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The yeast Ty3 retrotransposon contains a 5′–3′ bipartite primer-binding site and encodes nucleocapsid protein NCp9 functionally homologous to HIV-1 NCp7

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Retroviruses, including HIV-1 and the distantly related yeast retroelement Ty3, all encode a nucleoprotein required for virion structure and replication. During an *in vitro* comparison of HIV-1 and Ty3 nucleoprotein function in RNA dimerization and cDNA synthesis, we discovered a bipartite primer-binding site (PBS) for Ty3 composed of sequences located at opposite ends of the genome. Ty3 cDNA synthesis requires the 3′ PBS for primer $tRNA_i^{Met}$ annealing to the genomic RNA, and the 5′ PBS, *in cis* or *in trans*, as the reverse transcription start site. Ty3 RNA alone is unable to dimerize, but formation of dimeric $tRNA_i^{Met}$ bound to the PBS was found to direct dimerization of Ty3 RNA–$tRNA_i^{Met}$. Interestingly, HIV-1 nucleocapsid protein NCp7 and Ty3 NCp9 were interchangeable using HIV-1 and Ty3 RNA template–primer systems. Our findings impact on the understanding of non-canonical reverse transcription as well as on the use of Ty3 systems to screen for anti-NCp7 drugs.

Keywords: bipartite PBS/dimerization/HIV/NC/Ty

Introduction

Replication of retroviruses and retrotransposon elements proceeds by reverse transcription of genomic RNA followed by integration of the DNA copy into the host genome (Coffin, 1985; Varmus and Swanstrom, 1985; Boeke and Sandmeyer, 1991). Reverse transcriptase (RT), integrase, the RNA genome and primer tRNA that participate in this process are all packaged into a core nucleoprotein complex called a nucleocapsid (NC) (Chen *et al*., 1980; Hu and Temin, 1990; Katz and Skalka, 1994; Berkowitz *et al.*, 1996; Mak and Kleiman, 1997). For human immunodeficiency virus type 1 (HIV-1), nucleocapsid protein NCp7 is of one the major structural constituents of the virion core in which ~2000 NCp7 molecules cover the dimeric genome (Darlix *et al.*, 1990, 1995; Berkowitz *et al.*, 1993). NCp7 is a small basic protein possessing two conserved 'CCHC' zinc fingers with key functions in virus structure and replication (Aldovini and Young, 1990; Summers *et al.*, 1992; Dorfman *et al.*, 1993; Morellet *et al*., 1994; Darlix *et al.*, 1995; Berkowitz *et al.*, 1996). NCp7 appears to function as a nucleic acid chaperone, with RNA–DNA binding and annealing activities implicated in the specificity of reverse transcription initiation, and in DNA strand transfers required for generation of the long terminal repeats (LTRs) and completion of proviral DNA synthesis (Barat *et al.*, 1989; Allain *et al.*, 1994; Dannul *et al*., 1994; Peliska *et al*., 1994; Rodriguez-Rodriguez *et al*., 1995; Li *et al.*, 1996; Berthoux *et al*., 1997; Lapadat-Tapolsky *et al.*, 1997). In addition, NCp7 acts as an essential co-factor during dimerization, packaging, condensation and stability of genomic RNA (Housset *et al*., 1993; Berkowitz *et al.*, 1995; Ottmann *et al*., 1995; Zhang and Barklis, 1995).

Nucleoprotein complexes resembling the virion NC can be formed *in vitro* in the presence of NCp7, viral RNA and primer $tRNA^{Lys3}$, resulting in dimerization of viral RNA (provided the packaging E sequence is present) and annealing of primer tRNA to the primer-binding site (PBS). Interestingly, the viral RNA is protected from nuclease degradation in these nucleoprotein complexes, yet initiation of cDNA synthesis by RT can take place at the PBS followed by elongation and strand transfer (Lapadat-Tapolsky *et al*., 1993; Tanchou *et al.*, 1995), thus reproducing the early phases of proviral DNA synthesis (Coffin, 1985; Varmus and Swanstrom, 1985).

In view of the ubiquitous nature of NC protein among retroviruses and of its numerous key roles in virus structure and replication, we set out to compare the functions of NC proteins from two distantly related retroelements, namely HIV-1 and the yeast Ty3 retrotransposon. Ty3, an LTR-containing retroelement, encodes the nucleocapsid protein NCp9 which possesses a single zinc finger and is required for Ty3 transposition in yeast (Kirschner and Sandmeyer, 1993; Orlinsky and Sandmeyer, 1994), implying functional homology with HIV-1 NCp7. During an *in vitro* comparison of HIV-1 NCp7 and Ty3 NCp9 functions in genomic RNA dimerization and reverse transcription, we discovered an unusual bipartite PBS organization for Ty3 with segments of complementarity to primer $tRNA_i^{Met}$ located at opposite ends of the genome. Initiation of Ty3 cDNA synthesis requires the 3′ PBS for primer $tRNA_i^{Met}$ annealing and the 5′ PBS as the transcriptional start site. Furthermore, Ty3 RNA dimerization, unlike HIV-1 and other retroviral RNAs, appears to require and be mediated via dimeric $tRNA_i^{Met}$.

