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HIV-1 reverse transcriptase specifically interacts with the anticodon domain of its cognate primer tRNA

C.Barat, V.Lullien, O.Schatz[1], G.Keith[2], M.T.Nugeyre[3], F.Grüninger-Leitch[1], F.Barré-Sinoussi[3], S.F.J.LeGrice[1] and J.L.Darlix

Labo-Retro, Centre de Recherche de Biochimie et Génétique Cellulaires du CNRS, 118 route de Narbonne, F-31062 Toulouse Cedex, France, [1]Central Research Units, Hoffmann-La Roche & Co Ltd, CH-4002 Basel, Switzerland, [2]Institut de Biologie Moléculaire et Cellulaire, 15 rue R.Descartes, F-67084 Strasbourg Cedex and [3]Laboratoire de Biologie des Retrovirus, Institut Pasteur, 25 rue du Dr Roux, F-75724 Paris Cedex 15, France

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The virion cores of the replication competent type 1 human immunodeficiency virus (HIV-1), a retrovirus, contain an RNA genome associated with nucleocapsid (NC) and reverse transcriptase (RT p66/p51) molecules. *In vitro* reconstructions of these complexes with purified components show that NC is required for efficient annealing of the primer $tRNA^{Lys,3}$. In the absence of NC, HIV-1 RT is unable to retrotranscribe the viral RNA template from the tRNA primer. We demonstrate that the HIV-1 RT p66/p51 specifically binds to its cognate primer $tRNA^{Lys,3}$ even in the presence of a 100-fold molar excess of other tRNAs. Cross-linking analysis of this interaction locates the contact site to a region within the heavily modified anti-codon domain of $tRNA^{Lys,3}$.
Key words: HIV-1/nucleocapsid/reverse transcriptase/tRNA

Introduction

HIV-1 virions are small spheres ~100 nm in diameter, composed of an inner core surrounded by an outer envelope. The inner core can be subdivided into a shell of capsid protein molecules (CA, nomenclature according to Leis *et al.*, 1988) and the interior nucleocapsid (NC) (Di Marzo-Veronese *et al.*, 1987; Gelderblom *et al.*, 1987). By analogy with avian and mammalian retroviruses, this NC very likely is the site where the initiation of retrotranscription takes place (Benz and Dina, 1979; Chen *et al.*, 1980; Boone and Skalka, 1981). It consists of a large number of NC protein molecules probably wrapping the genomic RNA, some molecules of RT (p66/p51; Di Marzo-Veronese *et al.*, 1987) and a tRNA primer, most likely $tRNA^{Lys,3}$ (Raba *et al.*, 1979; Wain-Hobson *et al.*, 1985). HIV NC, like most other retroviral NC proteins, is derived from the carboxy-terminal end of the *gag* precursor. It is a small, basic protein containing two copies of the conserved Cys-His boxes (Cys-Xaa_2-Cys-Xaa_4-His-Xaa_4-Cys) sometimes implied in Zn^{2+} and nucleic acid binding (Covey, 1986; South *et al.*, 1989). Alteration of the Cys-His boxes of Moloney murine leukaemia virus (MoMuLV) and Rous sarcoma virus (RSV) NC by deletions or site-directed mutagenesis (Méric and Spahr, 1986; Karpel *et al.*, 1987; Gorelick *et al.*, 1988; Jentoft *et al.*, 1988; Méric *et al.*, 1988) abolishes the infectivity of these viruses, which also show a marked defect in packaging their genomic RNA. Exactly how these conserved domains are involved in nucleic acid and Zn^{2+} binding is still not clear, especially in view of reports claiming that oxidizing the cysteines of MoMuLV NC does not alter its binding constant for nucleic acids (Karpel *et al.*, 1987). Despite their relatively small size, NC proteins have a variety of other functions. For instance, it has been reported that they promote dimerization of the RNA template (Prats *et al.*, 1988), a process that appears necessary for synthesis of proviral DNA, since it has been shown that interstrand RNA template switching occurs during the early events of reverse transcription (Panganiban and Fiore, 1988). Furthermore, they also have to serve a structural role in packaging of the viral genome.

HIV-1 RT, a protein contained in the virion core, is matured from a 170-kd *gag/pol* polyprotein precursor translated via ribosomal frameshifting between the *gag* and *pol* genes (Jacks *et al.*, 1988). RT serves multiple functions in the synthesis of proviral DNA: from a tRNA primer annealed to the template RNA it primes minus strand DNA synthesis, in the course of which it has to switch templates (Panganiban and Fiore, 1988). In order to allow plus strand DNA synthesis, the RNA templates are then removed by a built-in RNaseH activity (Moelling *et al.*, 1971; Hansen *et al.*, 1988) in a poorly understood fashion that still leaves oligoribonucleotide primers for plus strand synthesis to occur. In both virions and bacterial expression systems HIV-1 RT is processed to 66- and 51-kd polypeptides when a functional HIV-1 protease (PR) is co-expressed (Lightfoote *et al.*, 1986; Di Marzo-Veronese *et al.*, 1988; Mous *et al.*, 1988). The two RT polypeptides are identical at their N termini which contain the DNA polymerase domain as characterized by sequence comparison and site-directed mutagenesis (Johnson *et al.*, 1986; Larder *et al.*, 1987, 1988); p51 RT lacks the C-terminal end spanning the RNaseH domain (Hansen *et al.*, 1988). The central polymerase domain contains, in addition to the two adjacent aspartic acids at the proposed catalytic site, a 'leucine zipper' motif, suggested to be important for dimer formation and DNA binding (Landschulz *et al.*, 1988; Gentz *et al.*, 1989; Turner and Tjian, 1989). Although many reaction parameters of HIV-1 RT function *in vitro* have been reported (Majumdar *et al.*, 1988; Huber *et al.*, 1989), most of these are derived using artificial primer-template systems which may not fully reflect the *in vivo* situation. In addition, the interactions occurring during the initiation of reverse transcription from the tRNA primer, an event that is crucial in the viral life cycle, is poorly documented. In an attempt to reconstruct the initiation of reverse transcription by HIV-1 RT *in vitro* more accurately, we have used a more appropriate primer-template system, namely bovine $tRNA^{Lys,3}$ (whose sequence is identical to its human homologue) as a primer, and an

