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Comparative nucleic acid chaperone properties of the nucleocapsid protein NCp7 and Tat protein of HIV-1

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ABSTRACT

RNA chaperones are proteins able to rearrange nucleic acid structures towards their most stable conformations. In retroviruses, the reverse transcription of the viral RNA requires multiple and complex nucleic acid rearrangements that need to be chaperoned. HIV-1 has evolved different viral-encoded proteins with chaperone activity, notably Tat and the well described nucleocapsid protein NCp7. We propose here an overview of the recent reports that examine and compare the nucleic acid chaperone properties of Tat and NCp7 during reverse transcription to illustrate the variety of mechanisms of action of the nucleic acid chaperone proteins.

1. Introduction: RNA chaperones to resolve RNA misfolding

RNA chaperones are proteins that interact with RNA molecules to solve the RNA folding problem (Herschlag, 1995; Cristofari and Darlix, 2002; Schroeder et al., 2004). RNA molecules are synthesized as linear polymers that require appropriate folding to reach their active (native) conformations. Along the folding trajectory, RNA navigates through a rugged funnel-like landscape biased toward few native structures (Fontana et al., 1993; Chen and Dill, 2000; Russell et al., 2002). Intermediate conformations with stabilities close to the native functional states usually coexist such that a fraction of the molecules rapidly folds into their native structures while many others are kinetically trapped in misfolded intermediate conformations (Fig. 1) (Thirumalai et al., 1997). RNA molecules are thus prone to adopt stable and persistent alternative secondary structures which have to overpass thermodynamic barriers to correctly fold (Treiber and Williamson, 1999). This is why assistance appears to be necessary to reach the active RNA conformations. RNA chaperones were thus postulated to direct the correct folding of RNA molecules and to resolve RNA misfoldings (Herschlag, 1995). Today's view is that RNA chaperones are nucleic acid binding proteins present in all living organisms, including viruses, where they perform multiple functions (Cristofari and Darlix, 2002; Schroeder et al., 2004). Questioning the structure-function paradigm (Wright and Dyson, 1999; Dyson and Wright, 2005), it was found that the prevalence of functional regions in RNA chaperones without a well defined 3D-structure was very high and such intrinsically disordered domains (IDD) were proposed to support the chaperone activity (Tompa and Csermely, 2004; Tompa, 2005). Since RNA chaperones do not share common sequences, motifs or structures, their identification is hardly predictable, and thus requires adequate assays to establish their chaperoning activity. Several in vitro assays are routinely used (Cristofari and Darlix, 2002; Rajkowitsch et al., 2005) and include annealing, helix destabilization, strand displacement (Cristofari and Darlix, 2002; Rajkowitsch et al., 2005, 2007; Rajkowitsch and Schroeder, 2007a, 2007b; Godet and Mély, 2010), *cis* or *trans*-splicing (Coetzee et al., 1994; Zhang et al., 1995; Mayer et al., 2002; Semrad et al., 2004; Belisova et al., 2005; Grohman et al., 2007) and hammerhead-ribozyme RNA cleavage (Bertrand and Rossi, 1994; Herschlag, 1995). Fewer in vivo assays also exist, such as intron folding trap or transcription antitermination (Clodi et al., 1999; Lorsch, 2002; Rajkowitsch et al., 2005). The latter tests investigate simultaneously several if not all the components that account for the chaperoning activity.

In vitro studies show that RNA chaperones function by repetitive transient interactions with RNA (Cruceanu et al., 2006a; Wu et al., 2010; Doetsch et al., 2011b). This property results in transient destabilization of the metastable NA conformations and in accelerating the annealing of complementary sequences. The fast binding kinetics is thus likely to be a critical component of the chaperoning activity. Monitoring the fast association and dissociation rates of

Abbreviations: HIV-1, human immunodeficiency virus-type I; NCp7, nucleocapsid protein of HIV-1; ZF, zinc finger; NA, nucleic acid; TAR, transactivation response element; PBS, primer binding site; AA, amino acids.