Results

Ty3 NCp9 promotes HIV-1 RNA dimerization and annealing of primer $tRNA^{Lys3}$

Ty3 NCp9 (Figure 1A) was synthesized by fmoc chemistry and purified by HPLC. Highly pure NCp9 thus obtained was found to complex an equimolar zinc cation with high

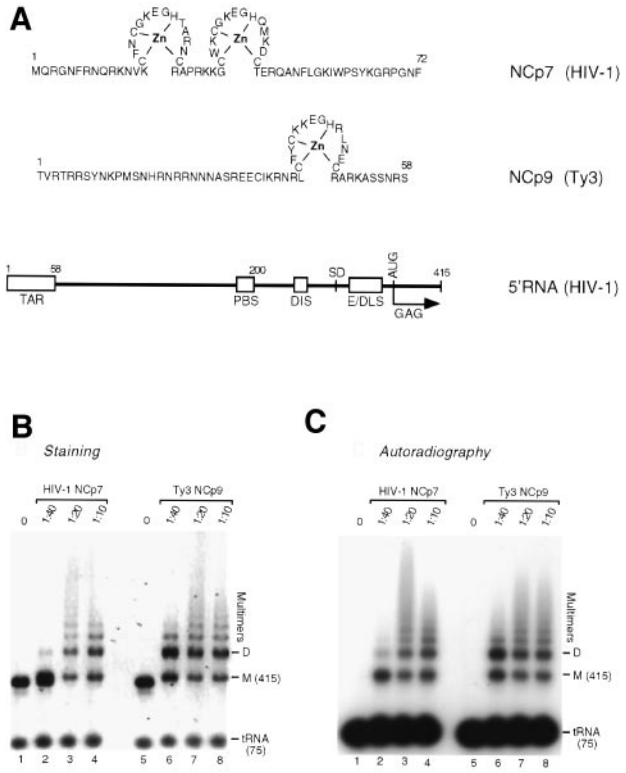

Fig. 1. Ty3 NCp9-directed dimerization of HIV-1 5′ RNA and annealing of replication primer $tRNA^{Lys3}$ to the PBS. (**A**) The amino acid sequences of HIV-1 NCp7 and Ty3 NCp9 are shown using the one-letter code. A zinc cation is shown within each zinc finger, coordinated by the conserved CCHC motif. Note the long N-terminal domain of NCp9. HIV-1 5′ RNA (position 1–415) used in these assays is shown schematically depicting TAR (trans-activation response region), PBS (primer $tRNA^{Lys3}$-binding site), DIS (dimer initiation sequence), SD (major splice donor site), E/DLS (packaging/dimerization element) and the initiator AUG of GAG. (**B**) Ethidium bromide staining of RNAs and (**C**) autoradiography to visualize ^{32}P-labelled tRNA. (A) and (B) show the same gel. Reactions with HIV-1 5′ RNA, *in vitro* synthesized $tRNA^{Lys3}$ and NC protein. A 0.16–1.77 kb RNA ladder was used for size markers (Gibco-BRL; not shown). M and D are monomeric and dimeric HIV-1 RNA respectively; sizes are in nucleotides. The NC protein to nucleotide molar ratios are indicated at the top of the figure. The data are representative of three independent experiments. Similar reactions were also performed with beef liver $tRNA^{Lys3}$ with very similar results (data not shown).

affinity, as does NCp7 of HIV-1 (Figure 1A, H.Deméné and D.Marion, unpublished data). In preliminary experiments, NCp9 exhibited strong nucleic acid binding and annealing activities, similar to HIV-1 NCp7, and was able to form nucleoprotein complexes with both Ty3 and HIV-1 RNA (data not shown; Tanchou *et al.*, 1995).

The ability of Ty3 NCp9 to promote HIV-1 RNA dimerization and annealing of HIV-1 primer $tRNA^{Lys3}$ to the PBS was examined using HIV-1 5′ RNA corresponding to the leader and part of the 5′ GAG domain (position 1–415, Figure 1A), containing the packaging–dimerization (E/DLS) as well as dimerization initiation (DIS) sequences (Paillart *et al.*, 1996). In accordance with published data (Barat *et al.*, 1989; Lapadat-Tapolsky *et al.*, 1993; Tanchou *et al.*, 1995), HIV-1 NCp7 promoted dimerization of the 5′ RNA (Figure 1B, lanes 2–4) and annealing of primer $tRNA^{Lys3}$ to the PBS (Figure 1C, lanes 2–4). Figure 1 shows that NCp9 of Ty3 promotes HIV-1 RNA dimerization (Figure 1B, lanes 6–8) and $tRNA^{Lys3}$ annealing (Figure 1C, lanes 6–8) as efficiently as does NCp7. Ty3 NCp9 was also found to function in place of HIV-1 NCp7 during *in vitro* strong stop cDNA synthesis and its transfer to the 3′ end of HIV-1 RNA (data not shown; for NCp7 see Barat *et al.*, 1989; Tanchou *et al.*, 1995).

A 5′–3′ bipartite PBS within Ty3 genomic RNA

The above data demonstrate functional homology of Ty3 NCp9 with HIV-1 NCp7. To pursue this observation, we devised an *in vitro* Ty3 system equivalent to that used for HIV-1 (Figure 1; Tanchou *et al.*, 1995; Lapadat-Tapolsky *et al.*, 1997). However, the PBS of Ty3 RNA, deduced from sequence complementarity to the primer tRNA in the region analogous to the retroviral PBS, is only eight nucleotides long and located, as is usual for canonical PBS sequences, adjacent to the 5′ LTR (position 121–128; Hansen and Sandmeyer, 1990). Interestingly, Ty3 5′ RNA (Figure 2A, position 1–355) alone did not support binding of replication primer $tRNA_i^{Met}$ (Figure 2C), dimerization (Figure 2B) or initiation of reverse transcription (Figure 3, lanes 1–3). This prompted us to perform a computer local alignment search for additional sequences within Ty3 RNA complementary to primer $tRNA_i^{Met}$. To our surprise, we found two adjacent sequences with high complementarity to $tRNA_i^{Met}$ located near the 3′ end of the Ty3 genomic RNA. The first, at position 4947–4959 in U3 of the RNA, is complementary to 12 nucleotides of the TΨC-arm of $tRNA_i^{Met}$ while the second, at position 4964–4974 in U3, is complementary to 11 nucleotides of the D-arm (Figure 2A). A Ty3 3′ RNA comprising nucleotides 4724–5011 was therefore generated *in vitro* (Figure 2A) and, as shown in Figure 2E, NCp9 efficiently promoted primer annealing to this RNA. Binding of $tRNA_i^{Met}$ to the 3′ RNA also resulted in partial dimerization (Figure 2D, lanes 5–8); however, Moloney murine leukaemia virus (MoMuLV) RT was unable to extend primer $tRNA_i^{Met}$ when annealed to this 3′ RNA by NCp9 (Figure 3A, lanes 4–6).

