outside the pbs (9, 11). Such observations suggest the ability of HIV-1 RT to disrupt tRNA-viral RNA complexes to initiate (-) strand DNA synthesis from pbs-bound tRNA^{Lys} is not shared by all enzymes. However, as will be discussed, such a property may be unique to HIV-1.

FIG. 5. Pausing during (-) strand DNA synthesis after initiation from tRNA^{Lys} annealed to the HIV, EIAV, and FIV genomes. (A) (-) strand DNA synthesis from primer tRNA^{Lys} annealed to HIV-1 pbs and pbsΔA RNA. (D) (-) strand DNA synthesis from primer tRNA^{Lys} on FIV pbs RNA, catalyzed by p66/p51 HIV-1 and p66/p66 FIV RT. (E) p66/p51 HIV and p66/p66 EIAV RT-catalyzed (-) strand DNA synthesis from tRNA^{Lys} on EIAV pbs RNA. B, C, and F schematically represent the computer-predicted secondary structures of the HIV-1 (shaded line) RNA, FIV RNA, and EIAV RNA, respectively, annealed to a tRNA^{Lys} extended by several deoxynucleotides (thick solid line), i.e., the number which induces RT pausing in A, D, and E, respectively.

With the exception of HIV-1 p66/p51, AMV, and SIV RT, no other enzyme initiated DNA synthesis from tRNA^{Lys} on HIV-1 pbs RNA. This, however, was not due to decreased affinity of these RTs for a ribonucleotide primer, because equivalent amounts of (-) strand DNA products were observed when synthesis was initiated from an 18-nt RNA primer on HIV-1 (Fig. 2B), FIV, and EIAV pbs RNA templates (data not shown). Thus, the additional 58 nt of tRNA^{Lys} must in some manner compromise certain heterologous RTs. This inhibition may well be imparted by tRNA^{Lys}-HIV-1 RNA interactions outside of the pbs, such as a complex involving the A-rich anticodon loop of tRNA^{Lys} and U-rich U5-IR loop of HIV-1 RNA (9, 11, 14). Disruption of this complex by pre-hybridization of a tRNA-DNA chimera or by deleting the A-rich loop of the viral RNA template restored tRNA-primed synthesis of (-) strand DNA to all enzymes. In a similar study, a mutant of HIV-1 RT (p66/p51Δ13 RT) failed to initiate HIV-1 (-) strand DNA synthesis from tRNA^{Lys}, while disruption of the loop-loop complex by a chimeric tRNA-DNA primer corrected this deficiency (17). HIV-1 RT, which interacts with the anticodon domain of tRNA^{Lys} through its connection and thumb subdomains (29), may disrupt the anticodon loop-U5-IR-loop complex prior to the initiation of HIV-1 (-) strand DNA synthesis. Although initiation of HIV-1 reverse transcription may not be directly dependent on this interaction, it is important to note that deletion of these four adenosines from the U5-IR loop in HIV-1 results in their gradual reappearance in this region after several virus passages (X.L. and M.A.W., unpublished work).

Although HIV-1 p66/p66 and p66/p51 RT have similar RNase H and DNA polymerase activities (30), the tRNA-