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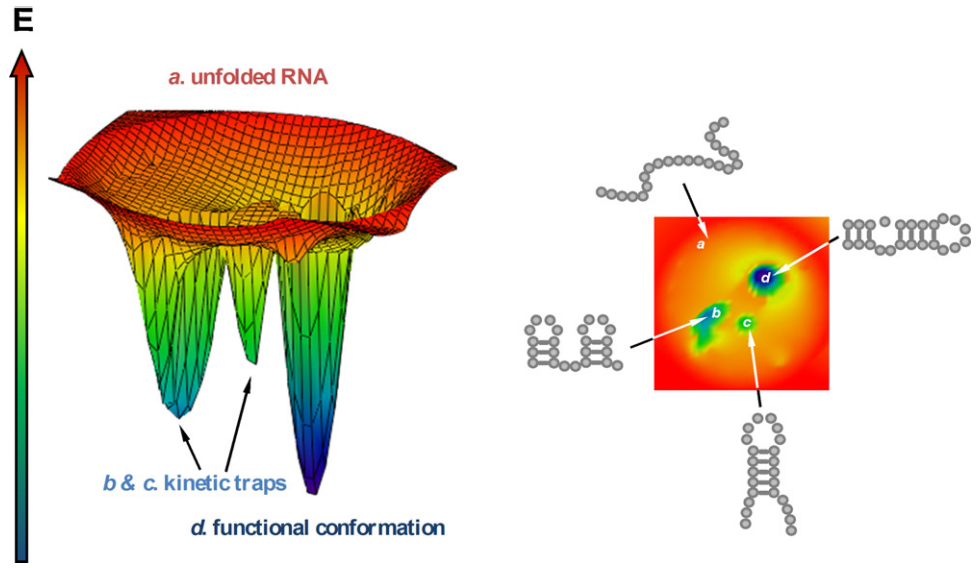

Fig. 1. Scheme illustrating the RNA energy landscape. RNA energy landscape is rugged with many local minima that correspond to suboptimal kinetic trap foldings (b and c). RNA-chaperones smoothen the landscape by destabilizing metastable conformations and lowering the activation energy between the different conformations, thus directing the folding of RNA towards the native conformation.

chaperones from NA substrates is a challenging issue but none of the currently used assays can yet provide direct measurements of the on- and off-binding rates. Indirect evidence of this property has been nevertheless gained from single molecule stretching experiments investigating the chaperone properties of HIV-1 Gag and NC (Williams et al., 2001; Cruceanu et al., 2006a), and the nucleocapsid protein of the yeast Ty3 retrotransposon (Chaurasiya et al., 2012).

At the same time, RNA chaperones promote molecular aggregation, also known as molecular crowding. RNA-chaperones usually present basic domains, ensuring that a significant part of the binding is provided by nonspecific electrostatic interactions. RNA chaperones can bind to highly diverse nucleic acid (NA) sequences and by so doing can coat NA molecules, which is required for function. This has been termed the window of RNA chaperone activity (reviewed in Darlix et al., 2011). This binding mode causes the formation of high molecular weight nucleoprotein complexes, where fuzziness appears to be a major trait in addition to molecular crowding (Fuxreiter and Tompa, 2012; Ivanyi-Nagy and Darlix, 2012). Fast binding kinetics, molecular crowding and fuzziness cause a high local concentration of the partners and a smoother energy landscape resulting in a large increase in the RNA folding rate (Woodson, 2010) (Fig. 1).

Multiple functions have been assigned to cellular RNA-chaperones, such as transcription regulation, RNP assembly, pre-mRNA processing, RNA nuclear export and translation, and in miRNA metabolism. RNA-chaperones are also present in the viral world, where they have major roles in virus structure and replication (Zúñiga et al., 2009). The first reported viral RNA-chaperones are the nucleocapsid (NC) proteins of avian and murine retroviruses (Prats et al., 1988). Later, a similar chaperone activity was shown for the HIV-1 NC protein (Darlix et al., 1990; De Rocquigny et al., 1992; Barat et al., 1993; Dib-Hajj et al., 1993; Lapadat-Tapolsky et al., 1993). The intense research efforts on HIV/AIDS benefited to the characterization of chaperones since a large part of our current understanding was gained during the past 20 years from studies on HIV-1 NC protein. Other virus-encoded RNA chaperones have since been characterized, notably the nucleoprotein N of Coronavirus (Zúñiga et al., 2007), the 3AB protein of Poliovirus (DeStefano and Titilope, 2006), the Core protein of Flaviviridae (Cristofari et al., 2004; Ivanyi-Nagy et al., 2006, 2008; Sharma et al., 2011), the nucleoprotein N of Hantavirus (Mir and Panganiban, 2005, 2006) or the small delta antigen SdAg of Hepatitis D Virus (Huang and Wu, 1998; Huang et al., 2003, 2004; Wang et al., 2003) (for a review see Zúñiga et al., 2009). Moreover, viruses are capable of highjacking cellular factors with RNA chaperoning activity in the course of viral RNA synthesis and translation (Zúñiga et al., 2009). All these examples highlight the variability of chaperone proteins selected through evolution to solve the folding problem and to assist the large nucleic acid rearrangements occurring throughout viral life cycles.

Here, we review the mode of action of HIV-1 NCp7 in parallel with that of the Trans-Activator of Transcription Tat, another potent nucleic acid chaperone encoded by HIV (Kuciak et al., 2008; Boudier et al., 2010) to illustrate the variety of the mechanisms of action of the nucleic acid chaperone proteins.

