Interaction of HIV-1 reverse transcriptase with a synthetic form of its replication primer, $tRNA^{Lys,3}$

Corinne Barat, Stuart F.J.Le Grice[2,+] and Jean-Luc Darlix[1,*]
CRBGC du CNRS, 118 Route de Narbonne, 31062 Toulouse, [1]LaboRetro, Ecole Normale Superieure de Lyon, 46 allée d'Italie, 69364 Lyon, France and [2]Central Research Units, F.Hoffmann-La Roche Ltd, Basel, Switzerland

Received November 30, 1990; Revised and Accepted January 21, 1991

ABSTRACT

Using synthetic oligonucleotides, a gene encoding the HIV-1 replication primer, $tRNA^{Lys,3}$, was constructed and placed downstream from a bacteriophage T7 promoter. *In vitro* transcription of this gene yielded a form of $tRNA^{Lys,3}$ which lacks the modified bases characteristic of the natural species and the 3′ -C-A-dinucleotide. Synthetic $tRNA^{Lys,3}$ annealed to a pbs-HIV1 RNA template can prime cDNA synthesis catalysed by recombinant HIV-1 reverse transcriptase. *Trans*-DDP crosslinking indicates that this synthetic tRNA is still capable of interacting with HIV-1 RT via a 12-nucleotide portion encompassing the anticodon domain. Gel-mobility shift and competition analyses imply that the affinity of synthetic tRNA for RT is reduced. In contrast to earlier observations, synthetic tRNA is readily competed from RT by natural $tRNA^{Pro}$. The reduced affinity of synthetic $tRNA^{Lys,3}$ for RT is not appreciably affected by mutations in positions within the loop of the anticodon domain. These results would imply that the overall structure of the anticodon domain of $tRNA^{Lys,3}$ is an important factor in its recognition by HIV-1 RT. In addition, modified bases within this, although not absolutely required, would appear to make a significant contribution to the enhanced stability of the ribonucleoprotein complex.

INTRODUCTION

Retroviral replication initiates from a tRNA molecule hybridised via its 3′ stem to a specific region at the 5′ end of the RNA genome, the primer binding site (pbs) (1). Examples of tRNAs required for initiation are $tRNA^{Trp}$ for Rous sarcoma virus (RSV) (2), $tRNA^{Pro}$ for murine leukaemia virus (MLV) (3), and $tRNA^{Lys,1,2}$ for mouse mammary tumour virus (MMTV) (4). In the case of the Human Immunodeficiency Virus (HIV), sequence homologies have implicated $tRNA^{Lys,3}$ as the replication primer (5). In a recent communication, we demonstrated by both gel-mobility shift and *trans*-DDP crosslinking analyses that a complex, involving the anticodon domain, could be formed between a recombinant form of p66/p51 HIV-1 RT and $tRNA^{Lys,3}$ (6). A specific interaction between HIV RT and its replication primer has also been demonstrated by Sallafranque-Andreola *et al.*, who showed inhibition of reverse transcription from a synthetic homopolymer template/primer by $tRNA^{Lys,3}$ but not $tRNA^{Val}$ (7), and later that a small oligonucleotide from the anticodon of $tRNA^{Lys,3}$ gave a similar inhibition (8). In the continuing search for improved anti-HIV agents, disruption of this specific RT/tRNA interaction might represent an alternative avenue for therapeutic intervention. Since RT-bound $tRNA^{Lys,3}$ cannot be competed from the enzyme with tRNA used as replication primers by related retroviruses (6), it is important to more precisely define the determinants controlling the affinity and specificity of this interaction. Obvious candidates for this would be the highly modified bases within and flanking the anticodon (9,10).

One means by which the interaction between HIV RT and its cognate replication primer might be assessed would be to study the interaction between the recombinant enzyme and a synthetic form of $tRNA^{Lys,3}$, the preparation of which in large quantities is possible by efficient *in vitro* transcription systems (11, 12). This possibility is strengthened by recent reports indicating that synthetic tRNA can still be charged with their cognate amino acid (13, 14), suggesting that overall structural integrity is preserved in these molecules. The availability to prepare large amounts of synthetic tRNA should thus allow a detailed study of its interaction with RT.

In this communication, we report the cloning and preparation of a synthetic form of $tRNA^{Lys,3}$ and its interaction with the heterodimeric (p66/p51) form of HIV-1 RT. *Trans*-DDP crosslinking studies indicate that, as with the natural tRNA species, a 12-nucleotide portion encompassing the anticodon domain is in close contact with the enzyme. In contrast to our previous findings (6), gel-mobility shift analyses indicate that natural $tRNA^{Pro}$ can displace synthetic $tRNA^{Lys,3}$ from RT. These combined observations would suggest that the overall structure of the anticodon domain of $tRNA^{Lys,3}$ contributes significantly to its interaction with RT, but that the affinity of this interaction may be further enhanced by the hyper-modified

* To whom correspondence should be addressed

+ Present address: Division of Infectious Diseases, Case Western Reserve University School of Medicine, 2109 Adelbert Road, Cleveland, Ohio 44106, USA

bases. Finally, $tRNA^{Lys,3}$ mutants containing alterations within the anticodon or modified bases display the same characteristics as the non-mutant synthetic species, indicating that the nature of the nucleotide at these positions might not be important to the interaction.

