Characterization of Active Reverse Transcriptase and Nucleoprotein Complexes of the Yeast Retrotransposon Ty3 in Vitro

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Gaël Cristofari†, Caroline Gabus‡, Damien Ficheux§, Marion Bona¶, Stuart F. J. Le Grice∥, and Jean-Luc Darlix###

From the †LaboRetro, Unité de Virologie Humaine, INSERM (#412), Ecole Normale Supérieure de Lyon, 69364 Lyon Cedex 07, France, ‡Institut de Biologie et Chimie des Protéines, 69367 Lyon, France, and ∥HIV Drug Resistance Program, Division of Basic Sciences, NCI-Frederick Cancer Research and Development Center, National Institutes of Health, Frederick, Maryland 21702

Human immunodeficiency virus (HIV) and the distantly related yeast Ty3 retrotransposon encode reverse transcriptase (RT) and a nuclear acid-binding protein designated nucleocapsid protein (NCp) with either one or two zinc fingers, required for HIV-1 replication and Ty3 transposition, respectively. In vitro binding of HIV-1 NCp7 to viral 5'-RNA and primer tRNA3'5'-ω catalyzes formation of nucleoprotein complexes resembling the virion nucleocapsid. Nucleocapsid complex formation functions in viral RNA dimerization and tRNA annealing to the primer binding site (PBS). RT is recruited in these nucleoprotein complexes and synthesizes minus-strand cDNA initiated at the PBS. Recent results on yeast Ty3 have shown that the homologous NCp9 promotes annealing of primer tRNA3'5'-Met to a 5'-3' bipartite PBS, allowing RNA:tRNA dimer formation and initiation of cDNA synthesis at the 5' PBS (1). To compare specific cDNA synthesis in a retrotransposon and HIV-1, we have established a model system comprising Ty3 RNA with the 5'-3' PBS, primer tRNA3'5'-Met, NCp9, and, for the first time, highly purified Ty3 RT. Here we report that Ty3 RT is as active as retroviral HIV-1 or murine leukemia virus RT using a synthetic template-primer system. Moreover, and in contrast to what was found with retroviral RTs, retrotransposon Ty3 RT was unable to direct cDNA synthesis by self-priming. We also show that Ty3 nucleoprotein complexes were formed in vitro and that the N terminus of NCp9, but not the zinc finger, is required for complex formation, tRNA annealing to the PBS, RNA dimerization, and primer tRNA-directed cDNA synthesis by Ty3 RT. These results indicate that NCp9 chaperones bona fide cDNA synthesis by RT in the yeast Ty3 retrotransposon, as illustrated for NCp7 in HIV-1, reinforcing the notion that Ty3 NCp9 is an ancestor of HIV-1 NCp7.

Retrotransposons and retroviruses are members of a large family of mobile elements called long terminal repeat-contain-

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** To whom correspondence should be addressed: LaboRetro, Unité de Virologie Humaine, INSERM (#412), Ecole Normale Supérieure de Lyon, 46 Allée d’Italie, 69364 Lyon, France. E-mail: Jean-Luc.Darlix@ens-lyon.fr.

† The abbreviations used are: RT, reverse transcriptase; NCp, nucleocapsid protein; Fmoc, N-(9-fluorenly)methoxycarbonyl; HIV, human immunodeficiency virus; PBS, primer binding site; SS-cDNA, strong stop complementary DNA; nt, nucleotide(s); HPLC, high performance liquid chromatography; MuLV, murine leukemia virus; PAGE, polyacrylamide gel electrophoresis; NC, nucleocapsid; MLV, murine leukemia virus; WT, wild type.
show that Ty3 nucleoprotein complexes are formed in vitro, allowing Ty3 RT to synthesize strong stop cDNA (ss-cDNA). Interestingly, the N-terminal domain of NCp9, but not the zinc finger, was found to be necessary for the formation of active Ty3 nucleoprotein complexes in vitro.

MATERIALS AND METHODS

RNA Substrates, NC Proteins, and Enzymes—Chimeric Ty3 5′-3′ RNA corresponding to nt 1–1355 and 4724–5011 with the repeat (R), the untranslated 5′ region (U5), the 5′ PBS, the polypurine tract (PPT), the 3′-untranslated region (3′ UTR), and the 3′ PBS were synthesized in vitro using pTy3-CG3 linearized by tRNAiSDS, 5 mM EDTA, treated with proteinase K (2 ind pmol; indicated molar protein-to-nt ratios. Reactions were stopped by 0.5% trichloroacetic acid, dried, and subjected to autoradiography. A percentage of primer tRNA annealing to HIV-1 or Ty3 RNA was determined by scanning densitometry.