To examine whether the putative 3′ PBS sequences of Ty3 RNA might act *in cis* to present the 3′ acceptor stem of $tRNA_i^{Met}$ to the 5′ PBS, thereby allowing initiation of reverse transcription, we generated a chimeric RNA containing both 5′ and 3′ sequences (Figure 2A). Ty3 NCp9 and HIV-1 NCp7 efficiently promoted annealing of primer $tRNA_i^{Met}$ to this 5′–3′ RNA (Figure 2G), resulting in high levels of RNA dimers (Figure 2F). Addition of MoMuLV RT and dNTPs to NCp9/5′–3′ RNA and NCp7/5′–3′ RNA complexes resulted in the synthesis of strong-stop cDNA, the initial product of reverse transcription (Figure 3, lanes 10–13).

Based on the above data, the distance between the 5′ and putative 3′ PBS sequences in the Ty3 genome is 4800 nucleotides (Hansen *et al.*, 1988). In order to probe the requirement for a physical link between these PBS sequences for strong-stop cDNA synthesis by RT, an experiment was devised in which 5′ RNA and 3′ RNA (Figure 2A) were used *in trans* in the presence of primer $tRNA_i^{Met}$, NC protein and MoMuLV RT. As reported (Figure 3A, lanes 7–9), high levels of strong-stop cDNA were synthesized in the presence of Ty3 NCp9 despite the