MATERIALS AND METHODS

Cloning and Preparation of Synthetic $tRNA^{Lys,3}$

A synthetic form of the $tRNA^{Lys,3}$ gene was prepared from a series of DNA oligonucleotides (figure 1A). The gene was preceded by a bacteriophage T7 promoter such that the 5′ G residue of the tRNA was the initiating nucleotide upon *in vitro* transcription. The complete transcriptional unit (i.e., promoter + tRNA gene) was flanked at its 3′ terminus with a *Bam*HI site and inserted into the plasmid pSP65 between the *Eco*RI and *Bam*HI sites to generate the plasmid pTL9. The integrity of the synthetic tRNA gene was determined by dideoxy sequencing (15) and shown to be consistent with the published sequence (9). Cleavage of pTL9 with *Ban*I allowed preparation of a 'run-off' transcript of $tRNA^{Lys,3}$ lacking the 3′ -C-A- dinucleotide (Figures 1 and 2).

HIV RT Preparation and Assays

Recombinant H-p66/H-p51 HIV-1 reverse transcriptase was purified from the *E.coli* strain M15::pDMI.1 harbouring the plasmid p6HRT-PROT as previously described (16). In addition to NTA-Sepharose (17), we used S-Sepharose ion exchange chromatography to remove trace contaminants. RT thus prepared was judged at least 95% pure by SDS/polyacrylamide gel electrophoresis, with a specific activity of 5000 U/mg on a poly r(C)/oligo d(G) template primer and buffer conditions previously described (16). 32P-internally labelled primer synthetic tRNA was prepared from Ban I digested pTL9. Template DNA was *Rsa*I-digested pCG1, which contains HIV-1 *mal* sequences (18) from nucleotides 30 to 310. Natural $tRNA^{Lys,3}$ was 5′ labelled as previously described (6). The primer and template RNA preparations were mixed, heated to 95°C, then incubated for 2 hours at 50°C. The primer/template complex was purified on a 5% polyacrylamide gel run in Tris/Borate/EDTA buffer. (19). RT assays contained 200ng of primer/template, 250μM dNTPs, 200ng p66/p51 HIV-1 RT in RT buffer (16). After 10min at 42°C, the reactions were stopped by addition of 20mM EDTA containing 0.2% SDS and heated 2min at 90°C. Extended tRNAs were analysed by electrophoresis through 8% polyacrylmide gels in 7M urea, and visualised by autoradiography.

Trans-DDP Crosslinking and Gel-Shift Experiments

Trans-DDP cross-linking experiments (20) of natural and synthetic $tRNA^{Lys,3}$ to p66/p51 HIV-1 RT were performed as previously described (6). Reactions contained 0.5μg p66/p51 RT (~4 pmoles) and 0.1μg of the respective tRNA (~4 pmoles, purified on 5% polyacrylamide/7M urea gels). After 30 min incubation with 250μM *Trans*-DDP at 20°C (in the dark), the complex was treated with 10U T1 RNase. T1 RNase-generated oligonucleotides were 5′labelled with polynucleotide kinase in the presence of ^{32}P-ATP. The RT/tRNA complex was then electrophoresed on an 8% polyacrylamide/SDS gel. Following electrophoresis, the gel was dried and subjected to autoradiography. Where the concentration of *trans*-DDP varied, this has been given in the text. Gel shift experiments were performed either on 1.0% agarose gels as before (6), or on 4% polyacrylamide gels (60:1 Acrylamide:Bis-acrylamide) in 0.5×Tris/Borate/EDTA. Acrylamide gels were pre-electrophoresed for 2 hours. Following electrophoresis, the gels were dried and subjected to autoradiography.

In vitro Mutagenesis of $tRNA^{Lys,3}$ Gene

The synthetic $tRNA^{Lys,3}$ gene was cloned between the *Pst*I and *Bam*HI sites of pUC8 to generate plasmid pTL6. pTL6 was