Reverse Transcription Assay—Reactions were incubated for 10 min at 28 °C in 10 mM Tris, pH 7.5, 30 mM NaCl, 0.2 mM MgCl2, 5 mM dithiothreitol, 0.01 mM ZnCl2, 1 to 587) was synthesized using T7 RNA polymerase. All RNAs were purified by spin column chromatography (Amersham Pharmacia Biotech S-300 HR) and dissolved at 1 mg/ml in sterile water. [32P]labeled tRNA was 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. Ty1 5′ RNA (nt 1 to 587) was synthesized in vitro using T7 RNA polymerase (1). Ribosomal 28 S RNA was extracted from mouse 3T3 cells and purified by agarose gel electrophoresis. Poly(A)oligo(dT) was from Roche Inc.

Highly pure NCp7 (72 amino acids, containing 2 Zn2+), Ty3 NCp9, and mutants of NCp9 were synthesized by the Fmoc-pentafluorophenyl ester chemical method and purified by HPLC as described for HIV-1 NCp7 (29). Wild type and mutant NCp9 as well as NCp7 stocks were reconstituted at 1 mg/ml in 20 mM Tris acetate, pH 6.5, 30 mM NaCl, and 1.5 eq of ZnCl2.

Ty3 RT was expressed from plasmid pHTy3RT as a 55-kDa protein containing a short polyhistidine extension at its N terminus. Recombinant protein was purified to near homogeneity by a combination of metal chelate and ion exchange chromatography as described previously (30) and shown to be free of contaminating nucleases. Recombinant HIV-1 RT was purified from Escherichia coli as described previously (30).

RESULTS

Characterization of the Retrotransposon Ty3 RT—Recombinant Ty3 RT was expressed in E. coli as a 55-kDa protein containing a short polyhistidine extension at its N terminus and purified to near homogeneity as described before (30). Using the synthetic poly(rA):oligo(dT) template-primer system, Ty3 RT was found to be as active as HIV-1 and MLV and HIV-1 RTs (Fig. 1). Interestingly, the poly(dT) products were found to have similar sizes with all three RTs assayed (from about 100 to 900 nt in length; data not shown).

One general feature of retroviral RTs is their potential to copy an RNA or a DNA template by means of a self-priming mechanism (20, 21). A number of different RNAs were used as templates in reverse transcription reactions (Fig. 2, A and B). In all cases examined Ty3 RT was found to be completely inactive, whereas HIV-1 and MLV RT were found to be very active. Reverse transcription by self-initiation of templates such as a 28 S rRNA, Ty1 and Ty3 RNAs was shown in Fig. 2, A and B. HIV-1 and MLV RTs were able to reverse-transcribe 28 S rRNA (Fig. 2A, lanes 3 and 4), but retrotransposon Ty3 RT was not (Fig. 2A, lanes 2 and 3; Fig. 2B, lanes 2 and 6). HIV-1 RT is also able to copy single-stranded DNA using a self-priming mechanism (21). Ty3 RT was found to be completely inactive on a single-stranded DNA in vitro (data not shown).

Characterization of Ty3 Nucleoprotein Complexes Formed with Wild Type Nucleocapsid Protein NCp9 and Deletion Mutants of NCp9—Ty3 NCp9 is a basic protein with a single canonical CCHC zinc finger and a long N-terminal domain, whereas the C terminus is short (Fig. 3A). Results on HIV-1 NCp7 and Moloney MLV NCp10 have shown that deletions in the N-terminal domain have profound effects on viral RNA dimerization, primer tRNA annealing to the PBS, and initiation of ss-cDNA synthesis. On the other hand, deleting the zinc fingers had only minimal effects on these processes in vitro (29, 31, 32). These results on two different retroviral NC proteins

2 M. Bona and S. Le Grice, unpublished information.
prompted us to progressively delete part or all of the N-terminal domain as well as the zinc finger of Ty3 NCp9. Fig. 3 reports sequences of NCp9 deletion mutants together with the Ty3 5’-3’ RNA used.