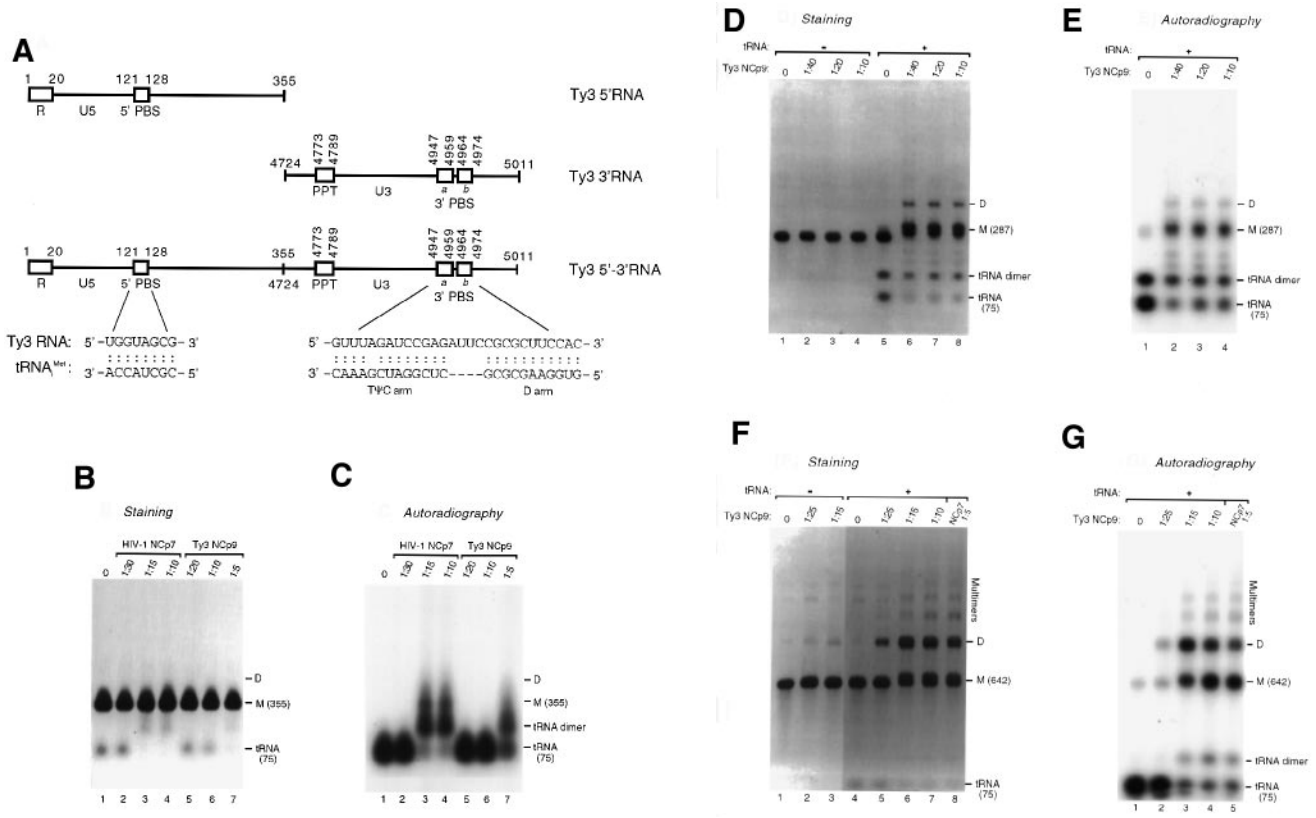

Fig. 2. Sequences near the 3′ end of Ty3 genomic RNA are required for annealing of primer tRNA$_i^{Met}$ and Ty3 RNA dimerization. (**A**) Schematic representation of RNAs utilized. Ty3 5′ RNA included the R repeat, the untranslated 5′ region (U5) and the 5′ PBS. Ty3 3′ RNA included the polypurine tract (PPT), the untranslated 3′ region (U3) as well as the putative 3′ PBS sequences. Chimeric 5′–3′ Ty3 RNA consisted of the above 5′ and 3′ RNAs joined to make a single RNA. Numbering is with respect to genomic RNA positions. Base complementarity between 5′ and putative 3′ PBS sequences and primer tRNA$_i^{Met}$ are indicated in detail. (**B**) Ethidium bromide staining of RNAs and (**C**) autoradiography to visualize ^{32}P-labelled tRNA. Ty3 5′ RNA dimerization and tRNA$_i^{Met}$ annealing, respectively. Note that 5′ RNA remained monomeric while tRNA$_i^{Met}$ underwent dimerization at elevated NC concentrations (C, lanes 3, 4 and 7). (B) and (C) represent the same gel. (**D**) Ethidium bromide staining of RNAs and (**E**) autoradiography to visualize ^{32}P-labelled tRNA. Ty3 3′ RNA dimerization and tRNA$_i^{Met}$ annealing, respectively. Note the large fraction of primer tRNA annealed to the 3′ RNA in the presence of NCp9 (E, lanes 2–4). (**F**) and **(G)** Ty3 5′–3′ RNA dimerization and tRNA$_i^{Met}$ annealing, respectively. Note that RNA dimer and multimer formation (F) and tRNA$_i^{Met}$ annealing (G) were efficient only in the presence of primer tRNA and NC protein. Note also the presence of tRNA$_i^{Met}$ dimers. These data are representative of more than three independent experiments. Incubations at 26 or 37°C gave similar results. Results were very similar using natural yeast tRNA$_i^{Met}$ (not shown). Pairs (D) and (E) and (F) and (G) represent the same gel. M and D are monomeric and dimeric Ty3 RNA, respectively; sizes are in nucleotides.

fact that the 5′ and the putative 3′ PBS sequences were on distinct RNAs. The level of strong-stop cDNA synthesis was similar to that seen with the 5′–3′ chimeric RNA (Figure 3A, lanes 10–13; B lanes 1–5) in which both PBS segments were present *in cis*. To confirm the requirement for the 5′ PBS, we used 5′–3′ RNA with a 5′ PBS deletion (5′ΔPBS–3′ RNA) shown to completely abolish Ty3 transposition *in vivo* (S.Sandmeyer, unpublished results). The 5′ PBS deletion was found to completely preclude strong-stop cDNA synthesis in the presence or absence of Ty3 NCp9 protein (Figure 3B, lanes 6–8).

We also examined the relative contribution of the two putative 3′ PBS sequences during primer tRNA$_i^{Met}$ annealing, Ty3 RNA–tRNA dimerization and initiation of reverse transcription by replacing either the first or the second putative 3′ PBS (3′PBSa or b, see Figure 2A) by a heterologous sequence (see Materials and methods). Results in Figure 4 show that deletion of either sequence strongly reduced RNA dimer formation (Figure 4A, compare lanes 2–4 with 6–8 and 10–12) as well as primer tRNA$_i^{Met}$ annealing (Figure 4B, compare lanes 3–4 with 7–8 and 11–12). Consistent with this, strong-stop cDNA synthesis by RT in the presence of NC protein was decreased ~10-fold by the 3′ PBSa mutation and 4- to 5-fold by the 3′ PBSb mutation (Figure 4C, lanes 4 and 5). Combining both the 3′ PBSa and b mutations precluded strong-stop cDNA synthesis *in vitro* (data not shown).

The effect of mutating the 3′ PBSa and 3′ PBSb sequences was also investigated in yeast cells using a Ty3 His+ element (see Materials and methods). Mutating segments a or b of the 3′ PBS, or both a and b caused a 6- to 7-fold, 4-fold and >20-fold decrease of transposition, respectively (see Materials and methods). These results clearly show that the 3′ PBS sequences are critical for transposition *in vivo*.

Mutations in primer tRNA$_i^{Met}$ that impair transposition result in an attenuation of Ty3 RNA–tRNA dimerization in vitro

Mutations in primer tRNA$_i^{Met}$ involving substitutions C3T and G70A in the acceptor stem previously were reported to inhibit Ty3 transposition *in vivo* (Keeney *et al.*, 1995).

We generated $tRNA_i^{Met}$ with the same point mutations and used it in tRNA annealing and tRNA-promoted dimerization, as well strong-stop cDNA synthesis assays *in vitro* (Figure 5). Annealing of $[^{32}P]tRNA_i^{Met}$ containing the C3T and G70A mutations to Ty3 RNA was essentially retained, but the majority of Ty3 RNA–tRNA was in a monomeric form (Figure 5A, compare lanes 2–3 with 5–6, and lanes 8–9 with 11–12). Interestingly, strong-stop cDNA synthesis was not affected by these mutations in primer $tRNA_i^{Met}$ (Figure 5B, contrast lanes 2–6 and 8–11).

Another set of mutations previously reported to inhibit Ty3 transposition *in vivo* (A54T and A60T, Keeney *et al.*, 1995) were introduced into the TΨC-arm of $tRNA_i^{Met}$. These mutations attenuated Ty3 RNA–tRNA dimerization by NC protein while again strong-stop cDNA synthesis was essentially unaffected (data not shown).