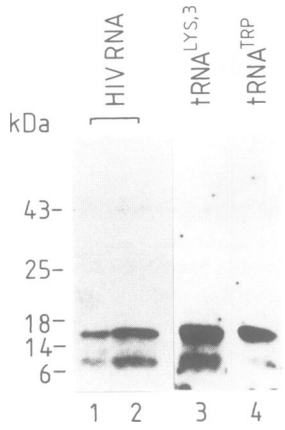

Fig. 1. North-western analysis of HIV-1 NC−RNA interactions. Virion proteins were fractionated by SDS−PAGE (10% gel), electroblotted onto nitrocellulose, then probed with either *in vitro* generated [^{32}P]HIV-1 viral RNA (**lanes 1** and **2**), [^{32}P]tRNA^{Lys,3} (**lane 3**) or [^{32}P]tRNA^{Trp} (**lane 4**). Amount of virion protein applied to the gel was: **lane 1**, 2 μg; **lane 2**, 7 μg; **lanes 3** and **4**, 5 μg. M, migration positions of protein mol. wt markers (in kd).

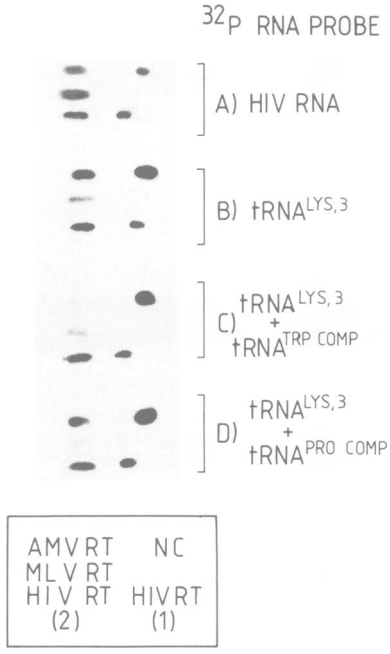

Fig. 2. RNA affinity assays of retroviral proteins by dot-blot analysis. The scheme below the figure represents the positions of the individual proteins. HIV NC (10 ng), AMV RT (200 ng), MoMuLV RT (200 ng) and HIV RT (100 or 200 ng) were spotted onto nitrocellulose and probed with the following ^{32}P-labelled samples: (**A**) 1330 nucleotide *in vitro* generated HIV-1 viral RNA; (**B**) tRNA^{Lys,3}; (**C**) tRNA^{Lys,3} and cold competitor tRNA^{Trp}; (**D**) tRNA^{Lys,3} and cold competitor tRNA^{Pro}.

SP6-derived HIV-1 viral RNA comprising most of the 5′ leader sequences plus the primer binding site (PBS) and the 5′ portion of the *gag* gene as a template.

Our results illustrate that HIV-1 RT, in the presence of NC and tRNA^{Lys,3}, accurately initiates reverse transcription from its viral RNA template. Furthermore, we show that the tRNA^{Lys,3} component of this complex is specifically bound to RT through its anticodon domain.

Results

NC and p66/p51 RT interact both with viral RNA and replication primer tRNA^{Lys,3}

To examine the interaction between HIV genomic RNA, primer tRNA^{Lys,3} and virion proteins, HIV-1 virions were disrupted, viral proteins separated by SDS−PAGE, renatured and blotted to a nitrocellulose membrane as previously described (Méric *et al.*, 1984). These proteins were then probed with ^{32}P-labelled HIV RNA. As can be seen in Figure 1, lanes 1 and 2, this North-western blot shows two major bands. From the apparent mol. wt of these proteins we conclude that they most likely correspond to the 15-kd NC and its 7-kd maturation product. This assumption was verified by a Western analysis using monoclonal antibodies against purified NC (data not shown). Probing the filters with ^{32}P-labelled tRNA^{Lys,3} and tRNA^{Trp} (the replication primers of HIV-1 and RSV respectively) illustrates that HIV-1 NC binds not only to tRNA^{Lys,3} but also to a somewhat lesser extent to tRNA^{Trp} (lanes 3 and 4). These results indicate that HIV-1 NC has a general affinity for RNA, probably by virtue of its basic character (Karpel *et al.*, 1987). However, as will be shown later, it also displays a clear specificity for the correct primer tRNA. With RSV and MoMuLV, it has also been demonstrated that selection of the correct viral RNAs for encapsidation is dependent on the presence of intact NC proteins (Méric and Spahr, 1986; Gorelick *et al.*, 1988).

In a second series of experiments, we determined whether HIV-1 RT might also impart some specificity to the core assembly process. For this, RNA binding assays were carried out with HIV-1 NC and RT of HIV-1 (p66/p51), avian myeloblastosis virus (AMV) and MoMuLV. These proteins were spotted onto a nitrocellulose membrane and probed either with HIV RNA, tRNA^{Lys,3} or a mixture of tRNA^{Lys,3} and a 20-fold excess of a competitor tRNA. Figure 2 shows that each of these viral proteins binds HIV RNA and tRNA^{Lys,3}. More importantly, neither tRNA^{Trp} nor tRNA^{Pro} are able to compete for tRNA^{Lys,3} binding to HIV-1 RT p66/p51 or HIV-1 NC. This observation is not due to lack of saturation of the immobilized proteins, since competition with the same excess of unlabelled tRNA^{Lys,3} reduces the signal by as much as 90% (data not shown). Note that tRNA^{Trp} but not tRNA^{Pro} competes for tRNA^{Lys,3} binding to AMV-RT as would be expected, given the affinity of AMV-RT not only for its primer tRNA^{Trp} but also tRNA^{Lys,3} (Panet *et al.*, 1975). MoMuLV RT shows little affinity for tRNA^{Lys,3}, although it does bind HIV RNA.