Complexes were formed by incubating 32P-labeled Ty3 5’-3’ RNA with NCp9 at increasing protein:nt stoichiometries at 30 °C under conditions reported under “Materials and Methods.” Equivalent ratios were used with deletion mutants Δ1, Δ2, Δ3, NCp9 dd, and Δ2-NCp9 dd. Nucleoprotein complexes were subsequently analyzed by PAGE in presence of 50 mM Tris borate but in the absence of a strong denaturing agent. Clearly wild type NCp9 was most effective in generating nucleoprotein complexes at a NCp9:nt ratio of 1:20 (Fig. 4, A and B, compare lanes 2, 6, 10, 14 in A and lanes 2, 6, and 10 in B). Completion of the assembly process was obtained upon increasing molar NCp9-to-nt ratios from 1:10 to 1:5 for Δ1, Δ2, and NCp9 dd (lanes 7–8 and 11–12 in Fig. 4A and 7–8 in Fig. 4B). For Δ3-NCp9, reactions were never complete (Fig. 4A, lane 16), whereas with Δ2-NCp9 dd, complexes appeared to be unstable (Fig. 4B, lane 12). These results indicate that the N-terminal domain of NCp9 is an important determinant for nucleoprotein complex formation.

Effects of NCp9 Deletions on Primer tRNA\textsuperscript{Met} Annealing and 5’-3’ RNA:tRNA Dimerization—As recently reported, dimerization of Ty3 RNA is mediated by NCp9-promoted annealing of primer tRNA\textsuperscript{Met} to the 5’-3’ PBS. The palindromic sequence at the 5’ end of tRNA\textsuperscript{Met} most probably directs dimerization since deletion of the 14 5’ nucleotides of tRNA\textsuperscript{Met} abolishes tRNA and Ty3 RNA:tRNA dimerization (data not shown; see Ref. 1). Primer tRNA annealing and Ty3 RNA dimerization assays were carried out with 32P-labeled tRNA\textsuperscript{Met}, Ty3 5’-3’ RNA, and either wild type NCp9 or deletion mutants using NCp9:nt ratios varying from 1:10 to 1:2 required for full complex formation (see Fig. 4). Subsequently, reaction mixtures were treated with proteinase K, phenol-extracted to remove NC protein, and analyzed by agarose gel electrophoresis (see “Materials and Methods”). Fig. 5A shows Ty3 RNA:tRNA dimerization, whereas Fig. 5B reports [32P]tRNA annealing. Clearly, NCp9 dd was as efficient as WT NCp9 in promoting tRNA annealing and Ty3 RNA:tRNA dimerization (Fig. 5, compare lanes 2–3 and 11–12). On the other hand Δ1, Δ2, and Δ2-NCp9 dd were much less efficient (lanes 5, 7, and 13–14). Δ3-NCp9 was found to be very poorly active in these processes (lanes 8–9). As previously shown, HIV-1 NCp7 was also able to promote primer tRNA annealing and Ty3 RNA dimerization (1).

Ty3 RT Is Active on Ty3 Nucleoprotein Complexes Formed in Vitro—We have previously shown that the addition of MuLV RT and dNTPs to Ty3 RNA:tRNA:NCp9 complexes resulted in the synthesis of ss-cDNA, the initial product of reverse transcription. It was also shown that Ty3 NCp9, and HIV-1 NCp7 were interchangeable using Ty3 and HIV-1 template/primer systems (1). In an attempt to reconstitute a complete homologous Ty3 reverse transcription system, we used Ty3 RT expressed as a 55-kDa protein containing a short polyhistidine extension at its N terminus (see “Materials and Methods” and Fig. 1). Nucleoprotein complexes were formed at NCp9:nt ratios of 1:20 to 1:4 to ensure that all RNA template has been recruited into nucleoprotein complexes (Figs. 4 and 5). Next, Ty3 RT was added at a molar RT to template/primer ratio of 1:3, and reverse transcription was allowed to proceed for 30 min at 30 °C. Reaction mixtures were treated twice with phenol:chloroform to remove all proteins, and cDNA products were heat-denatured and analyzed by 8% PAGE in 7 M urea. As a control without NCp9, primer tRNA\textsuperscript{Met} was heat-annealed to Ty3 5’-3’ RNA for 30 min at 60 °C. Without prior annealing of [32P]tRNA\textsuperscript{Met} and no NCp9, ss-cDNA could not be detected (Fig. 6, lane 1), while upon heat-annealing of primer tRNA a faint band corresponding in length to ss-cDNA was visualized (lane 2). The addition of NCp9 or NCp9 dd resulted in high levels of ss-cDNA synthesis (lanes 3–7). It should be noted that at an NCp9:nt ratio of 1:10, the level of ss-cDNA in the presence of NCp9 dd was consistently 30 to 40% less than that with wild type NCp9 (lanes 4–7). On the other hand, ss-cDNA synthesis was 10 to 15 times lower in the presence of Δ1-NCp9 (lanes 8 and 9) and undetectable with all other NCp9 deletion mutants (data not shown). Interestingly, ss-cDNA synthesis was also much lower with HIV-1 NCp7 (lanes 10 and 11), in contrast to our previous
The ubiquitous nature of the RT and NC protein among retroviruses and retrotransposons such as yeast Ty3 and Droso- phila Copia prompted us to analyze the reverse transcription process in Ty3 and compare it with that in HIV-1, a distantly related long terminal repeat-containing retroelement belonging to the lentivirus family.[12] Interestingly, Ty3 NCp9 and HIV-1 NCp7 are interchangeable in the Ty3 and HIV-1 template/primer systems, although Ty3 reverse transcription clearly differs from that of HIV-1 on the basis of NC protein and PBS sequences as well as on the mechanisms of RNA dimerization and initiation of minus-strand DNA synthesis.[1] In addition, anti-NCp7 compounds (56) were found to inhibit Ty3 reverse transcription (data not shown), suggesting that yeast Ty3 can be used to screen new anti-NCp7 inhibitors capable of impairing HIV-1 replication.