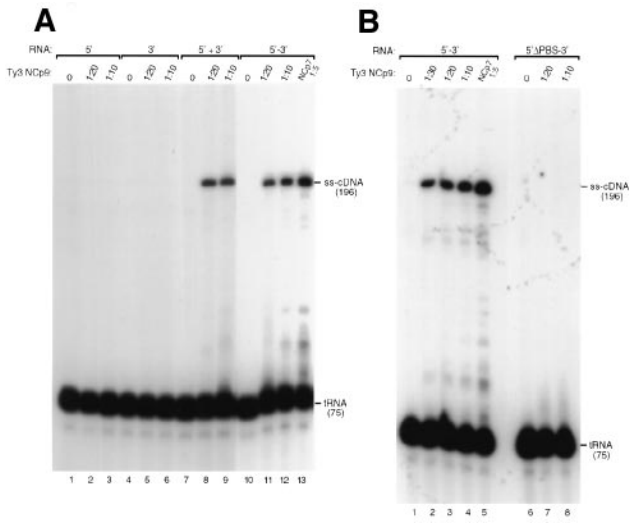

Fig. 3. 5′ and 3′ PBS sequences and NC protein are necessary for initiation of Ty3 reverse transcription. Formation of Ty3 RNA–NC protein complexes was as described in Figure 2. 5′ ^{32}P-labelled FX174 DNA *Hin*f markers (Promega) were used for size determination (not shown). Molar NC protein to nucleotide ratios are indicated at the top of the figure. For zero NCp9 controls, no strong-stop cDNA was detectable even after overexposure of the gels (not shown). 5′ + 3′ RNA reactions contained an equimolar mixture of 5′ and 3′ RNA; 5′–3′ reactions contained 5′–3′ chimeric RNA (see Figure 2A). Strong-stop cDNA includes the ^{32}P-labelled tRNA, resulting in a 196 nucleotide product. Results were the same for incubation temperatures of 26 or 37°C (not shown).

Discussion

Based on the data reported herein, the Ty3 retrotransposon possesses a bipartite PBS with sequences located at opposite ends of the Ty3 genomic RNA complementary to three domains of the primer $tRNA_i^{Met}$, namely the 3′ end, and TΨC- and D-arms. Each of these complementary segments is essential for efficient initiation of Ty3 reverse transcription. In the yeast retrotransposon Ty1, the 3′ end, TΨC- and D-arms of primer $tRNA_i^{Met}$ make contact with three PBS sequences all located near the 5′ end of the genomic RNA (Friant *et al.*, 1996, 1998), providing an interesting parallel with the bipartite PBS structure described herein for Ty3. Furthermore, mutations in Ty1 RNA that impair interactions with the TΨC and D-arms of $tRNA_i^{Met}$ severely attenuate transposition *in vivo* (Friant *et al.*, 1996, 1998). This situation in Ty3, and to a lesser extent in Ty1, is different from that prevailing in retroviruses where a canonical 5′ 18 nucleotide PBS is commonly the major binding site of primer tRNA as well as the start site for reverse transcription (Coffin, 1985;

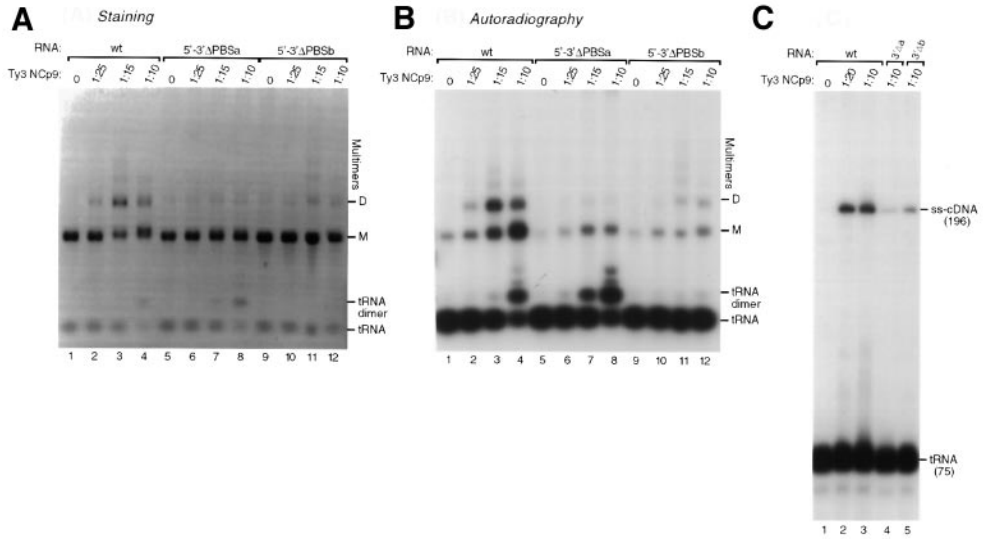

Fig. 4. Mutating the 3′ PBS sequences largely prevents primer $tRNA_i^{Met}$ annealing to the PBS, Ty3 RNA–tRNA dimerization and strong-stop cDNA synthesis. Ty3 RNA sequence complementary to the TΨC-arm (3′ PBSa) or D-arm (3′ PBSb) of $tRNA_i^{Met}$ was replaced by ACUAGU (see Materials and methods). Mutated 5′–3′ RNAs generated *in vitro* were used in RNA dimerization, $[^{32}P]$tRNA annealing and strong-stop cDNA synthesis assays under the conditions described in Materials and methods. (**A**) Ethidium bromide staining of RNAs and (**B**) autoradiography of ^{32}P-labelled tRNA. Dimerization and annealing of $tRNA_i^{Met}$ to Ty3 wild-type and 5′–3′ΔPBS RNA, respectively. Scanning densitometry showed that the 3′ PBS substitutions decreased primer tRNA annealing and dimerization from 5- to >10-fold (e.g. compare lanes 3, 7 and 11). (A) and (B) represent the same gel. (**C**) Strong-stop cDNA synthesis was decreased from 4- to 5-fold (Δ3′PBSb, lane 5) to >10-fold (Δ3′PBSa, lane 4). Mutating 3′ PBSa consistently had a more pronounced effect on dimerization, primer annealing and strong-stop cDNA synthesis than did the 3′ PBSb mutation. M and D are monomeric and dimeric Ty3 RNA–tRNA respectively; sizes are in nucleotides. NC protein to nucleotide molar ratios are indicated. Data are representative of several similar, but independent experiments.