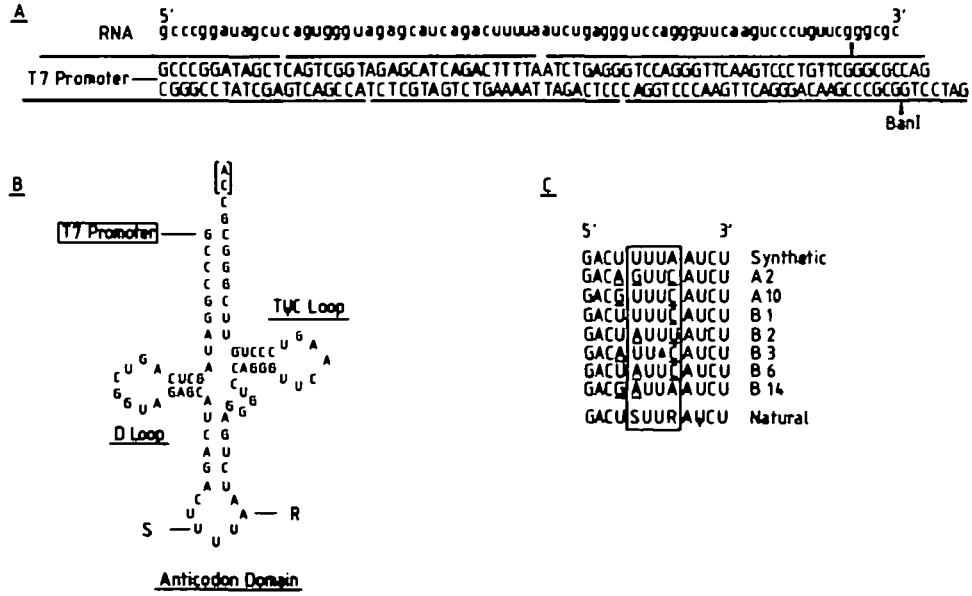

Figure 1. [A], DNA sequence of the synthetic gene coding for $tRNA^{Lys,3}$. The series of oligonucleotides constituting the tRNA gene, as well as the T7 promoter, are indicated by solid bars. The *Ban*I site on the template strand has been indicated below, and the corresponding RNA transcript from *Ban*I-digested pTL9 above, the DNA sequence. Note that $tRNA^{Lys,3}$ produced by this procedure lacks the terminal -C-A-dinucleotide. [B], Structure of synthetic $tRNA^{Lys,3}$ prepared by *in vitro* transcription. Positions of the highly modified bases S and R in the anticodon domain have been indicated. The 3′ terminal dinucleotides lacking after *in vitro* transcription have been bracketed. [C], Anticodon domain mutants of $tRNA^{Lys,3}$. Altered bases have been underlined. The sequence of the anticodon domain from synthetic and natural $tRNA^{Lys,3}$ are indicated at the top and bottom of the panel, respectively.

subsequently cleaved with *Ava*II, which cuts 3′ to the anticodon (Figure 1[A]). Mutagenesis was performed using the polymerase chain reaction (PCR) (21) and oligonucleotides A and B. A is **5′ GTAAAACGACGGCCAGT 3′** and represents positions 379–395 of pUC8 allowing amplification from the T7 promoter into the tRNA gene. B represents nucleotides 26–52 of the lower strand of the tRNA gene, encompassing the anticodon (positions 27–44).

Since oligo B is degenerated at three positions as shown in parentheses,

3′ TAGTCTG(A,T,C)(A,T,C)AAA(A,T,G)TAGACTCCC-AGGTCC 5′,

this allowed to prepare a random series of anticodon domain mutants. PCR was performed in 10mM Tris/HCl, pH 8.4, 25 mM $MgCl_2$, 30mM KCl, 0.05% Tween 20, 0.05% NP40, 0.2 mM of all four dNTPs, 1μM oligonucleotide, 20ng *Ava*II linearised pTL6 and 5U Taq DNA polymerase, in 100μl. 25 cycles comprising 1 min at 95°C, 2 min at 37°C and 2 min at 72°C were performed and the amplified DNA purified. Following *Pst*I/*Ava*II digestion, the resulting 68 bp fragment was purified by electrophoresis through low melting temperature agarose. Plasmid pTL6 was totally digested with *Pst*I and partially with *Ava*II, and a 2.7kb *Pst-Ava* fragment purified as above. This was ligated to the 68 bp *Pst*I-*Ava*II fragment. Mutant forms of tRNA were determined by sequencing several recombinant clones. Of 19 genes sequenced, the seven mutant forms indicated in Figure 1[C] were prepared.

Preparation of $tRNA^{Lys,3}$ and pbs-Containing RNA

Synthetic $tRNA^{Lys,3}$ was prepared by *in vitro* transcription of 5μg *Ban*I-linearised pTL9, or anticodon domain mutants, with T7 RNA polymerase. Template DNA was plasmid pMCG1 (6) linearised with *Rsa*I. Reactions (100μl) contained 40mM Tris/HCl, pH 7.5, 6mM $MgCl_2$, 2mM spermidine, 10mM NaCl, 10mM dithiothreitol, 0.5mM of each ribonucleoside triphosphate, ^{32}P-UTP at 20 Ci/mmol, 40 units T7 RNA polymerase and 50 units RNasin. After 2 hours at 37°C, the samples were treated with DNaseI. RNA was extracted with phenol, chromatographed over Sephadex G75, precipitated with ethanol and dissolved in double-distilled water. The concentration of RNA in the samples was determined spectrophotometrically. Natural $tRNA^{Pro}$ and $tRNA^{Lys,3}$ were from beef liver and essentially pure as indicated before (6,22).

Routine Chemicals

Radionuclides were purchased from Amersham, and restriction endonucleases from Boehringer Mannheim or Pharmacia. *Trans*-DDP and routine chemicals were from Sigma.

RESULTS

Preparation of Synthetic $tRNA^{Lys,3}$

Using the protocols outlined in Figure 1 (A), a synthetic gene coding for $tRNA^{Lys,3}$ was prepared from a series of overlapping oligonucleotides to generate the plasmid pTL9. This synthetic tRNA gene was preceded by a pair of oligonucleotides specifying a promoter for the RNA polymerase of bacteriophage T7, with appropriate spacing to the initiation site for transcription. Whilst this guarantees that the 5′ tRNA sequence is correct, preparation of a tRNA with correct 3′ sequences required that a restriction site be at or near this terminus, without changing the RNA sequence. As illustrated in Figure 1 (A), the endonuclease *Ban*I cleaves in the vicinity of the 3′ terminus of the tRNA gene. The template (lower) strand for *in vitro* transcription of *Ban*I-cleaved pTL9 extends to residue C74 to produce a $tRNA^{Lys,3}$ molecule lacking the -C-A- dinucleotide at the 3′ terminus (see figure 1B). Also a complete synthetic $tRNA^{Lys,3}$ was prepared by incubating the tRNA with the enzyme ATP (CTP);tRNA nucleotidyl-transferase (NTase) in the presence of CTP and ATP. Results of gel retardation assays indicated that tRNA binding to HIV RT was the same with either the full-length or 3′-CA deficient tRNA species (results not shown).