2. Two HIV-encoded proteins with chaperone activity

2.1. HIV-1 nucleocapsid protein NCp7

HIV-1 NCp7 is a small (55 amino acids) basic protein resulting from the protease-mediated cleavage of the Pr55Gag polyprotein precursor. NCp7 is characterized by two conserved '$CX_2CX_4HX_4C$' zinc fingers (ZFs) (Fig. 2A), flanked by small domains rich in basic residues. Interestingly the basic ZF linker and the N- and C-terminal domains appear to be disordered indicating that NCp7 belongs to the IDP family (Darlix et al., 2011). At the same time, a hydrophobic plateau can form at the top of the ZFs in the native protein (Morellet et al., 1992, 1994; Mély et al., 1994), which is composed of residues Val13, Phe16, Thr24 and Ala25 of the proximal ZF and residues Trp37, Gln45 and Met46 of the distal ZF. This ZF plateau plays a key role in the selection of the viral sequences during genomic RNA packaging and contributes to NCp7 chaperoning properties (Godet and Mély, 2010; Darlix et al., 2011). NCp7 can cause nucleic acid destabilization (Bernacchi et al., 2002; Azoulay et al., 2003; Beltz et al., 2003; Cosa et al., 2004, 2006) and can transiently interact with nucleic acids with fast binding and dissociation kinetics (Cruceanu et al., 2006a). NCp7 also activates the annealing of complementary sequences (Darlix et al., 1993, 1995; You and McHenry, 1994) and the cleavage of a cognate substrate by a hammerhead ribozyme (Bertrand and Rossi, 1994; Kuciak et al., 2008), and efficiently rescues the splicing of a group I intron mutant in the T4 phage td gene in vivo (Clodi et al., 1999).

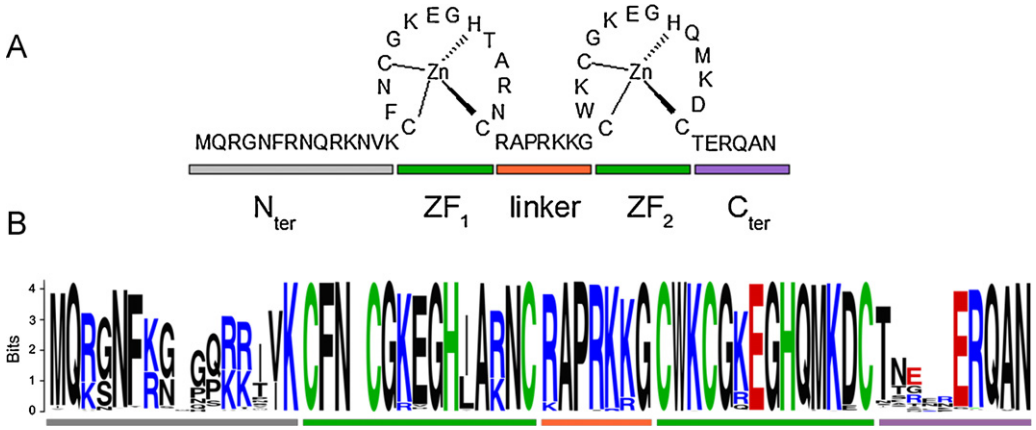

Fig. 2. Sequence (A) and sequence logo (B) of NCp7. NCp7 is a basic protein of 55 amino acids. The two zinc fingers, ZF_1 and ZF_2 (green) and the highly basic linker (orange) are strongly conserved whereas much more variability is allowed in the poorly folded N-terminal (grey) and C-terminal (purple) domains. In spite of the amino acid variability, the positions of the basic amino acids in the N-terminal domain are strictly conserved.

The destabilizing activity of NCp7 mainly relies on the ZFs (Beltz et al., 2005) while the nucleic acid annealing and condensation properties are largely dependent on the basic residues of the disordered regions (Fig. 2A) (De Rocquigny et al., 1992; Stoylov et al., 1997; Williams et al., 2001). The importance of the various NCp7 amino acids can be easily seen on the sequence logo plot resulting from the alignment of 3120 NCp7 HIV sequences of the Lanl HIV database (http://www.hiv.lanl.gov/). Fig. 2B shows that the information content associated to the amino acids composing the two ZFs and the linker are close to their theoretical maximum ($\log_2(20) \approx 4.32$ bits). In contrast, the N-terminal domain is less conserved although the positions of the positively charged amino acids are essentially conserved, suggesting that a precise distribution of basic amino acids in the N-terminal and basic linker domains is required for NC function (Darlix et al., 2011; Doetsch et al., 2011a).