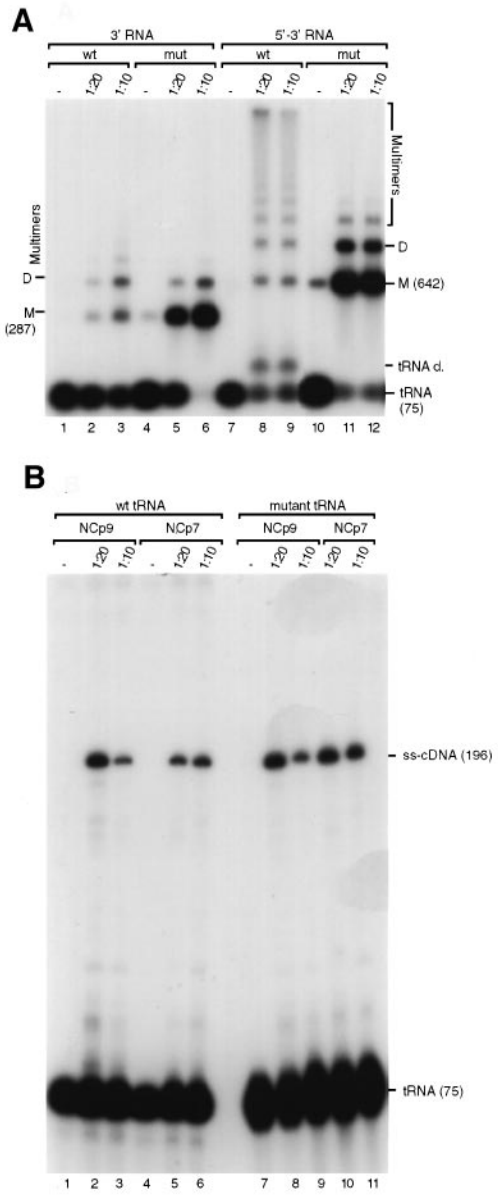

Fig. 5. Mutations in the acceptor stem of $tRNA_i^{Met}$ attenuate Ty3 RNA–tRNA dimerization. Reaction conditions for mutant [^{32}P]$tRNA_i^{Met}$ annealing to Ty3 RNA and strong-stop cDNA synthesis were as described in Figures 2 and 3, respectively.
(**A**) Autoradiography of ^{32}P-labelled tRNA. Annealing of mutant $tRNA_i^{Met}$ (Keeney *et al.*, 1995) to Ty3 3′ RNA (lanes 1–6) and 5′–3′ RNA (lanes 7–12). Note that mutations in the acceptor stem of $tRNA_i^{Met}$ strongly attenuate formation of Ty3 3′ RNA–tRNA dimers (compare lanes 3 and 6) and 5′–3′ RNA–tRNA dimers and multimers (compare lanes 9 and 12) since the majority of mutant tRNA was annealed to monomeric Ty3 RNA (lanes 5, 6, 11 and 12). Data for the 5′–3′ RNA were quantified by scanning densitometry: the ratio of radioactivity in dimers + multimers, versus monomers, was 3:1 for wild-type (lanes 8–9) and 0.5:1 for the mutant (lanes 11–12), after correction for total radioactivity in each lane. Note that mutant tRNA did not form dimers with itself (compare lanes 8 and 11). (**B**) cDNA synthesis. Strong-stop cDNA synthesis was essentially unaffected by mutations in the acceptor stem of primer $tRNA_i^{Met}$. M and D are monomeric and dimeric Ty3 RNA–RNA, respectively; sizes are in nucleotides; tRNA d. are primer tRNA dimers. NC protein to nucleotide molar ratios are indicated. Data are representative of two independent experiments.

Darlix *et al.*, 1993, 1995; Berkowitz *et al*., 1996; reviewed in Mak and Kleiman, 1997).

Using 3′ PBS mutants found to attenuate transposition (see Results), we show that annealing of $tRNA_i^{Met}$ to Ty3 RNA is essential for the formation of dimeric Ty3 RNA–$tRNA_i^{Met}$ complexes (Figure 4). In agreement with this, mutations in the acceptor stem of $tRNA_i^{Met}$ shown to impair transposition strongly (Keeney *et al.*, 1995) were found to decrease the level of dimeric Ty3 RNA– $tRNA_i^{Met}$ *in vitro* (Figure 5 and data not shown) (note $tRNA_i^{Met}$ dimerization in Figures 2–5, whereas $tRNA^{Lys3}$ remained monomeric in Figure 1). Moreover, preliminary experiments indicate that the genomic RNA of the yeast retrotransposon Ty1 also undergoes dimerization dependent upon binding of $tRNA_i^{Met}$ to the PBS (data not shown; Friant *et al*., 1996). Dimerization of $tRNA_i^{Met}$ probably occurs by interaction between the 5′ ends since this domain contains a 12 nucleotide palindrome (position 2–13, GCGCCGUGGCGC) (Keeney *et al.*, 1995), and a DNA oligonucleotide complementary to the palindrome or a deletion of the 15 5′ nucleotides completely inhibits Ty3 RNA and $tRNA_i^{Met}$ dimerization by NC protein (data not shown; see also Figure 5).

A plausible dimerization model for the Ty3 RNA–$tRNA_i^{Met}$ complex is illustrated schematically in Figure 6. In this structure, the primer tRNA is key to both dimer formation and initiation of reverse transcription. Due to the proposed proximity of the 5′ and 3′ ends of Ty3 RNA imposed by dimerization, the tRNA would also indirectly play a role in the 5′ to 3′ transfer of strong stop DNA during minus strand DNA synthesis. Taken together, these data suggest that dimerization of the Ty3 RNA–$tRNA_i^{Met}$ complex is coincident with positioning of the primer tRNA on the RNA template (see also Sandmeyer and Menees, 1996).

The fact that both Ty3 NCp9 and HIV-1 NCp7 were shown to be functionally equivalent in these two distantly related systems favours the notion that Ty3 NCp9 is an ancestor of HIV-1 NCp7. In support of this, preliminary experiments have shown that Ty3 reverse transcription can be inhibited by anti-NCp7 compounds (Rice *et al.*, 1993), raising the possibility that the Ty3 genetic system might be used to screen potential inhibitors of HIV replication.