Figure 2. [A], Analysis of synthetic $tRNA^{Lys,3}$ and anticodon domain variants by high resolution gel electrophoresis. *Ban*I-digested plasmids were transcribed with T7 RNA polymerase in the presence of ^{32}P-UTP, heat-denatured and analysed on 8% polyacrylamide gels containing 7M urea. As a comparison, a sample of end-labelled $tRNA^{Pro}$ was included. Size markers on the left of the panel are in nucleotides. The non-mutant form of $tRNA^{Lys,3}$ produced from plasmid pTL6 is identical with that from pTL9. [B], Strong-stop cDNA synthesis primed by either natural (Lanes 1–3) or synthetic $tRNA^{Lys,3}$ (Lanes 4–5) hybridised to a pbs-HIV-1 RNA template. Lane 1, marker DNA; Lanes 2,4, no RT; Lanes 3,5 reverse transcription in the presence of p66/p51 HIV-1 RT. The extension of the tRNA primer is indicated at the bottom of the figure.

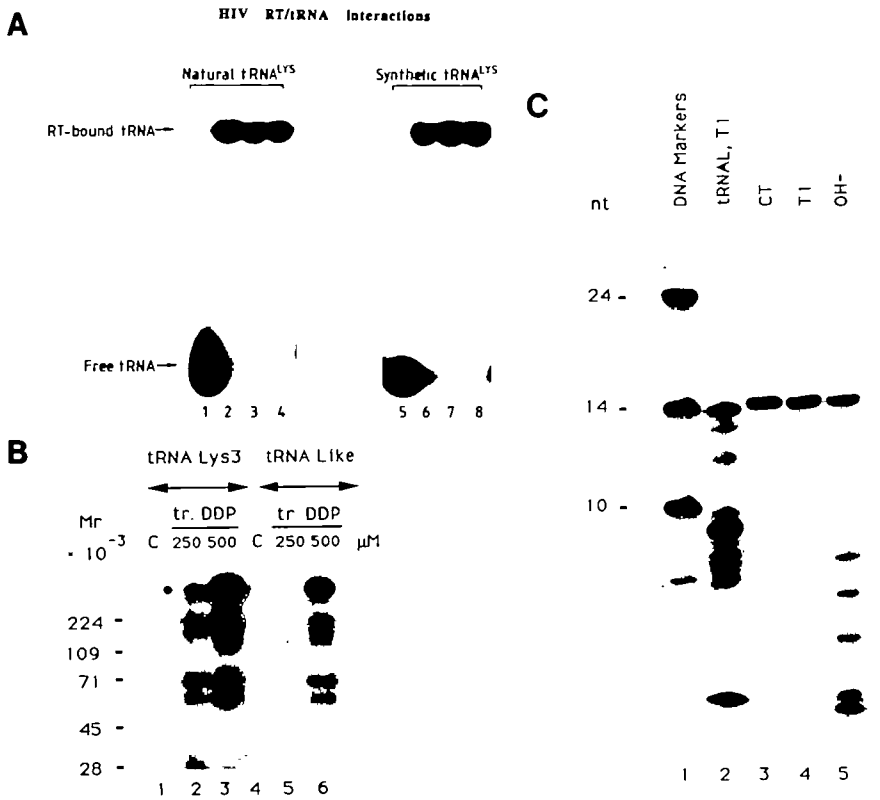

Figure 3. [A] Gel-mobility shift analyses of HIV-1 p66/p51 RT complexed to the natural (Lanes 1–4) and synthetic forms of tRNA^{Lys,3} (Lanes 5–8). Gel shift experiments were performed on a 1% agarose gel. Lanes 1 and 5 are free tRNA. 20ng (0.8 pmoles) of natural or 30ng (1.2 pmoles) synthetic tRNA^{Lys,3} was used, and complexed with 2.5 (Lanes 2 and 6), 5.0 (Lanes 3 and 7) or 7.5 (Lanes 4 and 8) pmoles recombinant p66/p51 HIV-1 RT. Positions of free and complexed tRNA are indicated. [B], *Trans*-DDP crosslinking of HIV-1 p66/p51 RT to natural and synthetic tRNA^{Lys,3}. The concentrations of cross-linking agent (tr. DDP) used are indicated above each panel. C, control reaction lacking *trans*-DDP. The autoradiogram depicts a cross-link between RT and an oligoribonucleotide generated by T1 RNase digestion of the RNA/protein complex (6). Molecular weight markers are given in kDa. [C], Analysis of the RNase T1-generated oligoribonucleotide bound to RT after reversal of the cross-link. **tRNAL,T1**, T1 RNase treatment of full-length synthetic tRNA^{Lys,3} to localise the 12 nucleotide portion representing the anticodon domain; **T1**, further treatment of the purified oligoribonucleotide with T1 RNase; **CT**, purified RT-bound oligoribonucleotide; **OH-**, alkaline hydrolysis of the purified fragment. Molecular weight single-stranded DNA markers (in nucleotides) are indicated in the leftmost panel. Note that RNA and DNA species migrate at slightly different rates. Samples were electrophoresed through a 20% polyacrylamide/7M urea gel.