The Gag precursor also exhibits nucleic acid chaperone activity (Rein, 2010; Wu et al., 2010). The comparative chaperone activities of recombinant Gag (or more precisely Gag ΔP6 (Campbell and Rein, 1999)) and of the differently processed cleavage products have been investigated. Gag can bind nucleic acids with higher affinity than NCp7, while in contrast, the nucleic acid chaperone activity improves greatly as the Gag precursor is progressively processed to give NCp15, NCp9 and NCp7 (Cruceanu et al., 2006b; Rein, 2010; Wu et al., 2010). Modification of the chaperone activity of NC during the viral cycle likely accounts for the modifications in the virus structure and function consecutive to the Gag cleavage events during maturation (Mirambeau et al., 2010). In particular, critical architectural changes of the viral core result from the nucleoproteic complex condensation as Gag is progressively processed (Mirambeau et al., 2007). The progressive decrease of binding affinity as Gag is processed is likely ascribed to the loss of the specific high affinity binding mode required for selective vRNA encapsidation to favour a less specific and transient binding mode mandatory to facilitate the nucleic acid rearrangements during reverse transcription. The mature NCp7 is characterized by highly dynamic binding properties (Cruceanu et al., 2006a). Effective strand annealing activity is notably correlated with NC's ability to rapidly bind and dissociate from nucleic acids. Indeed, NC variants with slow on/off rates are poorly efficient in rearranging nucleic acids, even though they are still capable of binding with high affinity to nucleic acids and to aggregate nucleic acids (Cruceanu et al., 2006b; Stewart-Maynard et al., 2008).

In the inner core of the mature viral particle, approximately 1500–2000 copies of NCp7 are coating the genomic RNA (Briggs et al., 2004; Chertova et al., 2006; Chen et al., 2009), thus corresponding to an average occupancy of 5–7 nt per NCp7 molecule. Such an occupancy value was found to be required for optimal NC chaperoning activities in vitro (reviewed in Darlix et al., 2011), notably in the annealing reactions taking place during viral DNA synthesis by RT. During reverse transcription which occurs in this viral nucleoprotein assemblage called the reverse transcription complex (RTC), NCp7 is thought to direct most of the critical nucleic acid rearrangements, namely primer tRNALys,3 annealing to the viral PBS (Barat et al., 1989, 1993; De Rocquigny et al., 1992; Hargittai et al., 2004), the first and second DNA strand transfers (You and McHenry, 1994; Lapadat-Tapolsky et al., 1995; Auxilien et al., 1999; Ramalanjaona et al., 2007; Godet et al., 2011) and to assist the formation of the central DNA flap (Charneau et al., 1994; Hameau et al., 2001). In addition, NC appears to assist the viral DNA polymerase activity of RT throughout viral DNA synthesis, notably by relieving RT pausing at the initiation step corresponding to ss-cDNA synthesis (Liang et al., 1998; Rong et al., 2001; Bampi et al., 2004; Liu et al., 2010), by increasing the polymerization rate and stimulating the RT-RNase H activity (Bampi et al., 2006; Grohmann et al., 2008), and by promoting the fidelity of plus-strand priming (Jacob and DeStefano, 2008; Post et al., 2009) as well as that of (−) and (+) strand DNA synthesis (Kim et al., 2012). Thus, the overall efficiency of reverse transcription is increased by NCp7 which by forming a condensed but highly mobile ribonucleoproteic complex increases the molecular crowding of the nucleic acids generated during reverse transcription and thus, facilitate their annealing (Lapadat-Tapolsky et al., 1995; Tanchou et al., 1995). However, direct demonstration of these nucleic acid rearrangements in the viral context is still missing.

2.2. The transcription trans-activator Tat

Tat is a small basic protein that has multiple key roles in virus replication and pathogenesis (Karn, 1999). Tat is encoded for by two exons and is composed of 101 (clinical isolates) or 86 amino acids (laboratory isolates). The full-length Tat is composed of six different regions which composition and functions are briefly described below (Fig. 3). Region I (aa 1–20) is acidic and proline rich and is involved in Tat-mediated immune suppression (Wrenger et al., 1997). Region II (aa 21–40) contains seven conserved Cys residues, where all of them but Cys31 are required for transcription transactivation (Kuppuswamy et al., 1989; Jeang et al., 1999). These Cys residues interact with two Zn^{2+} ions (Frankel et al., 1988a, 1988b), conferring to Tat the property to trigger apoptosis (Egelé et al., 2008). Region III (aa 41–48) represents the highly conserved core

