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Zinc finger function of HIV-1 nucleocapsid protein is required for removal of 5′-terminal genomic RNA fragments: A paradigm for RNA removal reactions in HIV-1 reverse transcription

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ABSTRACT

During (−) strong-stop DNA [(−) SSDNA] synthesis, RNase H cleavage of genomic viral RNA generates small 5′-terminal RNA fragments (14–18 nt) that remain annealed to the DNA. Unless these fragments are removed, the minus-strand transfer reaction, required for (−) SSDNA elongation, cannot occur. Here, we describe the mechanism of 5′-terminal RNA removal and the roles of HIV-1 nucleocapsid protein (NC) and RNase H cleavage in this process. Using an NC-dependent system that models minus-strand transfer, we show that the presence of short terminal fragments pre-annealed to (−) SSDNA has no impact on strand transfer, implying efficient fragment removal. Moreover, in reactions with an RNase H^{-} reverse transcriptase mutant, NC alone is able to facilitate fragment removal, albeit less efficiently than in the presence of both RNase H activity and NC. Results obtained from novel electrophoretic gel mobility shift and Förster Resonance Energy Transfer assays, which each directly measure RNA fragment release from a duplex in the absence of DNA synthesis, demonstrate for the first time that the architectural integrity of NC's zinc finger (ZF) domains is absolutely required for this reaction. This suggests that NC's helix destabilizing activity (associated with the ZFs) facilitates strand exchange through the displacement of these short terminal RNAs by the longer 3′ acceptor RNA, which forms a more stable duplex with (−) SSDNA. Taken together with previously published results, we conclude that NC-mediated fragment removal is linked mechanistically with selection of the correct primer for plus-strand DNA synthesis and tRNA removal step prior to plus-strand transfer. Thus, HIV-1 has evolved a single mechanism for these RNA removal reactions that are critical for successful reverse transcription.

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1. Introduction

The human immunodeficiency virus type 1 (HIV-1) nucleocapsid protein (NC) is a small, basic nucleic acid binding protein having two zinc-binding domains or zinc fingers (ZFs), connected by a short, flexible basic linker. Each finger contains the invariant metal-ion binding motif, CCHC, and both ZFs are required for virus replication (Bampi et al., 2004; Darlix et al., 1995; Levin et al., 2005, 2010; Rein et al., 1998; Thomas and Gorelick, 2008). NC is also a nucleic acid chaperone, which remodels nucleic acid structures so that the most thermodynamically stable conformations are formed (Tsuchihashi and Brown, 1994; reviewed in Bampi et al., 2004; Godet and Mély, 2010; Levin et al., 2005, 2010; Rein et al., 1998). This activity is essential for many events in virus replication, including a crucial role in facilitating efficient and specific reverse transcription (Darlix et al., 2011; Godet and Mély, 2010; Levin et al., 2005, 2010; Rein et al., 1998).

Abbreviations: HIV-1, human immunodeficiency virus type 1; NC, nucleocapsid protein; ZFs, zinc fingers; RT, reverse transcriptase; (−) SSDNA, (−) strong-stop DNA; R, repeat; EMSA, electrophoretic mobility shift assay; FRET, Förster resonance energy transfer; PPT, polypurine tract; WT, wild-type; SP, self-priming; TAR, transactivation response element; FAM, 6-carboxyfluorescein.

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† This article is dedicated to the memory of our dear colleague Dr. Jianhui Guo. A tribute can be found on p. 356 of this issue.

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Reverse transcription consists of a complex series of reactions that culminate in conversion of the single-stranded viral RNA genome into a linear, double-stranded DNA copy that is ultimately integrated into host chromosomal DNA (reviewed in Herschhorn and Hizi, 2010; Sarafianos et al., 2009). This process is catalyzed by the viral reverse transcriptase (RT) enzyme (Baltimore, 1970; Mizutani et al., 1970). The first DNA product synthesized by RT is a short DNA copy of the 5′-end of the viral RNA genome, known as (−) strong-stop DNA [(−) SSDNA]. As the DNA is being synthesized, the RNase H activity of RT degrades the viral RNA template. When RT reaches the 5′-end of the template, 5′-terminal RNA fragments, ranging in size from 14 to 18 nt remain annealed to (−) SSDNA (Fu and Taylor, 1992) because RNase H cleavage of blunt-ended substrates is inefficient (Champoux, 1993). However, these RNA fragments must be removed or the next step in the pathway, minus-strand transfer, will be blocked. Although it is known that NC stimulates secondary RNase H cleavage of blunt-ended duplexes (Wisniewski et al., 2002) and plays a role in 5′-RNA fragment release (Chen et al., 2003b; Peliska et al., 1994), the precise mechanism has not been investigated.

Minus-strand transfer is required to generate full-length minus-strand DNA (Gilboa et al., 1979; Telesnitsky and Goff, 1993) and is dependent upon NC's nucleic acid chaperone activity (Darlix et al., 2011; Godet and Mély, 2010; Levin et al., 2005, 2010; Rein et al., 1998). Two separate, but not mutually exclusive, mechanisms have been proposed to explain how transfer occurs. The first of these is known as "end terminus transfer", i.e., transfer of the 3′ end of full-length (−) SSDNA to the 3′ end of viral RNA (acceptor RNA), in a reaction facilitated by base pairing of the complementary repeat (R) regions (Telesnitsky and Goff, 1993). Indeed, cell-based assays performed with murine leukemia virus, spleen necrosis virus, and HIV-1 showed that a majority of the transfer events occurred after synthesis of (−) SSDNA (Klaver and Berkhout, 1994; Kulpa et al., 1997; Lobel and Goff, 1985; Ohi and Clever, 2000; Ramsey and Panganiban, 1993; Yin et al., 1997), demonstrating that end terminus transfer is an important mechanism for minus-strand transfer in vivo.