In order to determine whether specific bases within the anticodon domain contributed to either the specificity or affinity of the RT/tRNA interaction, the series of anticodon domain mutations outlined in Figure 1 (C) was prepared. Since degenerate oligonucleotides were used in the preparation of these mutants, multiple alterations were introduced into the anticodon domain. Plasmids containing each of these tRNA mutants were linearised by digestion with *Ban*I, and transcribed with T7 RNA polymerase in the presence of 32P-UTP. The RNA products of each reaction were heat denatured and purified by polyacrylamide gel electorophoresis in 7M urea. As illustrated in Figure 2[A], each transcription reaction yielded a tRNA species of the expected size with minimal degradation.

The proficiency of both natural and synthetic tRNA^{Lys,3} as a primer was also determined in an *in vitro* reverse transcription assay, using a truncated RNA template containing the HIV-1 pbs sequence. With the exception that the nucleocapsid protein (NC) is absent, this experiment closely mimics initiation of minus strand DNA synthesis in the virion. As shown in Figure 2 [B], both the natural and synthetic tRNA^{Lys,3} are efficiently hybridised to the pbs and serve as a primer from which cDNA synthesis can be initiated. Despite the ability to serve as a primer, the results of Figure 2 do not address whether the correct structure has been imparted into synthetic tRNA^{Lys,3}. In the following sections, other aspects of RT/tRNA intreractions verifying the structural integrity of synthetic tRNA^{Lys,3} are addressed.

Interaction of Synthetic tRNA^{Lys} with HIV-1 RT

In a preliminary series of experiments, the affinity of the synthetic tRNA^{Lys,3} for a recombinant form of p66/p51 heterodimer HIV-1 RT was determined by gel-shift experiments (see figure 3A). In the absence of any competitor, synthetic tRNA^{Lys,3} appeared as capable as the natural species of binding to RT and eliciting a gel mobility shift. However, despite this gel mobility shift, the results of Figure 3 (A) address neither the specificity nor affinity of synthetic tRNA^{Lys,3} for HIV-1 RT. In order to determine whether the specificity of the interaction had been preserved, *trans*-DDP cross-linking experiments were performed as before (6), and the nature of any RT-bound oligoribonucleotide determined. Figure 3 (B) indicates that, at slightly elevated concentrations of cross-linking agent, a small oligoribonucleotide of the synthetic tRNA can indeed be linked to both the 66 and 51 kDa subunit of the enzyme. Following reversal of the cross-link with thiourea, high resolution gel electrophoresis indicated that a 12-nucleotide RNA fragment was released. In a control experiment, ^{32}P-labelled synthetic tRNA^{Lys,3} was digested with T1 RNase and run in parallel to identify the 12-nucleotide portion containing the anti-codon domain (Figure 3[C]). Since this is the

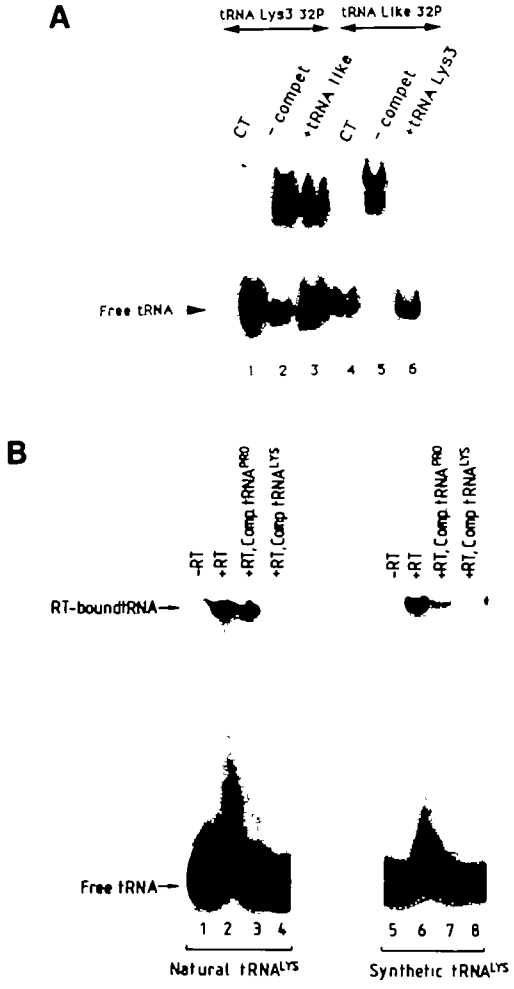

Figure 4. [A], Competition of natural and synthetic $tRNA^{Lys,3}$ from p66/p51 RT with their snythetic (Lanes 1–3) and natural (Lanes 4–6) counterpart. Lanes 1 and 4 contain uncomplexed tRNA. The tRNA species bound to RT and competing species are illustrated at the top of the panel. In either case, the competing tRNA was added in a 10-fold excess over the bound species. [B], Competition of natural (Lanes 1–4) and synthetic $tRNA^{Lys,3}$ (Lanes 5–8) bound to RT with homologous or non-homologous natural tRNAs. The nature of competitor is illustrated above each panel. Lanes 1–4 contained 0.6 pmoles tRNA (Lane 1) complexed with 1.2 pmoles RT (Lane 2), in the presence of either 24 pmoles $tRNA^{Pro}$ (Lane 3) or 8 pmoles $tRNA^{Lys,3}$ (Lane 4). For the synthetic species, 0.6 pmoles tRNA (Lane 5) was complexed with 1.2 pmoles RT (Lane 6) and competed with either 24 pmoles $tRNA^{Pro}$ (Lane 7) or 8 pmoles $tRNA^{Lys,3}$. Gel shift experiments were performed on a 4% polyacrylamide gel (Panel A) and on a 1% agarose gel (Panel B).