To analyse the affinity of primer tRNA^{Lys,3} for HIV-1 RT p66/p51 in more detail, and estimate the number of primer tRNA^{Lys,3} molecules bound to each molecule of RT, we used a gel-retardation assay, the results of which are presented in Figure 3. RT−tRNA complexes were formed by incubation in reverse transcription buffer lacking dNTPs then subjected to electrophoresis through agarose gels. These complexes migrate slower in relation to free tRNA, which is found near the bottom of the gel. When 0.3 pmol RT is incubated with 0.2 pmol tRNA^{Lys,3}, all of the tRNA is complexed with RT (lane 10). Increasing the amount of tRNA^{Lys,3} to 0.5 pmol or 1.0 pmol results in ~30 or ~70% of the material as free tRNA respectively (lanes 11 and 12, panel 'tRNA^{Lys,3} titration'). Assuming that the HIV-1 RT is present as a dimer, we estimate that ~0.8−1 mol tRNA^{Lys,3} is bound per mol of RT.

Figure 3 furthermore illustrates that, in this assay, a

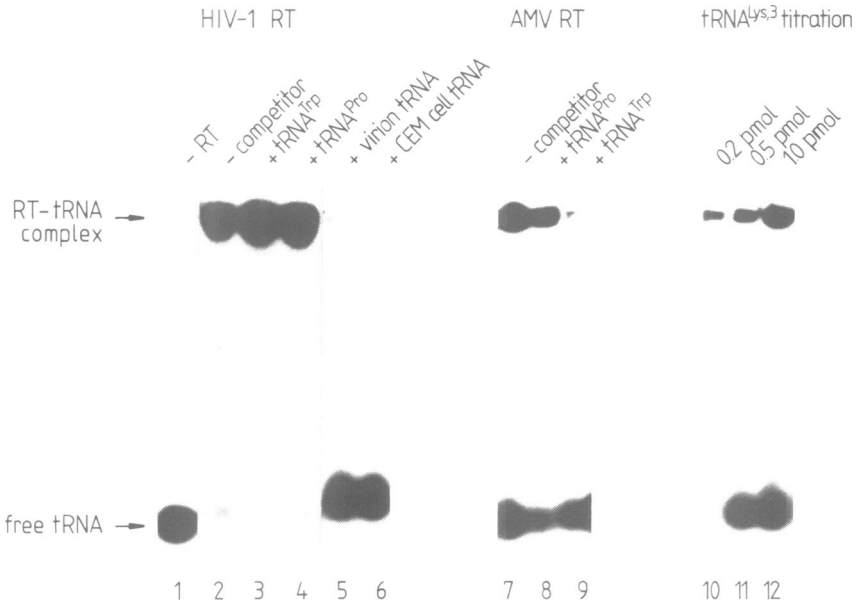

Fig. 3. Gel-retardation assays of primer tRNA^{Lys,3}–RT complexes. Complexes were electrophoresed through 0.8% agarose gels as described in Materials and methods, dried and subjected to autoradiography. 0.8 pmol p66/p51 HIV-1 RT (**lanes 2–6**) or AMV RT (**lanes 7–9**) and 0.5 pmol [^{32}P]tRNA^{Lys,3} were supplemented with the following prior to electrophoresis: **lane 3**, competitor tRNA^{Trp}; **lane 4**, competitor tRNA^{Pro}; **lane 5**, complete virion tRNA; **lane 6**, CEM cell tRNA; **lane 8**, competitor tRNA^{Pro}; **lane 9**, competitor tRNA^{Trp}. **Lanes 10–12** indicate titration of the HIV-1 enzyme with primer tRNA^{Lys,3} (amounts of tRNA^{Lys,3} are given above each lane). **Lanes 2** and **7** are HIV-1 and AMV RT–tRNA^{Lys,3} complexes lacking competitor tRNA. **Lane 1**, tRNA^{Lys,3}, no RT. Migration positions of complexed and free tRNA^{Lys,3} are indicated.

100-fold excess of tRNA^{Trp} or tRNA^{Pro} does not compete for the binding of ^{32}P-labelled tRNA^{Lys,3} to HIV-1 RT (lanes 3 and 4). However, a 10- and 200-fold excess of HIV virion or cellular tRNA from the human acute lymphoblastic leukaemia cell line CEM, respectively, do compete very efficiently (>90%, lanes 5 and 6). The requirement for less HIV than CEM tRNA for competition may reflect that virions are enriched for tRNA^{Lys,3}, but that it is a minor species in total cellular tRNA. A second example of selection of the correct primer tRNA is shown with AMV RT (lanes 7–9). The interaction of AMV RT with tRNA^{Lys,3} can be competed with its cognate primer tRNA^{Trp}, but not with tRNA^{Pro}. In conclusion, these data indicate that both HIV-1 NC and RT p66/p51 bind to viral RNA and primer tRNA^{Lys,3}. Moreover, the competition assays also suggest that primer tRNA^{Lys,3} is selectively recognized by HIV-1 RT. In light of this, we designed further experiments to study the RT–tRNA interaction in more detail.