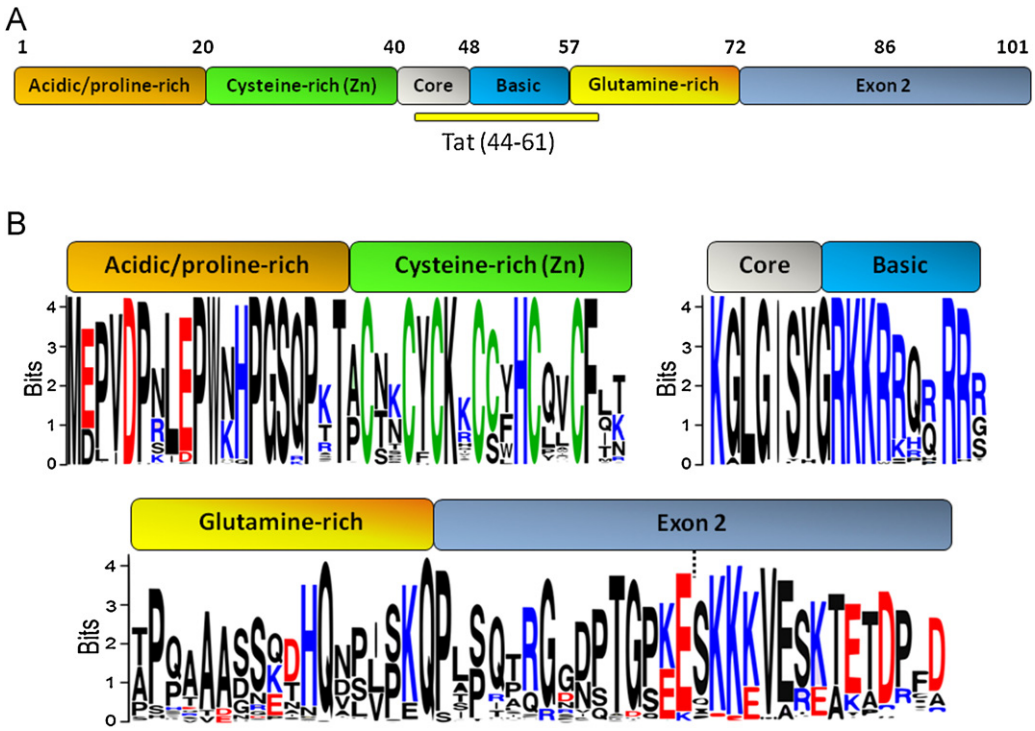

Fig. 3. Domains and sequence logo of Tat. Sequence logo generated from the alignment of 1143 Tat sequences. Tat is composed of 6 regions differently conserved.

that is critical for Tat binding to tubulin (Chen et al., 2002), which triggers the mitochondrial pathway of apoptosis and neuronal cytoskeletal changes leading to AIDS-associated dementia (Chen et al., 2002; Giacca, 2005). Region IV (aa 49–64) is Arg-rich and mediates the binding of Tat to the 5′ TAR sequence (Kuppuswamy et al., 1989; Betti et al., 2001). This region also contains the Tat nuclear localization signal (Vivès et al., 1997). The C-terminal region contains a glutamine-rich domain (aa 57–72) that contributes to the transactivating activity of Tat (Kuppuswamy et al., 1989). This region is also involved in induction of apoptosis in T-lymphocytes upon binding to tubulin (Campbell et al., 2004). Finally, region VI (aa 73 to 86 or 101) can interact with the integrin-mediated sites of cellular adhesion and with integrin and fibronectin receptors. It is also involved in the cell penetration properties of Tat (Barillari et al., 1993).

Tat binding to TAR RNA (Trans Acting Responsive element) activates viral DNA transcription initiation and elongation from the 5′ LTR promoter (Laspia et al., 1989; Feinberg et al., 1991). The Tat/TAR nucleoprotein complex promotes the recruitment of a series of transcription factors leading to the formation of very active elongating transcription machinery (Chun and Jeang, 1996; Okamoto et al., 1996). Beyond its role in viral DNA transcription, Tat/RNA interactions were reported to influence viral mRNA capping and splicing, and translation (Chiu et al., 2002; Berro et al., 2006; Charnay et al., 2009). Moreover, Tat could possibly act as a RNA silencing suppressor via interactions with DICER, TRBp, siRNA and mRNA (Bennasser et al., 2005; Bennasser and Jeang, 2006).

Tat belongs to the IDP's family (Shojania and O'Neil, 2006). Conformational disorder and flexibility may confer to Tat its ability to interact with numerous viral and cellular partners (Dunker et al., 2005; Dosztányi et al., 2006). Together with its nucleic acid binding properties, the disordered nature of Tat suggests that this protein is also a nucleic acid chaperone (Kuciak et al., 2008). Tat and several Tat-derived peptides were found to efficiently activate DNA annealing, ribozyme-mediated RNA cleavage and RNA trans-splicing in vitro (Kuciak et al., 2008). Tat was also found to induce the displacement of an imperfect DNA strand by a perfectly complementary sequence in a DNA exchange assay (Kuciak et al., 2008), while no strand-displacement activity was found in assays using complementary RNA sequences (Doetsch et al., 2011a), suggesting a nucleation-limited strand exchange activity. The inability of Tat to exchange RNA strands is likely explained by the limited ability of Tat to destabilize RNA structures. Therefore, Tat likely promotes nonspecific nucleic acid annealing reactions when destabilization is not or poorly required. Amino acids responsible for the chaperone activity spanned from residues 44 to 61. Tat(44–61) was found to be the shortest known sequence with nucleic acid chaperoning activity. Interestingly, a panel of alanine-scanning mutations from amino acids 45 to 54 evidenced a striking correlation between the conservation of these amino acids (Fig. 3) and the positions of the mutations that prevent virus to initiate the natural endogenous reverse transcription (NERT) in a cell-free virus supernatant assay (Apolloni et al., 2003). The selection pressure on the central stretch of basic amino acids may thus be related to its implication in reverse transcription. The substantial nucleic acid chaperone properties exhibited by Tat may account for its ability to promote the annealing of the primer tRNA to the viral RNA (Kameoka et al., 2002) and intervene in the first strand transfer (Boudier et al., 2010) and by this way, to stimulate RTion as does NCp7 (Harrich et al., 1997; Ulich et al., 1999; Apolloni et al., 2007).