To extensively analyze the reverse transcription process of a retrotransposon, namely yeast Ty3, and to compare it with that of HIV-1, we devised a functional in vitro Ty3 template/primer system consisting of a bipartite Ty3 5′-3′ RNA template, the cognate tRNA primer (tRNA_A[^344]), and NCp9. A purified, recombinant 55-kDa version of Ty3 RT was included in the system, since it was highly active on a synthetic poly(rA):oligo(dT) template/primer (Fig. 1). In addition Ty3 RT was found to be more specific than that of MLV or HIV-1, since it was unable to copy a RNA or a DNA template by self-priming (Fig. 2 and data not shown). However, it is important to point out that Ty3 NCp9 (data not shown), like MLV NCp10 or HIV-1 NCp7, can only inhibit cDNA synthesis by self-priming using MLV or HIV-1 RT (21). Trivial explanations for this finding might have included either nuclease contamination and destruction of the RNA templates or an inability of the Ty3 RT to recognize and polymerize from an RNA 3′ hydroxyl. The former notion appears unlikely, based on observations that incubation of Ty3 NCp9 with RNA or DNA templates by self-priming (Fig. 2 and data not shown). However, it is important to point out that Ty3 NCp9 (data not shown), like MLV NCp10 or HIV-1 NCp7, can only inhibit cDNA synthesis by self-priming using MLV or HIV-1 RT (21). Trivial explanations for this finding might have included either nuclease contamination and destruction of the RNA templates or an inability of the Ty3 RT to recognize and polymerize from an RNA 3′ hydroxyl. The former notion appears unlikely, based on observations that incubation of Ty3 NCp9 with RNA or DNA templates by self-priming (Fig. 2 and data not shown). However, it is important to point out that Ty3 NCp9 (data not shown), like MLV NCp10 or HIV-1 NCp7, can only inhibit cDNA synthesis by self-priming using MLV or HIV-1 RT (21). Trivial explanations for this finding might have included either nuclease contamination and destruction of the RNA templates or an inability of the Ty3 RT to recognize and polymerize from an RNA 3′ hydroxyl. The former notion appears unlikely, based on observations that incubation of Ty3 NCp9 with RNA or DNA templates by self-priming (Fig. 2 and data not shown). However, it is important to point out that Ty3 NCp9 (data not shown), like MLV NCp10 or HIV-1 NCp7, can only inhibit cDNA synthesis by self-priming using MLV or HIV-1 RT (21). Trivial explanations for this finding might have included either nuclease contamination and destruction of the RNA templates or an inability of the Ty3 RT to recognize and polymerize from an RNA 3′ hydroxyl. The former notion appears unlikely, based on observations that incubation of Ty3 NCp9 with RNA or DNA templates by self-priming (Fig. 2 and data not shown). However, it is important to point out that Ty3 NCp9 (data not shown), like MLV NCp10 or HIV-1 NCp7, can only inhibit cDNA synthesis by self-priming using MLV or HIV-1 RT (21). Trivial explanations for this finding might have included either nuclease contamination and destruction of the RNA templates or an inability of the Ty3 RT to recognize and polymerize from an RNA 3′ hydroxyl. The former notion appears unlikely, based on observations that incubation of Ty3 NCp9 with RNA or DNA templates by self-priming (Fig. 2 and data not shown). However, it is important to point out that Ty3 NCp9 (data not shown), like MLV NCp10 or HIV-1 NCp7, can only inhibit cDNA synthesis by self-priming using MLV or HIV-1 RT (21). Trivial explanations for this finding might have included either nuclease contamination and destruction of the RNA templates or an inability of the Ty3 RT to recognize and polymerize from an RNA 3′ hydroxyl. The former notion appears unlikely, based on observations that incubation of Ty3 NCp9 with RNA or DNA templates by self-priming (Fig. 2 and data not shown). However, it is important to point out that Ty3 NCp9 (data not shown), like MLV NCp10 or HIV-1 NCp7, can only inhibit cDNA synthesis by self-priming using MLV or HIV-1 RT (21). Trivial explanations for this finding might have included either nuclease contamination and destruction of the RNA templates or an inability of the Ty3 RT to recognize and polymerize from an RNA 3′ hydroxyl. The former notion appears unlikely, based on observations that incubation of Ty3 NCp9 with RNA or DNA templates by self-priming (Fig. 2 and data not shown). However, it is important to point out that Ty3 NCp9 (data not shown), like MLV NCp10 or HIV-1 NCp7, can only inhibit cDNA synthesis by self-priming using MLV or HIV-1 RT (21).
This unexpected property of Ty3 RT together with the chaperoning activity of NCp9 (Figs. 4 and 5; see also below) may have evolved to ensure that in intracellular virus-like particles, retrotransposon genomic RNA is selectively reverse-transcribed. This concept is presently under investigation.