HIV-1 RT recognizes the anticodon domain of primer tRNA^{Lys,3}

To map the domain of tRNA^{Lys,3} recognized by HIV-1 RT we cross-linked these with *trans*-diamine-dichloro-platinum (*trans*-DDP; Tukalo *et al.*, 1987). This method has been proven to be very reliable, since binding positions are specific on both RNA (purine residues) and protein (predominantly at cysteine and methionine, and to a lesser extent histidine; Tukalo *et al.*, 1987). Due to the chemical properties of the co-ordination linkages between platinum and the acceptors on the macromolecules, cross-links can either be kept stable or easily reversed by addition of stronger acceptors. These properties facilitate the analysis of the cross-linked regions.

Complexes between HIV-1 RT and tRNA^{Lys,3} were formed (under reverse transcription conditions) and platinated at various concentrations of *trans*-DDP. We found that 0.3 mM *trans*-DDP gave high coupling efficiency without degrading the macromolecules. After platination, the incubation mixture was subjected to T1 RNase digestion. The resulting fragments were labelled at their free 5′ termini with [γ-^{32}P]ATP and T4 polynucleotide kinase. T1 oligoribonucleotides complexed to HIV-1 RT were purified from the free RNA fragments by gel filtration over Sephadex G-50, where oligoribonucleotide–RT complexes eluted in the void volume (Figure 4A). Controls without RT (closed circles) or without *trans*-DDP (small crosses), or HIV-1 RT and tRNA^{Trp} instead of tRNA^{Lys,3} (not shown) showed <10% of the radioactivity in the void volume when compared to HIV-1 RT–tRNA^{Lys,3} complexes. The HIV-1 RT–tRNA^{Lys,3} oligoribonucleotide complex obtained by this procedure was then analysed by SDS–PAGE, from which it is clear that both the 66- and 51-kd subunits of HIV-1 RT were labelled to about the same extent (Figure 4B). The migration positions of these polypeptides was shifted by ~3 kd relative to non-complexed p66 and p51 RT, indicative of a small cross-linked oligoribonucleotide. Furthermore, the presence of both p66 and p51 RT in the high mol. wt radiolabelled complex (data not shown) suggests that it may contain two p66/p51 molecules complexed with tRNA.

To analyse the oligoribonucleotide cross-linked to HIV-1 RT, the RT–tRNA complex was dissociated with thiourea and the ^{32}P-labelled oligoribonucleotide recovered by ethanol precipitation and purification on a 15% urea/polyacrylamide gel. The major band observed, which was 12 nucleotides in length (Figure 5A), was subsequently excised and subjected to sequence analysis using T1, U2 and pancreatic RNases. The sequence ladder obtained (Figure 5B) shows an A at positions 1 and 8, C or U at positions 2, 3, 5, 6, 9, 10 and 11 and two 'holes' at positions 4 and 7. These 'holes' most likely correspond to modified nucleotides which are not readily accessible to the RNases.

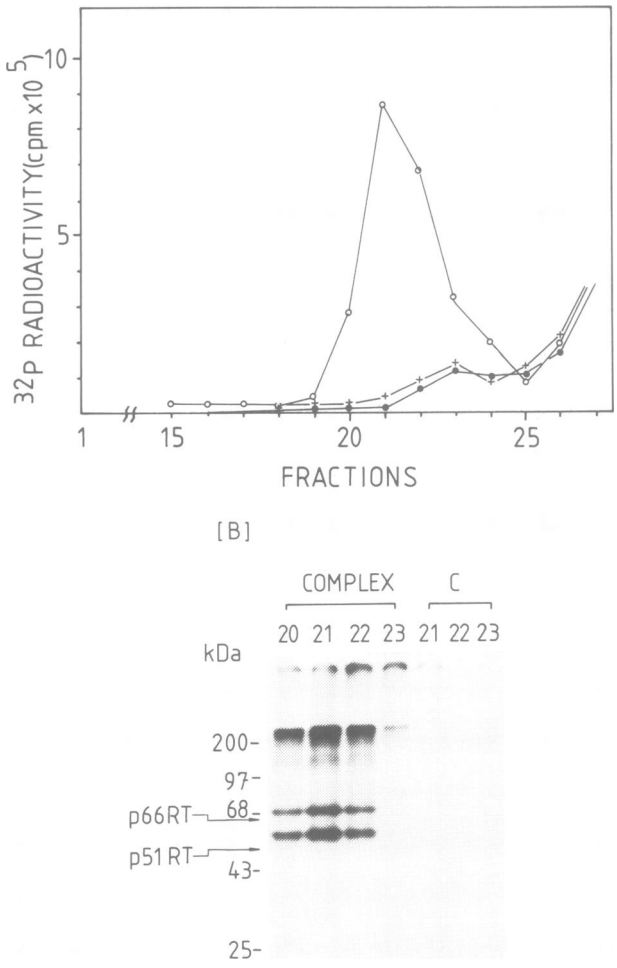

Fig. 4. Analysis of cross-linked HIV RT−tRNA complexes. (**A**) Sephadex G-50 filtration of complexes following T1 ribonuclease digestion and 5′ ^{32}P-labelling of the remaining RNA fragment. (○) HIV RT−tRNA^{Lys,3}; (●) tRNA^{Lys,3} alone; HIV RT−tRNA^{Lys,3} minus *trans*-DPP. Radioactivity in column fractions was determined by Cerenkov counting. (**B**) SDS−PAGE (8%) of HIV RT−tRNA^{Lys,3} T1 oligonucleotide complex isolated by Sephadex G-50 gel filtration. Presented is an autoradiogram of the stained and dried gel. Aliquots (5 μl) of column samples were heated at 60°C with 1% SDS in the absence of β-mercaptoethanol (to preserve cross-link). The migration positions of HIV-1 RT p66 and p51 were determined from a sample of enzyme run on the same gel. Protein mol. wt markers are given in kd. CT represents a similar analysis of the gel filtration run with tRNA^{Lys,3} alone.