3. Chaperoning reverse transcription

During reverse transcription, major and complex nucleic acid rearrangements are required to allow the full-length genomic dsDNA, also called vDNA, to form. All along these steps, the nucleic acid chaperone activity of NCp7 was evidenced to assist and to facilitate the synthesis of the vDNA. Tat was also found to promote most of these steps in vitro. In the following sections, we will briefly outline the chaperone properties of Tat and NCp7 regarding their mechanism to chaperone in vitro the rearrangements of the nucleic acid sequences involved in critical steps of reverse transcription.

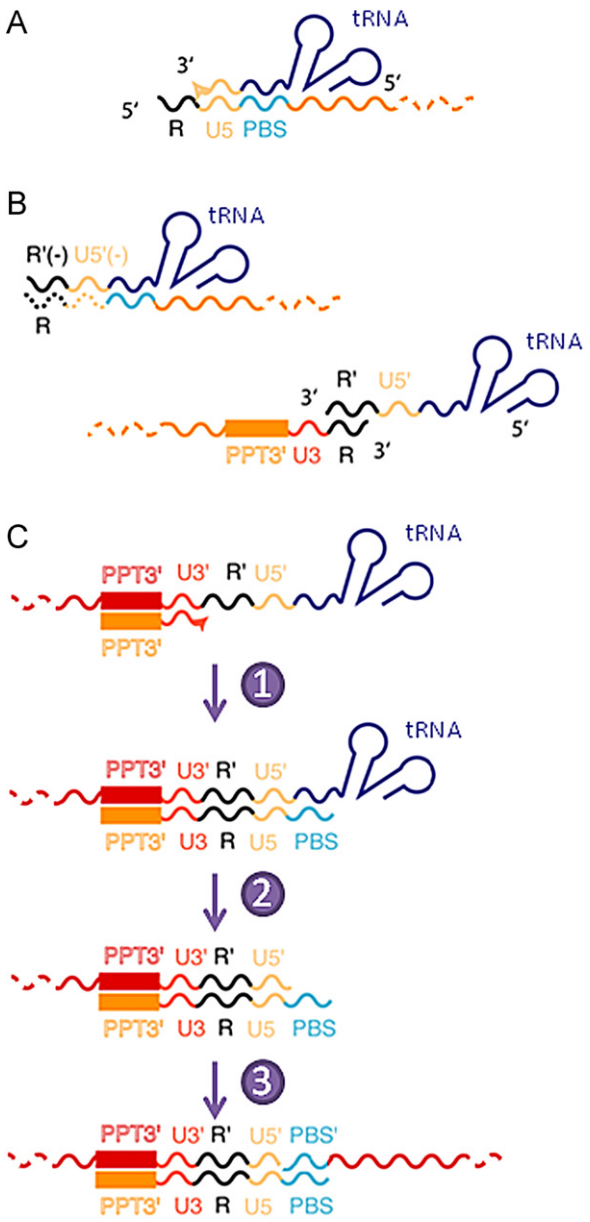

Fig. 4. Schematic diagram of particular events occurring in reverse transcription. A. During initiation, the $tRNA^{Lys,3}$ is placed on the vRNA and serves as a primer to be further elongated by RT. B. During the first strand transfer, the minus-strand strong-stop DNA is translocated to the 3′ end of the viral RNA genome, in a reaction mediated by base-pairing of the repeat R sequences at the 3′ ends of the RNA (containing TAR) and cDNA (containing cTAR) reactants, to allow reverse transcription to resume.
C. The second strand transfer involves (1) the synthesis of plus strand strong-stop DNA that terminates after copying the 3′ end of the tRNA sequence; (2) $tRNA^{Lys,3}$ primer removal and (3) the annealing of (−)PBS to the (+)PBS sequence. The annealing of the two complementary PBS DNA stem-loops enables RT to complete viral DNA synthesis.