Understanding the role of the NC protein, the detailed nature of the bipartite Ty3 PBS and the utilization of multiple molecular determinants of a primer tRNA for reverse transcription initiation are of importance for our comprehension of the mechanism by which non-retroviral and cellular RNAs might be reverse transcribed in cells containing active retroelements (Klenerman *et al.*, 1997). It is tempting to evoke a mechanism in which Ty3-like RNA presents tRNA 3′ sequences (via a 3′ PBS ‘grip’) for interaction with non-canonical RNAs *in trans*, resulting in encapsidation and reverse transcription of such RNAs. This hypothesis currently is under investigation.

Materials and methods

RNA substrates, NC proteins and enzymes

HIV-1 5′ RNA corresponding to nucleotides 1–415 was generated *in vitro* as previously described (Barat *et al.*, 1989). HIV-1 3′ RNA (position 8583–9208 of the HIV-1 genome) with a poly(A) tail was prepared by

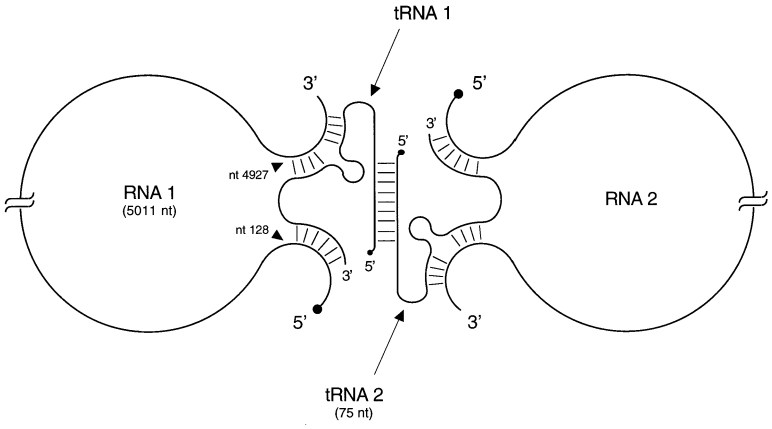

Fig. 6. Proposed schematic model for the Ty3 RNA–$tRNA_i^{Met}$ dimeric structure. Two Ty3 RNA molecules, each with primer tRNA annealed via 5′ and 3′ PBS interactions, are joined via tRNA–tRNA 5′ palindrome complementarity. Extensive and simultaneous interaction of primer tRNA with Ty3 RNA as well as with itself is thus proposed as the central element during Ty3 RNA dimerization. The anticodon arm of primer tRNA appears to be the sole domain not involved in tRNA annealing (see also Figure 2A and evidence not shown) and Ty3 RNA dimerization since mutations in the anticodon domain of $tRNA_i^{Met}$ have little or no effect on Ty3 transposition (Keeney *et al.*, 1995). The spatial proximity of the 5′ and 3′ ends of Ty3 RNA may greatly facilitate minus strand DNA transfer. Regions of interaction and general proportions are not to scale.

transcription as described (Darlix *et al.*, 1993). DNA encoding Ty3 5′ RNA under T7 promoter control was generated by PCR using pEGTy3-1 (Kirschner and Sandmeyer, 1993), a 5′ oligonucleotide containing T7 Po, an *Eco*RI site and 16 nucleotides of the 5′ end of Ty3 RNA, and a 3′ oligonucleotide complementary to position 1106–1121 of Ty3 RNA and containing a *Hin*dIII site. DNA was cloned into pSP64 and controlled by sequencing (pTy3-CG1). Template DNA was linearized at position 355 (*Eco*RV) and 5′ RNA synthesized *in vitro*. DNA encoding Ty3 3′ RNA was generated after PCR using pETGY3-1, a 5′ oligonucleotide containing a *Hin*dIII site and 16 nucleotides (position 4724–4738) of Ty3, a 3′ oligonucleotide complementary to position 4994–5011 and containing an *Nhe*I site, cloned into pBS and controlled by sequencing (pTy3-CG2). Template DNA was linearized with *Sma*I for *in vitro* synthesis of 3′ RNA (T3 pol). For chimeric Ty3 5′–3′ RNA, pTy3-CG1 was cut with *Eco*RV and *Nhe*I to remove sequences downstream of position 355, and treated with *Taq* DNA polymerase to add a 3′ dT (method described in Marchuk *et al.*, 1991), allowing direct insertion of the 3′ DNA PCR fragment digested with *Nhe*I. The construct was verified by sequencing (pTy3-CG3) and linearized by *Nhe*I to generate RNA *in vitro*.

5′ΔPBS–3′ RNA was generated the same way except that pKO271 (S.Sandmeyer, unpublished; nucleotides 77–172 deleted) was used as the template for the first round of PCR. The construct was verified by sequencing (pTy3-CG4).

Ty3 sequence complementary to the TΨC-arm (3′ PBSa) or D-arm (3′ PBSb) of $tRNA_i^{Met}$ was replaced by ACUAGU (a *Spe*I site) using a method based on two successive rounds of PCR (Mikaelian and Sergeant, 1992) and the pTy3-CG3 clone (see above) as template. Mutations were verified by sequencing (pTy3-CG5 and pTy-CG6 for 3′ΔPBSa and 3′ΔPBSb, respectively) and mutated 5′–3′ RNAs synthesized *in vitro* (T7 pol).