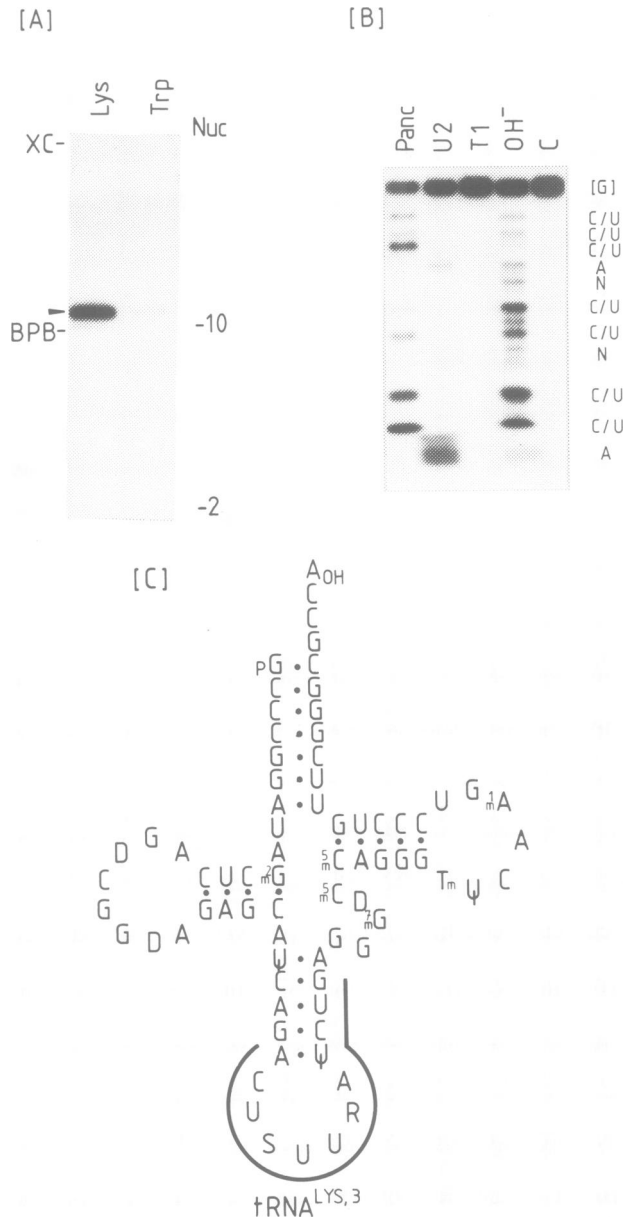

Fig. 5. Determination of the RT binding site on primer tRNA^{Lys,3}. (**A**) High-resolution gel analysis of T1 oligonucleotide recovered from fractions 20−23 in Figure 4, following reversion of cross-link. **Lanes Lys** and **Trp** refer to HIV-RT complexes with tRNA^{Lys,3} or tRNA^{Trp} respectively. Positions of xylene cyanol (XC) and bromophenol blue (BB) marker dyes are indicated. nt, nucleotides. (**B**) Sequence analysis of tRNA^{Lys,3} T1 oligonucleotide. **Lanes U2** and **T1**, sequence-specific ribonucleases; **Panc**, pancreatic RNase;**OH**$^-$, alkaline hydrolysate; **C**, control. (**C**) Nucleotide sequence of tRNA^{Lys,3}, showing the position of the T1 oligonucleotide bound to HIV-1 RT.

The last residue must be a G, since the oligoribonucleotide was derived from a complete T1 digestion. tRNA^{Lys,3} contains only one T1 fragment whose size and sequence are compatible with the data presented in Figure 5A and B, namely the region from positions 31 to 42 comprising the anticodon domain (5′-A-C-U-S-U-U-R-A-Ψ-C-U-G-3′, where S is 2-thio-5-carboxy-methyl uridine methyl ester and R, 2-methylthio-N^6-threonyl-adenosine (Raba *et al.*, 1979, Figure 5C).

NC is needed to anneal tRNA^{Lys,3} onto the primer binding site (PBS) of HIV genomic RNA

Having demonstrated that HIV-1 RT can (i) form a specific and stable complex with the replication primer tRNA^{Lys,3} and (ii) bind to HIV genomic RNA, we were interested to learn whether this complex could initiate reverse transcription of HIV genomic RNA from the primer binding site (Wain-Hobson *et al.*, 1985). Consquently, we incubated the HIV-1 RT−tRNA^{Lys,3} complex with a 1.3-kb PBS-containing viral RNA (spanning position 30−1333 in the HIV genome; Alizon *et al.*, 1986) in the presence of all four dNTPs in reverse transcription buffer. Correct initiation of cDNA synthesis from the primer binding site would predict synthesis of a product of 280 nucleotides (cDNA