3.1. Chaperoning the initiation of reverse transcription

At the early events of vDNA synthesis, the reverse transcriptase (RT) elongates a primer tRNA annealed to the viral primer-binding site (PBS), a 18-nt conserved sequence in the 5′ leader region of the genomic RNA, to later synthesize the minus-strand strong-stop cDNA (minus ss-cDNA) (Fig. 4A). These steps are facilitated by different viral chaperones.

3.1.1. Positioning the replication primer tRNA onto genomic RNA

During assembly, cellular $tRNA^{Lys}$ isoacceptors are selectively incorporated into virions (Jiang et al., 1993; Mak and Kleiman, 1997; Pavon-Eternod et al., 2010). The chaperone properties of the NC domain in Pr55gag are thought to facilitate the specific placement of the $tRNA^{Lys,3}$ primer on the primer binding site (PBS) of the viral RNA (Cen et al., 1999; Feng et al., 1999; Cruceanu et al., 2006b; Guo et al., 2009; Wu et al., 2010). In vitro, NCp7 directs the annealing of the $tRNA^{Lys,3}$ primer to the PBS (Li et al., 1996), by facilitating the strand exchange at the level of the tRNA acceptor stem and by unlocking in the presence of the complementary genomic RNA sequence, the highly stable interactions at the level of the TΨC loop (Chan and Musier-Forsyth, 1997; Tisné et al., 2003, 2004; Hargittai et al., 2004; Tisné, 2005; Barraud et al., 2007). The kinetics of the $tRNA^{Lys,3}$ annealing on the PBS sequence follow a nucleation-limited bimolecular reaction. The reaction is enhanced by five orders of magnitude by NCp7, largely due to its ability to strongly promote duplex nucleation (Hargittai et al., 2004). The NC ZFs specifically interact with the TΨC loop. Although NC ZFs do not promote unwinding of $tRNA^{Lys3}$, the truncated form of NCp7 was found to destabilize two base pairs that could serve as nucleation points for the annealing of tRNALys3 to the viral RNA (Tisné et al., 2001). Contrary to NC ZFs, a SSHS mutant of NC, which lacks the folded ZFs, poorly destabilizes the tRNA tertiary core (Hargittai et al., 2001). It was nevertheless able to anneal more efficiently than NCp7 the $tRNA^{Lys,3}$ primer onto the PBS (Hargittai et al., 2004). Mutants with complete ZF deletions are also able to efficiently anneal $tRNA^{Lys,3}$ primer to the PBS, provided that the basic N- or C-terminal domains are present (De Rocquigny et al., 1992). These ZF mutants suggest that the destabilization of the tRNA core does not appear to be critical in vitro and that multivalent cationic peptides might be sufficient for efficient tRNA primer annealing to the PBS. This conclusion is also supported by the minimal alterations of the annealing kinetics induced by mutations which alter the secondary or tertiary structure or the stability of the tRNA (Hargittai et al., 2004) and by the greater tRNA-annealing activity of an N-terminal extended form of NCp7 protein in vitro (Roldan et al., 2005). Though the ZFs appear dispensable in vitro, it cannot be excluded that as for the second strand transfer (see below), the ZFs can induce a specific reaction pathway that is critical for a timely and controlled $tRNA^{Lys,3}$/PBS annealing reaction.

In full line with its ability to facilitate RNA annealing, Tat was also reported to increase the efficiency of primer tRNA placement onto genomic RNA. In vitro, Tat could even replace NCp7 at this step (Kameoka et al., 2002) in a largely electrostatically driven tRNA annealing promotion. This was confirmed by mutational analysis showing that deletion of the basic domains of Tat resulted in the loss of the annealing property (Kameoka et al., 2002). Nonetheless, initiation of reverse transcription from Tat-annealed $tRNA^{Lys,3}$ occurred less efficiently than from heat-annealed $tRNA^{Lys,3}$ (Kameoka et al., 2002). Specific formation of the initiation complex is mediated by extended interactions between the HIV-1 RNA and $tRNA^{Lys,3}$ (Goldschmidt et al., 2002). Although variable among HIV strains (Goldschmidt et al., 2004), these extended interactions (Isel et al., 1996, 1998, 1999, 2010; Li et al., 1996; Beerens and Berkhout, 2002; Huthoff et al., 2003) are decisive for the efficiency of the initiation of reverse transcription. Formation of the initiation complex requires rearrangements in the 5′UTR vRNA that may be promoted by the fully processed NC (Iwatani et al., 2003; Guo et al., 2009). Indeed, $tRNA^{Lys,3}$ annealed by Gag exhibits a strongly reduced ability to initiate reverse transcription and binds less tightly to viral RNA than the NCp7-annealed $tRNA^{Lys,3}$ (Guo et al., 2009). This necessary chaperoning of reverse transcription priming thus appears as a key regulatory step which can only be catalysed when a significant amount of NCp7 has been processed.