More recently, an alternative "acceptor invasion-driven mechanism" has been proposed. This mechanism is based on data from studies of NC-dependent minus-strand transfer in reconstituted RT assay systems, which show that transfer can begin before completion of (−) SSDNA synthesis (Chen et al., 2003a,b; reviewed in Basu et al., 2008; Piekna-Przybylska and Bambara, 2011). In this case, RNase H cleavages at internal sites within the viral RNA template create gaps (invasion sites). These gaps make it possible for acceptor RNA to displace template RNA fragments and subsequently anneal to complementary sequences in (−) SSDNA. Removal of 5′-terminal RNA fragments in the final step has been suggested to occur by strand displacement and release of uncleaved 5′-terminal RNA (Chen et al., 2003b). Although there are clearly differences in the two mechanisms for minus-strand transfer, it should be emphasized that the requirement for fragment removal is independent of whether transfer occurs after completion of or concomitant with (−) SSDNA synthesis.

In the present study, we focus on the molecular mechanism of 5′-terminal RNA removal and the roles of RNase H and NC in this process. Using a reconstituted minus-strand transfer system containing a short RNA annealed to (−) SSDNA, we show that the rate and extent of strand transfer were the same regardless of whether the RNA fragment was present or absent. The reaction did not require RNase H cleavage, but strand transfer was most efficient in the presence of both RNase H and NC. For the first time, we also provide direct evidence that NC's helix destabilizing activity, which is associated with the native ZFs, is absolutely required for release of terminal RNA fragments. Most importantly, our results also demonstrate that HIV-1 uses the same mechanism for RNA removal reactions that occur during reverse transcription: (i) removal of 5′-terminal RNAs, which remain annealed to (−) SSDNA, as described here; (ii) removal of potential plus-strand RNA primers to block mispriming by non-polypurine tract (PPT) RNAs, thereby ensuring the fidelity of plus-strand DNA synthesis (Jacob and Destefano, 2008; Post et al., 2009); and (iii) removal of the 3′-terminal tRNA sequence covalently linked to minus-strand DNA prior to plus-strand transfer (Guo et al., 2000; Wu et al., 1999).

2. Materials and methods

2.1. Materials

All RNA and DNA oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA) (Table 1). SUPERaseIN (an RNase inhibitor), T4 polynucleotide kinase, proteinase K, and Gel Loading Buffer II were obtained from Applied Biosystems (now Invitrogen) (Foster City, CA). [γ-^{32}P]ATP and [γ-^{33}P]ATP (each 3000 Ci/mmol) were obtained from PerkinElmer (Shelton, CT). HIV-1 RT was purchased from Worthington Biochemical Corp. (Lakewood, NJ). The HIV-1 RNase H^{-} RT used for this work was the E478Q point mutant (Schatz et al., 1989). Wild-type (WT) and ZF mutant NC proteins were prepared as recombinant proteins and were purified as described previously (Carteau et al., 1999; Wu et al., 1996). The sequences of the NC proteins and nucleic acids used in this study were derived from the HIV-1 pNL4-3 clone (GenBank accession no. AF324493) (Adachi et al., 1986).

2.2. Reconstituted minus-strand transfer assay

The minus-strand transfer assay described previously (Heilman-Miller et al., 2004; Wu et al., 2010) was modified as follows. Where specified, 0.2 pmol of a 128-nt DNA representing (−) SSDNA (DNA 128), labeled at its 5′ end with ^{32}P or ^{33}P (Guo et al., 1995), was annealed at 65 °C for 5 min in annealing buffer (50 mM Tris–HCl, pH 8.0, and 75 mM KCl) to 0.4 pmol of a 14- or 20-nt RNA oligonucleotide (RNA 14 or RNA 20, respectively) (Fig. 1); the RNA is complementary to the 3′-terminal 14 or 20 nt of (−) SSDNA, respectively (Table 1). After gradual cooling to 37 °C, the annealed (−) SSDNA-RNA hybrid was incubated with 0.5 U of SUPERaseIN and 0.2 pmol of RNA 148 (acceptor RNA) for 5 min at 37 °C. HIV-1 WT or mutant NC protein was then added at the indicated concentrations together with 0.2 pmol of HIV-1 WT or RNase H^{-} RT. Reactions (final volume, 20 μl) were initiated by addition of 4 μl of "start" buffer (50 mM Tris–HCl, pH 8.0, 75 mM KCl, 0.1 mM each of the four dNTPs, and 7 mM $MgCl_2$). Control reaction mixtures lacking a small RNA oligonucleotide were subjected to a mock annealing step. For kinetic experiments, reactions were scaled up as appropriate and 10-μl aliquots were removed at the specified time points. Note that since (−) SSDNA is provided in this system (and is not synthesized using a donor RNA template), RNase H activity is not required in the absence of a short 5′-terminal RNA fragment.

Termination of the reactions, polyacrylamide gel electrophoresis (PAGE) in 8% denaturing gels, visualization of the DNA products, and PhosphorImager analysis were performed as described previously (Wu et al., 2007). The percentage of transfer product formation was calculated by dividing the amount of full-length transfer product by the total signal found in the gel lane (i.e., the sum of the transfer product, self-priming (SP) products (Beltz et al., 2005; Driscoll and Hughes, 2000; Guo et al., 1997, 2000; Heilman-Miller et al., 2004; Lapadat-Tapolsky et al., 1997; Levin et al., 2005,

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