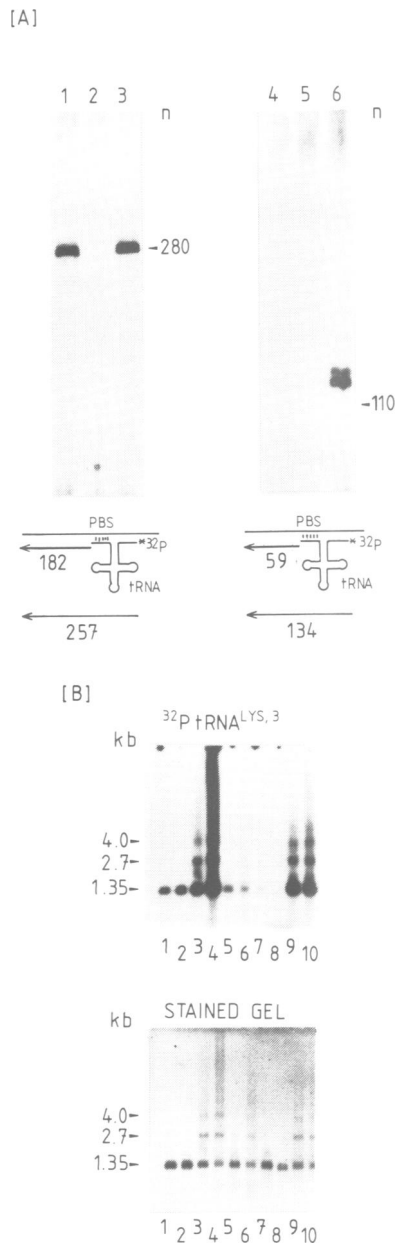

Fig. 6. Annealing of $tRNA^{Lys,3}$ to HIV RNA at the primer binding site and its extension by RT in the presence of NC protein. (**A**) Reverse transcription from RNA−$tRNA^{Lys,3}$−NC complexes. Viral RNA template in **lanes 1−3** was the *in vitro* generated 1330-nucleotide viral RNA fragment with the PBS 192 nucleotides from the 5′ end. In **lanes 4−6**, a slightly truncated RNA, with PBS 59 nucleotides from the 5′ end (see Materials and methods), was used. **Lanes 2** and **4** contain HIV-1 RT, but lack NC. **Lanes 1** and **3** contain NC of HIV and RSV respectively. The reactions depicted in lanes 4−6 were carried out in the absence of NC and RT (**lane 4**), NC (**lane 5**) or presence of RT and NC (**lane 6**). Note that the size of the reverse transcripts corresponds to the distance to the 5′ end of the template RNA plus 75 nucleotides of $tRNA^{Lys,3}$. This is schematically represented under each panel, where PBS represents the primer binding site. nt, nucleotides. (**B**) NC-mediated $tRNA^{Lys,3}$−RNA interaction. A 1330-nucleotide, PBS-containing, viral RNA, [^{3P}]$tRNA^{Lys,3}$ and the proteins indicated were electrophoresed through a 0.8% agarose gel, which was either stained with ethidium bromide (**panel 'Stained Gel'**), or dried and subjected to autoradiography (**panel '^{32}P-$tRNA^{Lys,3}$'**). **lane 1**, RNA/tRNA alone, in the presence of 0.3 M NaCl; **lane 2**, 300 ng HIV-1 RT; **lane 3**, 10 ng HIV NC; **lane 4**, 30 ng HIV NC; **lane 5**, 50 ng MoMuLV NC; **lane 6**, 100 ng MoMuLV NC; **lane 7**, RNA/tRNA alone, no NaCl; **lane 8**, 1 μg T4 gene 32 protein; **lane 9**, 30 ng RSV NC; **lane 10**, 60 ng RSV NC.

+ $tRNA^{Lys,3}$) from the viral RNA generated in our *in vitro* system. In the absence of NC, initiation of reverse transcription was not observed (Figure 6A, lanes 2 and 5). When HIV NC was added to the reaction, we observed the correct extension product (lane 1). Interestingly, in this assay, RSV NC could substitute for HIV-1 NC (lane 3). As further proof of correct initiation of HIV-1 reverse transcription, we used a second *in vitro* generated viral RNA which was truncated by ~120 nucleotides at its 5′ end. In the presence of HIV-1 NC and $tRNA^{Lys,3}$, this RNA is copied to give the correspondingly truncated cDNA (Figure 6A, lane 6).

A possible explanation for the failure of HIV-1 RT to reverse transcribe the RNA template from the $tRNA^{Lys,3}$ primer in the absence of NC was that the tRNA simply could not stably anneal to the template. This was addressed by the gel shift experiment outlined in Figure 6B. ^{32}P-Labelled primer $tRNA^{Lys,3}$ was incubated together with a 1.3-kb genomic RNA fragment in the presence or absence of RT and NC proteins. The mixture was then extracted with phenol/chloroform and the RNAs separated on an agarose gel. Panel '^{32}P$tRNA^{Lys,3}$' shows an autoradiogram of this gel, where bands correspond to labelled $tRNA^{Lys,3}$ that has bound to the 1.33-kb genomic RNA fragment or multimers thereof (free tRNA runs off the gel). The panel 'stained gel' is the same gel stained with ethidium bromide prior to autoradiography. In the absence of any proteins, $tRNA^{Lys,3}$ binds inefficiently to the 1.3-kb RNA (lanes 7). Addition of HIV-1 RT improves annealing (lanes 2), although to a much lesser extent than HIV-1 NC (lanes 3 and 4). In this assay, RSV NC can substitute for HIV NC, both in $tRNA^{Lys,3}$ annealing and dimerization of the RNA template (lanes 9 and 10). On the other hand, MoMuLV NC could promote dimer formation, but was inefficient in binding of $tRNA^{Lys,3}$ to the viral RNA (lanes 5 and 6). In contrast, the phage T4 gene 32 protein promoted neither function (lanes 8). Interestingly, pre-incubating the RNA mixture in high salt at 42°C also gives some annealing of the tRNA (lane 1). In all cases annealing is dependent on the presence of an intact primer binding site, because no annealing is observed when a PBS−RNA is incubated with $tRNA^{Lys,3}$. A variety of other viral and nucleic acid binding proteins (e.g. HIV-1 matrix and capsid proteins, MAαCA, RSV CA and *Escherichia coli* recA protein) were tested in this assay, none of which could efficiently promote annealing of $tRNA^{Lys,3}$ to HIV RNA.