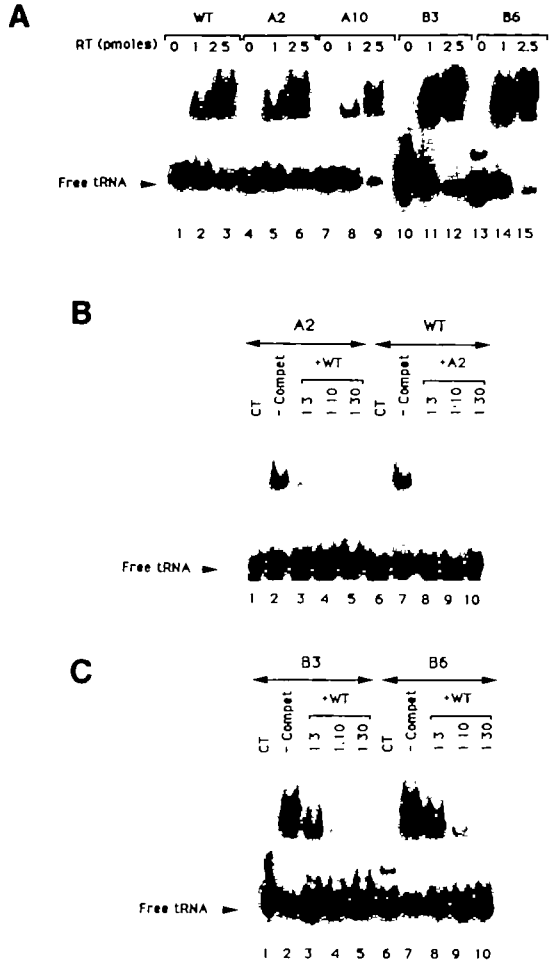

Figure 5. [A], Gel-mobility shift experiments with synthetic tRNA molecules mutated in their anticodon domain. Each reaction contained 1pmole of ^{32}P-tRNA and the indicated amounts of RT (in pmoles). Gel shift experiments were performed on 4% polyacrylamide gels. The anticodon domain mutants have been indicated above the panel. Positions of free tRNA is indicated by the arrow. [B], Reciprocal competition assays between the A2 mutant (Lanes 1–5) and synthetic $tRNA^{Lys,3}$ (WT, Lanes 6–10) pre-bound to HIV RT. [C] Competition experiments between synthetic tRNALys ,3 and the B3 (Lanes 1–6) and B6 mutant (Lanes 6–10).**CT**, no RT; **-Compet**, tRNA + RT. The molar ratio of competing synthetic tRNA or its mutant equivalent is indicated above lanes 3–5 and 8–10, respectively (Panels **B** and **C**).

only 12-nucleotide fragment which results from T1 RNase digestion, the data of Figure 3[C] strongly suggest that the specificity of the tRNA/RT interaction has been preserved in the synthetic molecule, despite the fact that this species lacks the highly modified bases characteristic of natural $tRNA^{Lys,3}$ that are S and R, being 5-(Methoxycarbonyl-methyl)-2-thiouridine and N-(N-(9-B-D-Ribofuranosyl-2 methylthiopurin-6-yl)threonine, respectively, (9,10)

Natural $tRNA^{Pro}$ and $tRNA^{Lys,3}$ Compete with Synthetic $tRNA^{Lys,3}$ for Binding to HIV RT

In Figure 4 (A), reciprocal competition experiments were performed with either the synthetic or natural form of $tRNA^{Lys,3}$, using in each case a 10:1 ratio of competitor to bound tRNA. From these results, it is clear that synthetic tRNA has difficulty competing with the natural species for binding to RT. In contrast, the synthetic species is easily competed from the enzyme by its natural counterpart. Thus, although the specificity of synthetic $tRNA^{Lys,3}$ for HIV-1 RT is still maintained through its anticodon domain, results with the natural tRNA species indicate that additional features of the anticodon domain appear necessary to maintain the affinity.

In a further series of experiments, the affinity of synthetic $tRNA^{Lys,3}$ for RT was determined in the presence of competing natural $tRNA^{Lys,3}$ and $tRNA^{Pro}$ (the replication primer utilised by MLV RT) (see figure 4B). As was shown in the previous experiment, an excess of non-radiolabelled natural $tRNA^{Lys,3}$ competes both the natural and synthetic species previously bound to RT. However, an unusual observation was the differential effect of competitor $tRNA^{Pro}$ on each of the RT/$tRNA^{Lys,3}$

complexes. We have recently demonstrated that $tRNA^{Pro}$ is incapable of competing natural $tRNA^{Lys,3}$ bound to to HIV-1 RT (6). In contrast to these earlier observations, synthetic $tRNA^{Lys,3}$ appears in this case to be well displaced from RT by the non-homologous $tRNA^{Pro}$. Since only the anticodon domain of tRNA appears to be involved in the interaction with RT, the results from Figure 4 once more suggest that additional structural features within this make a significant contribution to the interaction.