To further examine the chaperoning properties of Ty3 NCp9 and compare them to HIV-1 NCp7, we synthesized NCp9 deletion mutants and examined their ability to (i) promote nucleoprotein complexes, (ii) direct primer tRNA\textsuperscript{iMet} annealing to the PBSs, and (iii) catalyze Ty3 RNA:tRNA dimerization. We also examined the activity of Ty3 RT to specifically direct minus-strand strong-stop cDNA synthesis, the early product of the reverse transcription process, in Ty3 nucleoprotein complexes.

Functions of the NCp9 deletion mutants in nucleoprotein complex formation, tRNA annealing and Ty3 RNA dimerization, and minus-strand cDNA synthesis are summarized in Table I. Clearly, only wild type NCp9 was optimal in all these functional assays. Nevertheless, deletion of the zinc finger (NCp9\textsuperscript{dd}) only had a moderate inhibitory impact on nucleoprotein complex formation, tRNA annealing, Ty3 RNA dimerization, and tRNA-primed reverse transcription (Figs. 4, 5, and 6). On the other hand, deleting N-terminal residues (\delta\textsuperscript{3}-NCp9) resulted in an almost complete loss of activity in vitro. This is reminiscent of the findings with HIV-1 NCp7 where deletion of the two zinc fingers only had a moderate inhibitory effect on \psi+ activity.
RNA dimerization, tRNA\^[\text{30s}]
 annealing, and tRNA-primed cDNA synthesis, whereas N-terminal deletion resulted in a drastic inhibition of functions in vitro (29, 31).

Retroviral NC protein has an important chaperoning function in reverse transcription in directing specific tRNA-primed cDNA synthesis. This appears to be achieved in two ways: first, by inhibiting nonspecific self-primed reverse transcription of the genome and of cellular RNAs and nonspecific replication of newly made DNA products (20, 21); second, by promoting primer tRNA annealing and minus-strand DNA synthesis (15, 29). Retrotransposon Ty3 NCp9 was also found to chaperone specific Ty3 RNA reverse transcription by a retroviral RT in a manner similar to HIV-1 NCp7 and MLV NCp10 (data not shown; Ref. 21). Using MLV RT, this takes place with maximal efficiency with WT NCp9 (Table I). As has been documented for HIV-1 NCp7, the N-terminal and zinc fingers domains are essential for nucleic acid binding (17, 22, 29, 37, 38), and this is presently under investigation.

Ty3 is an ancestor of HIV-1, whereas Ty1 is probably more distant (36, 42, 43). Last, these data on NCp9 reinforce the notion that Ty3 is an ancestor of HIV-1, whereas Ty1 is probably more ancient (44), and characterization of an NC-like activity in Ty1 is presently under investigation.

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