Discussion

Although initiation of proviral DNA synthesis is a crucial step in replication of retroviruses, surprisingly little is known about this initial step of reverse transcription. In order to study initiation of HIV reverse transcription *in vitro*, we have tried to reconstruct more accurately the *in vivo* situation using purified virion proteins, the authentic primer $tRNA^{Lys,3}$ and an RNA template whose sequence is identical to a genomic HIV-1 RNA fragment encompassing most of the 5′ leader (including the primer binding site) and the 5′ part of the *gag* gene. This experimental strategy has allowed us to study some very important interactions and speculate on their role in the life cycle of HIV-1. We have demonstrated that HIV-1 RT specifically and stably binds to its replication primer $tRNA^{Lys,3}$. Furthermore, we have delineated a region in this

tRNA, the anti-codon domain, which is most likely the contact site recognized by HIV-1 RT. A similar pattern of tRNA recognition has been reported for AMV RT, which binds primer $tRNA^{Trp}$ (Araya *et al.*, 1980; Garret *et al.*, 1984). An interesting observation from Figure 5 is that a similarly sized T1 RNase generated oligoribonucleotide can be obtained with an HIV-1 RT–$tRNA^{Trp}$ complex, albeit in extremely low amounts. The fact, however, that this can be detected may suggest that RT molecules may have a tRNA binding cleft which makes the primary selection, and a secondary selection mechanism whereby the correct tRNA is retained over others. These findings would explain how retroviruses manage to select even minor tRNA species from a large pool of different cellular tRNAs. Virions of various retroviruses are enriched in their specific replication primer tRNA, e.g. RSV virions in $tRNA^{Trp}$ (Peters and Hu, 1980) or mouse mammary tumour virus (MMTV) virions in $tRNA^{Lys\,1,2}$ (Peters and Glovers, 1980). It may well be that the RTs help to encapsidate the correct replication primer tRNA to ensure rapid and efficient priming of reverse transcription. However, there are also RTs, e.g. the single subunit enzyme of MoMuLV, which do not display specific tRNA binding. Conceivably, selective recognition of tRNAs might be dependent on the existence of two subunits. Our observation that both the p66 and the p51 polypeptides are cross-linked to $tRNA^{Lys,3}$ would be consistent with this assumption.

Preliminary experiments furthermore suggest that neither purified p51 nor p66 HIV-1 RT (the latter of which is extremely active on synthetic templates) can form the specific complex with $tRNA^{Lys,3}$ (J.Darlix, unpublished), suggesting in this context a role for the p66/p51 heterodimer. At present, we do not know whether the ability to form a tRNA–RT complex is relevant *in vivo*, but one means of resolving this issue would be to study the *in vivo* effects of a mutational inactivation of the tRNA binding region in HIV-1 RT. In this respect, we are presently attempting to locate the amino acids that make contact with $tRNA^{Lys,3}$.

As we have seen, complex formation between HIV-1 RT and primer $tRNA^{Lys.3}$ alone is not sufficient to properly initiate reverse transcription. Only when HIV-1 or RSV NC was included in the reaction was reverse transcription observed (see also Prats *et al.*, 1988). What, then, is the role of the NC in the initiation of reverse transcription? Since NC seems to have an affinity for both HIV-1 RNA and $tRNA^{Lys,3}$ (Figure 2), the simplest explanation would be that it binds to and unwinds the RNA template, thus facilitating the annealing of the tRNA primer. In contradiction to this proposal, other proteins that are known to bind to nucleic acids or have a similar structure perform very poorly in this NC function. These results would suggest that the annealing of the tRNA primer is a specific process rather than a simple unwinding of RNA secondary structure. The fact that the binding of NC to $tRNA^{Lys,3}$ is not competed by excess $tRNA^{Trp}$ or $tRNA^{Pro}$ would also argue in favour of a specific RNA binding. This specificity is possibly mediated by the conserved cysteines in the Cys-His boxes as suggested for the NC proteins of RSV and MoMuLV (Méric and Spahr, 1986; Gorelick *et al.*, 1988). Exactly how NC acts in promoting reverse transcription is still an open question. We are currently investigating whether NC is physically associated with RT so as to elucidate whether both may form part of a putative replication complex.

Materials and methods

Plasmid constructions

Escherichia coli strain 1035 (recA$^-$) was used for plasmid manipulation and preparation. For generation of viral RNA by *in vitro* transcription, two derivatives of plasmid pSP64 (Pharmacia) were constructed, namely (i) HIV-1 provirus pmal (Alizon *et al.*, 1986, and cloned in pBR322), digested with *Sma*I (position +30 in genomic RNA, i.e. 152 nucleotides 5′ to the primer binding site) and *Eco*RI (position +4223), generating plasmid pmal CG1; and (ii) an *Xba*I fragment of pmal (positions +152 to +4005, i.e. 30 nucleotides 5′ to the PBS), generating plasmid pmal CG2. The PBS$^-$ construction, pmal CG3, was created by insertion of a *Hin*cII–*Eco*RI fragment (spanning positions +521 to +4223) into pSP64. Note that in the experiments depicted in Figure 6A, the reverse transcripts are longer than the corresponding polylinker region of the pSP64 vector. Plasmid DNAs were isolated by alkaline lysis, followed by RNase treatment and phenolization.

Proteins and enzymes

p66/p51 HIV-1 RT was produced in *E.coli* from the recombinant plasmid p6HRT-PROT (F.Gruninger-Leitch and S.F.J.Le Grice, submitted), and purified by Ni^{2+}-NTA (Hochuli *et al.*, 1988), DEAE–Sephacel and poly(rC) Sepharose chromatography. Scanning microdensitometry of the purified protein indicated it to be >95% pure. MoMuLV RTp80 was from Pharmacia. HIV-1, RSV and MoMuLV NCs were purified from their respective virions according to Moelling *et al.* (1979). Starting from 2 mg packed virions (Bru isolate) HIV-1 NC yield was ~50 μg. This corresponds to a recovery of 30–40% assuming that 7% of total protein is nucleocapsid (Moelling *et al.*, 1979). Purified HIV-1 MA and CA proteins, produced in *E.coli*, were a kind gift of Dr H.V.J.Kolbe, Transgene, Strasbourg. RSV CA was purified by acid–urea electrophoresis and recovered by electroelution into 20 mM sodium acetate, pH 7.0.