Binding of $tRNA^{Lys,3}$ Mutants to HIV-1 RT

Since a specific interaction between synthetic $tRNA^{Lys,3}$ and HIV-1 RT could be demonstrated, it was possible to determine whether specific bases within the anticodon domain could be altered without disrupting this interaction. Figure 5 (A) illustrates a series of gel-mobility shift experiments between p66/p51 HIV-1 RT and synthetic $tRNA^{Lys,3}$ molecules with a variety of alterations in their anticodon domain. Very little difference is observed between the synthetic tRNA and mutants A2 and A10, both of which contain substitutions at the positions of the hyper-modified bases of natural $tRNA^{Lys,3}$, i.e. at a 2.5:1 ratio of enzyme to tRNA, virtually all the tRNA is complexed to the enzyme. In, contrast, mutants B3 and B6 display an apparently higher affinity for RT, although slightly smaller amounts of mutant tRNA in the assays might account for this. Experiments with other tRNA mutants show essentially the same result (C. Barat, *unpublished)*. In figure 5 (B), reciprocal competition experiments were performed between mutant A2 and the non-mutant synthetic tRNA. In either case, the bound, radiolabelled tRNA can be competed from the enzyme by cold competitor, suggesting an equal affinity for either species. Additional competition experiments were performed between mutants B3 and B6 and the non mutant synthetic tRNA. In figure 5 (C) it is reported that radiolabelled B3 and B6 tRNA bound to RT can be competed by unlabelled non mutant tRNA in a manner similar to the reciprocal competion experiments described in figure 5 (B).The combined results of Figure 5 would thus suggest that individual as well as multiple substitutions can be tolerated within the anticodon domain without markedly impairing the interaction with HIV RT. These results are again suggestive of the notion that a stem-loop structure within the anticodon domain suffices to direct the tRNA to RT.

DISCUSSION

The previous demonstration that $tRNA^{Lys,3}$ was capable of forming a stable complex with HIV-1 RT (6–8) suggests that this interaction may present a novel avenue for chemotherapeutic intervention. This specific interaction is not limited to HIV-1 RT, but can be demonstrated for RT of the Rous sarcoma virus (RSV) which requires $tRNA^{Trp}$ for initiation of transcription (22, 23). Although natural $tRNA^{Lys,3}$ can be prepared, the quantities of radiolabelled tRNA required for high flux screening would present a formidable problem. The same problem arises when considering biophysical studies on RT in the presence of its replication primer. An alternative approach would be the substitution of an *in vitro* synthesised tRNA, since studies with both $tRNA^{Phe}$ (13) and $tRNA^{Arg}$ (14) have indicated that they have the structural integrity required for charging with their cognate tRNA. In this communication, we have used these precedents to prepare a synthetic form of $tRNA^{Lys,3}$ as well as several variants with mutations in their anticodon domain, and analysed their interaction with recombinant p66/p51 HIV-1 RT.

As might have been expected, $tRNA^{Lys,3}$ prepared by *in vitro* transcription serves as a primer from which cDNA synthesis can be initiated when hybridised to a pbs-HIV1 RNA template. In recent experiments, cDNA synthesis can be more efficiently initiated from template/primer formed in the presence of a recombinant form of the HIV-1 nucleocapsid protein (Darlix, unpublished data). Despite these observations on initiation of reverse transcription, a more encouraging is the finding that synthetic tRNA can also bind to RT and elicit a gel-mobility shift. In addition, *trans*-DDP cross-linking experiments indicate that the 12-nucleotide T1 RNase fragment encompassing the anticodon domain is involved in this interaction, as was similarly demonstrated for the natural species (6). From these latter analyses, we can therefore conclude that the conformational integrity of our tRNA prepared by *in vitro* transcription has been preserved, as was demonstrated for other tRNA species similarly prepared (13, 14). In a related study, we are monitoring the structure of HIV-1 RT by neutron solution scattering (H. Heumann, S. Le Grice *et al., in preparation*). The ability to produce synthetic $tRNA^{Lys,3}$, RT, NC and the pbs-HIV RNA in large quantities should now permit a study of the HIV enzyme in the presence of its replication primer, either prior to, or shortly after, initiation of minus strand DNA synthesis.

Whilst the general structure of the anticodon domain would appear to be a pre-requisite for the RT/tRNA interaction, an interesting observation with synthetic $tRNA^{Lys,3}$ has arisen from competition experiments with the natural form of either the homologous or heterologous species. Natural $tRNA^{Lys,3}$ appears to readily compete the synthetic species from heterodimer RT. This result would appear to strengthen previous postulations (6–8) that the highly modified anticodon bases S and R provide additional stability to the complex, possibly 'docking' the tRNA onto the enzyme in the correct configuration. More surprising was the observation that the synthetic tRNA could also be competed from RT by natural $tRNA^{Pro}$, which was previously shown to be incapable of displacing natural $tRNA^{Lys,3}$ from the enzyme. The observations with $tRNA^{Pro}$ might suggest that the modifications in its anticodon domain have the potential to dock this more stably on RT than synthetic $tRNA^{Lys,3}$, but less stably than the natural species. tRNA binding to RT may thus involve two distinct steps, i.e. initial placement of the anticodon domain within a cleft of RT, followed by correct orientation, mediated by the modified bases.