Primer $tRNA^{Lys3}$ (Barat *et al*., 1993) and $tRNA_i^{Met}$ were kindly provided by G.Keith and B.Ehresmann (Strasbourg). Synthetic $tRNA^{Lys3}$ was generated *in vitro* using T7 RNA polymerase (Barat *et al.*, 1993). Plasmid DNA HG300 encoding $tRNA_i^{Met}$ (Senger *et al*., 1992) and 5′ and 3′ mutated oligonucleotides (5′ containing the substitution C3→T and 3′ containing G70→A; see Keeney *et al*., 1995) were used for DNA amplification. Mutation of the TΨC-arm of $tRNA_i^{Met}$ (A54→C and A60→T; see Keeney *et al*., 1995) was performed using the PCR strategy outlined above. Mutant tRNAs were verified by sequencing (pΨMet,i-CG2 and 3) and synthesized *in vitro* (T7 pol). All RNAs were purified by spin column chromatography (Pharmacia S-300 HR) and dissolved at 1 mg/ml in sterile water. [^{32}P]UMP-labelled $tRNA^{Lys3}$ and $tRNA_i^{Met}$ were synthesized *in vitro* using T7 RNA polymerase, purified by polyacrylamide gel electrophoresis (PAGE) in 7 M urea, recovered and dissolved at 0.1 mg/ml in sterile water.

Highly pure NCp7 (72 amino acids, containing two Zn^{2+}) was prepared by peptide synthesis as described previously (de Rocquigny *et al.*, 1991). A Ty3 NCp9 variant (P23→A and R31 insertion, relative to Hansen *et al.*, 1988) (Figure 1A) was synthesized by the fmoc/opfp chemical method and purified by HPLC in conditions previously described for HIV-1 NCp7 (de Rocquigny *et al.*, 1991, 1992); 50 mg of >98% pure Ty3 NCp9 was obtained. NCp9 and NCp7 stocks were at 1 mg/ml in 20 mM Tris–acetate pH 6.5, 30 mM NaCl, 5 mM dithiothreitol (DTT) and 1.5 equivalents of $ZnCl_2$. HIV-1 RT (p66/p51), purified from *Escherichia coli* (Le Grice and Grüninger-Leitch, 1990), was provided by S.Le Grice. MoMuLV RT purified from *E.coli* was from Gibco-BRL.

tRNA annealing assay

Reactions with HIV-1 or Ty3 RNA, *in vitro* synthesized ^{32}P-labelled tRNA, or natural primer 5′ [^{32}P]tRNA and NC protein were for 10 min at 37°C (HIV-1) or 28°C (Ty3) in 10 µl containing 20 mM Tris–HCl pH 7.5, 30 mM NaCl, 0.2 mM $MgCl_2$, 5 mM DTT, 0.01 mM $ZnCl_2$, 5 U of RNasin (Promega), 1.5 pmol of RNA, 3 pmol of *in vitro* synthesized tRNA (or natural tRNA) and NCp7 or NCp9 at the indicated molar protein to nucleotide ratios. Reactions were stopped by SDS/EDTA (0.5%/5 mM), treated with proteinase K (2 µg) for 10 min at room temperature, phenol–chloroform extracted and RNA analysed by 1.3% agarose gel electrophoresis in 50 mM Tris–borate pH 8.3 and visualized by ethidium bromide staining followed by gel fixation in 5% trichloroacetic acid (TCA), drying and autoradiography. A 0.16–1.77 kb RNA ladder was used for size determination. The percentage of primer tRNA in HIV-1 or Ty3 RNA annealing was determined by densitometric scanning of the autoradiograph.

Reverse transcription assays

The reactions were performed basically as described previously (Barat *et al.*, 1989; Darlix *et al.*, 1993; Allain *et al.*, 1994). After 5 min at 30°C for the nucleic acid-binding assay in 10 µl (see above), the reaction volume was increased to 25 µl by addition of 2 pmol of HIV-1 RT or MoMuLV RT (Gibco-BRL), 0.25 mM each dNTP, 60 mM NaCl and 2.5 mM $MgCl_2$. Incubation was for 20 min at 30°C (or 37°C with HIV-1 RT), and they were stopped and processed as for the analysis of tRNA annealing (see above), except that after phenol extraction nucleic acid was ethanol precipitated, recovered by centrifugation, dissolved in formamide, denatured at 95°C for 2 min and analysed by 8% PAGE in 7 M urea and 50 mM Tris–borate pH 8.3. 5′ ^{32}P-labelled FX174 DNA *Hin*f markers (Promega) were used for size determination (not shown). The levels of cDNA synthesized by RT were quantified by scanning densitometry.

Transposition assays

The effect of 3′ PBS mutations on Ty3 transposition was examined using a low-copy helper plasmid and a high-copy Ty3 plasmid encoding HIS (Kirschner and Sandmeyer, 1993). The yeast strain yTM443 (derivative of TMy18: *MAT***a**, *trp1-H3, ura3-52, his3-200, ade2-101, lys2-1, leu1-12, can1-100, bar1::his G, GAL3*$^+$, Δ*Ty3*) was transformed with one HIS3-marked high-copy donor plasmid (pKO254 for the wild-type plasmid) (3′ PBS sequences of pKO254 were replaced by a *Spe*I site, ACTAGT, to generate plasmids pCG100 with 3′ΔPBSa, PCG101 with 3′ΔPBSb and pCG102 with 3′ΔPBSab) and with one unmarked low-copy helper plasmid to supply the necessary Ty3 proteins *in trans*

(pJK312). The transformation was done with a lithium acetate protocol at 30°C. Transformants were selected on synthetic medium minus uracil and tryptophan at 30°C.

Two transformants of each type were streaked onto selective medium containing either 2% glucose, to repress, or 2% galactose, to induce, transcription of the Ty3 elements once at 24°C and other times at 30°C. After 3 days, five colonies from each transformant were patched onto YPD (+glucose) to allow for loss of the plasmids at 30°C. Three days later, these patches were replicated onto minus-histidine, 5-fluoroorotic acid (5-FOA)-containing medium (+glucose) to select for cells which lost the URA3-marked donor plasmid but retained the HIS3-marked Ty3 element (at 30°C). To distinguish growth which was due to transposition of the marked Ty3 element from background recombinant events, the cells grown on galactose were compared with the cells grown on glucose. Experiments were repeated four times, and HIS+ colonies were counted. Galactose-induced HIS+ colonies obtained with the wild-type HIS+ plasmid were found to be 10–20 times more abundant than background colonies and much bigger.

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