RNA preparation

For *in vitro* generation of viral RNA, 5 μg of pmal CG1 or pmal CG2 were linearized at either the *Hae*III (+413), *Pvu*II (+705) or *Ava*II site (+1333) and transcribed with SP6 RNA polymerase under conditions suggested by the manufacturer. Following DNase treatment, RNA was phenol extracted, chromatographed over Sephadex G75, ethanol precipitated and dissolved in double-distilled water. Beef liver $tRNA^{Trp}$, $tRNA^{Pro}$ and $tRNA^{Lys,3}$ were purified as described by Fournier *et al.* (1976). $tRNA^{Lys,3}$ sequence was determined and conforms to that of Raba *et al.* (1979). 5′-Labelled $tRNA^{Lys,3}$ was prepared using T4 polynucleotide kinase and [γ-^{32}P]ATP, and the labelled RNA purified on a 10% urea–polyacrylamide gel. Cellular ribosomal and tRNA as well as HIV-1 tRNAs were purified from 1% low melting temperature agarose gels.

Cell culture and virion production

MoMuLV-infected NIH 3T3 and HIV-1-infected (Bru isolate; Barré-Sinoussi *et al.*, 1983) CEM cells (a human acute lymphoblastic leukaemia cell line) were cultured in DMEM and RPMI-1640 media (Gibco/BRL) respectively, containing 5–10% fetal calf serum at 37°C in the presence of 5% CO_2. Virions were purified by two rounds of centrifugation and the amount of virion proteins estimated from Coomassie Blue staining or immunoblotting following electrophoresis (Burnette, 1981).

NC assays

These were performed in 10 μl reactions comprising 40 mM Tris–HCl, pH 7.0, 50 mM NaCl, 5 mM DTT, 0.01 mM $ZnCl_2$, 0.3–0.5 μg *in vitro* generated viral RNA, 10 ng 5′ ^{32}P-labelled $tRNA^{Lys,3}$ and 10–100 ng of NC at 37°C for 15 min. Reactions were terminated by addition of SDS to 0.5% and 1 μg proteinase K. After 5 min at 37°C, the samples were phenolized and RNA analysed by electrophoresis through a 0.8% agarose gels run in 50 mM Tris–borate, pH 8.3, 1 mM EDTA and at 7 V/cm. Gels were then washed with water and the RNA visualized by eithidium bromide staining. For autoradiographic analysis, the gels were fixed with 5% TCA, then dried.

RT assay

RT assays were performed at 40°C in 20 μl mixture comprised of the following: 40 mM Tris–HCl, pH 8.3, 60 mM NaCl, 6 mM $MgCl_2$, 5 mM DTT, 0.2 mM deoxyribonucleotide triphosphates, with viral RNA, $tRNA^{Lys,3}$ and NC added as described in the text. Reactions were terminated by addition of 10 μl formamide containing 0.1% SDS and 50 mM EDTA. After heating at 90°C for 1 min, the reaction products were fractionated through 7% polyacrylamide gels containing 8 M urea. Following

electrophoresis, gels were subjected directly to autoradiography without drying.

Gel-retardation assays

These were performed in 20 μl reactions under RT assay conditions with the exception that the dNTPs were omitted. Electrophoresis of the RT–$tRNA^{Lys,3}$ complexes was through a 0.8% agarose gel in 50 mM Tris–acetate buffer, pH 7.2, containing 2 mM $MgCl_2$ and 5 mM 2-mercaptoethanol at 3 V/cm for 3 h, with buffer circulation. Following electrophoresis, gels were fixed with 5% TCA for 10 min, dried and subjected to autoradiography.

RNA–protein crosslinking

Crosslinking experiments were performed with *trans*-diaminedichloroplatinum(II) (*trans*-DDP) according to Tukalo *et al.* (1987), in 10 μl RT reaction mix containing 1–3 μg RT, 0.3–1.0 μg tRNA and 30 μM DTT. After 10 min at 37°C, *trans*-DDP was added to a final concentration of 0.3 mM (reaction volume 25 μl) and incubation continued a further 60 min at 20°C in the dark. From 0.3 to 1 unit T1 RNase was then added, and digestion allowed to proceed 60 min at 4°C. After thermal denaturation of T1 RNase (1 min, 100°C) the RNA protein complex and free oligonucleotides were 5′ labelled by the T4 polynucleotide kinase reaction in the presence of [γ-^{32}P]ATP, in a buffer lacking DTT. Reaction products were subsequently passed over a Sephadex G-50 column, equilibrated with 10 mM Tris–HCl, pH 7.0, 1 mM EDTA. Column fractions containing radioactivity were subsequently analysed by SDS–PAGE to determine those containing the tRNA–RT complexes. These fractions were subsequently pooled and the *trans*-DDP cross link reversed by addition of 1 M thiourea and incubation for 2 h at 37°C. Radiolabelled RNA nucleotides were ethanol precipitated (>95% of the radioactivity was recovered) and purified by 15% polyacrylamide/7 M urea gels.

RNA sequence analysis

The sequence of ^{32}P-labelled T1 oligonucleotides purified by urea–PAGE was determined by enzymatic digestion with G-specific T1 RNase (0.01 U/μg RNA), A-specific U2 RNase (0.3 U/μg RNA) and pyrimidine-specific (U,C) RNaseA (0.001 U/μg RNA) as described by Donis-Keller *et al.* (1977). A partial alkaline hydrolysate was obtained by treatment at pH 9.5. Enzymatic and alkaline hydrolysis-generated fragments were fractionated through 15% polyacrylamide/7 M urea gels in 75 mM Tris–borate, 1 mM EDTA, pH 8.3.

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