The observation that synthetic $tRNA^{Lys,3}$ binds with the correct specificity to RT would predict that base substitutions within the anticodon domain would minimally perturb this interaction, provided they maintained the general structure of the anticodon domain. This is indeed what has been experimentally observed, where four forms of $tRNA^{Lys,3}$ modified in the anticodon domain elicit a similar gel shift, although the experimental systems presented here might not detect small changes in affinity for RT. In summary, we have demonstrated that a synthetic form of $tRNA^{Lys,3}$ mimics the natural species sufficiently to permit its use for further biochemical and biophysical studies in complex with HIV RT. Although the first series of mutations have not yielded any information on features determining the affinity or specificity of the interaction, several other features of the synthetic molecule which might influence not only initial binding but also subsequent steps in initiation of reverse transcription can now be attempted. Since the complex has sufficient stability in gel-shift assays, this might be considered a potential system for screening of antiviral agents to inhibit HIV RT before it can embark upon the first step of the replication cycle.

ACKNOWLEDGEMENTS

This work was supported in part by the French effort against AIDS (ANRS). The excellent technical assistance of C. Gabus is acknowledged, as well as that of K.J. Howard for proof-reading the manuscript. We are furthermore indebted to Dr. G. Keith, Strasbourg, for the gifts of purified bovine $tRNA^{Lys,3}$,$tRNA^{Pro}$ and NTase.

REFERENCES

1. Taylor, J.M. , and Illmensee, R. (1975) *J. Virol.* 16, 553–558.
2. Harada, F., Sawyer, R.C. , and Dahlberg, J.E. (1975) *J. Biol. Chem.* **250**, 3487–3497.
3. Harada, F., Peters, G.G., and Dahlberg, J.E. (1979) *J. Biol. Chem.* **254**, 10979–10985.
4. Peters, G.G. , and Glover, C. (1980) *J. Virol.* **33**, 708–716.
5. Wain-Hobson, S., Sonigo, P., Danos, O. , and Alizon, M. (1985) *Cell* **40**, 9–17.
6. Barat, C., Lullien, V., Schatz, O., Keith, G., Nugeyre,M.T., Gruninger-Leitch, F., Barre-Sinoussi, F., Le Grice, S.F.J. , and Darlix, J.L. (1989) *EMBO J.* **8**, 3279–3285.
7. Sallafranque-Andreola, M.L., Robert, D., Barr, P.J., Fournier, M., Litvak, S., Sarah-Cottin, L. , and Tarrago-Litvak, L. (1989) *Eur. J. Biochem.* **184**, 367–374.
8. Bordier, B., Tarrago-Litvak, L., Sallafranque-Andreola, M-L., Robert, D., Tharud, D., Fournier, M., Barr, P.J., Litvak, S, and Sarih-Cottin, L. (1990) *Nuc. Acids Res.* **18**, 429–436.
9. Raba, M., Limburg, K., Burghagen, M., Katze, J.R., Simsek, M., Heckman, J.E., Rajbhandary, U.L. and Gross, H.J. (1979) *Eur.J. Biochem.* **97**, 305–318.
10. Dirheimer, G (1983) *Recent Results in Cancer Research* **84**, 15–46.
11. Langer. P.R., Waldrop, A.A. , and Ward, D.C. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 6633–6637.
12. Krieg, P.A. , and Melton, D.A. (1987) *Methods Enzymol.* **155**, 397–415.
13. Sampson, J.R, and Uhlenbeck, O.C. (1988) *Proc. Natl, Acad, Sci, USA* **85**, 1033–1037.
14. Perret, V., Garcia, A., Grosjean, H., Ebel, J-P., Florentz, C. and Giege, R. (1990) *Nature* (London) **344**, 787–789.
15. Sanger, F., Nicklen, S. and Coulson, A.R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
16. Le Grice, S.F.J. and Grueninger-Leitch, F. (1990) *Eur. J. Biochem.* **187**. 307–314.
17. Hochuli, E., Bannwarth, W., Doebeli, H., Gentz, R. & Stueber, D. (1989) *BIO/TECHNOLOGY* 6, 1321–1325.
18. Alizon, M., Wain-Hobson, S., Montagnier, L. and Sonigo, P. (1986) *Cell*, **46**, 63–74.
19. Sambrook, J., Fritsch, E.F. and Maniatis, T. (Eds.) (1989) 'Molecular Cloning, A Laboratory Manual' Cold Spring Harbor Laboratory Press, Cold Spring Harbor. N.Y.
20. Tukalo, M.A., Kubler, M.D., Kern, D., Mougele, M., Ehresmann, C., Ebel, J.P., Ehresman, B. and Giege, R. (1987) *Biochemistry*, **26**, 5200–5208.
21. Mullis, K.B. and Faloona, F. (1987) *Meth Enzymol.* **155**, 335–347.
22. Prats, A.C., Sarih, L., Gabus, C., Litvak, S., Keith, G. and Darlix, J.L. (1988) *EMBO J.* **7**, 1777–1783