primed (–) strand synthesis assay employed here shows that only the heterodimer is capable of initiating (–) strand DNA synthesis from the cognate tRNA_{3^{lys}} primer annealed to HIV-1 RNA. In the homodimer, the subunit assuming the “p51-like” structure of the heterodimer (31) may not provide the correct structural backbone for the “polymerase-active” p66 subunit to interact correctly with tRNA_{3^{lys}} for initiation on HIV-1 RNA. However, this feature of the HIV-1 p66/p66 homodimer cannot explain the inability of HIV-2 heterodimer RT to use tRNA_{3^{lys}} as primer on HIV-1 RNA. Recent studies suggest that the HIV-2 enzyme employed in this study (21) may contain a C-terminal deletion in the small subunit. Fan *et al.* (32) have presented convincing evidence that the HIV-2 p68 subunit is cleaved between Met-484 and Ala-485 by its cognate protease and not between Phe-440 and Tyr-441, as with HIV-1 protease (31, 32). As described earlier (17), deleting as little as 13 residues from the C terminus of p51 generated a mutant of HIV-1 RT (p66/p51Δ13 RT) incapable of initiating tRNA-primed (–) strand DNA synthesis on HIV-1 RNA. Therefore, removal of 44 C-terminal residues from the smaller HIV-2 subunit may remove sequences required for interactions with the tRNA_{3^{lys}}-HIV-1 RNA complex and subsequent initiation of (–) strand DNA synthesis. Currently, it is unclear why the p66/p66 homodimer of SIV RT supports (–) strand synthesis selectively from the natural tRNA primer, but serves to further underscore subtle differences between closely-related retroviral enzymes. The ability of AMV RT to productively initiate (–) strand DNA synthesis from tRNA_{3^{lys}} may reflect the ability of this enzyme to unwind nucleic acid duplexes (7).

FIV and EIAV RT failed to initiate (–) strand DNA synthesis from tRNA_{3^{lys}} on HIV-1 RNA, while doing so from tRNA_{3^{lys}} on their respective genomes. The structures of EIAV and FIV RNA in the vicinity of the pbs are significantly different than that of HIV-1 RNA (Fig. 1). No A-rich loop exists within 500 nt of the FIV and EIAV. Disruption of a tRNA-viral RNA barrier may therefore not be necessary for initiation of (–) strand DNA synthesis in FIV and EIAV. Alternatively, a tRNA-EIAV or tRNA-FIV RNA structure may exist that EIAV and FIV RT is capable of disrupting to initiate (–) strand DNA synthesis. Considering that all enzymes were capable of using primer tRNA_{3^{lys}} on FIV and EIAV, it is unlikely that retrovirus-specific RNA structural barriers were present in tRNA_{3^{lys}}-EIAV RNA and tRNA_{3^{lys}}-FIV RNA duplexes.

Recent studies have described pause products on HIV-1 RNA 1–6 nt from the tRNA primer terminus (27, 28). Isel *et al.* (28) attributed these to an initiation complex formed through specific interactions between tRNA_{3^{lys}}, HIV-1 RNA, and HIV-1 RT. This complex was less dependent on tRNA sequence, since reduced RT pausing occurred at these sites when DNA synthesis was initiated from an oligoribonucleotide primer (Fig. 2B and ref. 28). In this study, we observed (–) strand DNA pause products near the primer terminus on FIV, EIAV, and HIV-1 RNA templates. The position of these pause products was directly related to the presence or formation of a stable RNA stem in the viral RNA genome as (–) strand DNA synthesis proceeds from the tRNA (Fig. 5). Pausing near the primer terminus was also observed under conditions where the tRNA anticodon loop and U5-IR loop could not interact, i.e., with either an 18-nt RNA primer or when tRNA_{3^{lys}} was hybridized to the HIV-1 pbsΔA genome. However, we failed to detect pausing near the primer terminus in a reaction where both the U5-IR stem and the loop-loop complex were disrupted, i.e., an 85 nt tRNA-DNA chimeric primer annealed to HIV-1 pbs RNA (Fig. 3D). These results suggest that pausing during initiation of (–) strand DNA synthesis may be more attributable to template structure and sequence than to properties intrinsic to the RT.

In summary, it appears that most retroviral RNAs and their cognate tRNA primer do not form the intermolecular inter-

action involving the tRNA anticodon loop observed in the HIV-1 system (11, 14, 28). Such a complex may not be necessary for initiation of HIV-1 (–) strand DNA synthesis, but may be a requirement for efficient tRNA_{3^{lys}} placement on the viral genome, or co-packaging of tRNA with viral RNA. Different tRNA-viral RNA structures appear to have evolved for other retrovirus and may serve a similar function to the tRNA anticodon-U5-IR-loop complex of HIV-1 (7–11).

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