3.1.2. Primer tRNA extension

Once the primer is placed, the subsequent synthesis of vDNA can start. The primer is initially extended by 6 nt in a slow and distributive initiation phase where the DNA polymerization is characterized by a rapid dissociation of RT and early kinetic pausing events at positions +3 or +5 nt (Isel et al., 1996; Lanchy et al., 1996, 1998). NCp7 reduces RT pauses at these positions, although with different efficiencies. Interestingly, an additional pause at +1 nt was observed in vitro when the tRNA was heat-annealed on PBS but not when the annealing reaction was performed using NCp7. In contrast, the pause at +1 position was not affected with ZF mutants of NC, suggesting again that all the chaperone properties of the native NCp7 protein are important for promoting an active tRNALys,3/vRNA initiation complex (Rong et al., 2001). On the contrary to the +1 nt pause, NC was not able to help RT to escape the +3 pausing event (Liang et al., 1998; Rong et al., 2001; Liu et al., 2010). The strong +3 nt pause has been proposed to originate from the template structure and notably from the folding of the A-rich stem-loop located upstream the PBS (Liang et al., 1998; Liu et al., 2010). NCp7 induced a partial and transient disruption of the stem secondary structure, as evidenced by a decrease of the high-throughput SHAPE footprinting reactivity and a broadening of the stem end-to-end FRET distribution (Wilkinson et al., 2008; Liu et al., 2010), but this destabilization appeared nevertheless not sufficient to prevent the +3 nt pause event. In sharp contrast to NCp7, multimerization of Gag or Gag-related proteins dramatically compromises reverse transcription since the cooperative binding and the slow dissociation rate of the multimerized Gag proteins impaired RT processivity (Wu et al., 2010). Similarly, full-length two-exon Tat (86- or 101-amino acid) but not the truncated one-exon Tat (72 amino acid) was found to suppress the elongation of the $tRNA^{Lys,3}$/vRNA initiation complex (Kameoka et al., 2001, 2002). Interestingly, peptides resulting from the cleavage of Tat by the HIV-1 protease were able to enhance the synthesis of the (−)ssDNA in a natural endogenous reverse transcription assay (Apolloni et al., 2003). Thus, as envisioned for Gag, the protease cleavage of Tat is also needed for promoting primer elongation in vitro. Tat was therefore hypothesized as a reverse transcription accessory factor involved in the spatio-temporal control of the reverse transcription. Whether or not these findings are biologically relevant, they clearly underline the need for small basic proteins which can bind transiently and remain mobile on the nucleic acid lattice to allow primer extension.

Taken together, these observations suggest that if tRNA positioning can be promoted by different partners, primer extension requires a structurally well-defined initiation complex and a dynamic reverse transcription complex, which are likely promoted by the highly dynamic NCp7 (Cruceanu et al., 2006a). Initiation of reverse transcription could thus constitute a key regulatory step of which timing is finely regulated by the processing of the Gag precursor. This hypothesis is ascertained by observations showing that most of the annealed $tRNA^{Lys,3}$ in immature extracellular particles are only extended by a few nucleotides (Oude Essink et al., 1996; Huang et al., 1997).

3.2. Chaperoning minus strand DNA transfer

The first strand transfer constitutes a critical step in reverse transcription. This transfer occurs from region R at the 5′-end of the genome to a redundant R region at the 3′-end (Fig. 4B). In HIV-1, the R region consists of two strongly structured hairpins, namely the TAR and poly(A) hairpins (Baudin et al., 1993; Watts et al., 2009). TAR is especially critical for efficient strand transfer (Berkhout et al., 2001). Indeed, the antisense cTAR DNA (further referred as cTAR) of the (−)ssDNA has to anneal to the complementary TAR RNA sequence located at the 3′ end of the vRNA to allow DNA synthesis to resume on the acceptor strand (Negroni and Buc, 2000; Basu et al., 2008). cTAR and TAR are imperfect stem-loops defined by double-stranded segments separated by numerous conserved bulges, mismatches and an internal loop (Baudin et al., 1993). Reacting TAR and cTAR in vitro does spontaneously lead to the cTAR/TAR duplex, but at an extremely slow rate (You and McHenry, 1994; Godet et al., 2006; Vo et al., 2006, 2009). Through its chaperone activity, NCp7 plays a major role in promoting the annealing reaction (Tsuchihashi and Brown, 1994; You and McHenry, 1994; Rein et al., 1998; Guo et al., 2000) by enhancing the annealing rate by about 3000-fold at physiological temperature and salt conditions (Darlix et al., 1993; You and McHenry, 1994; Driscoll and Hughes, 2000; Urbaneja et al., 2002). The stability of TAR and cTAR local structures appeared of key importance to modulate the strand transfer (Wu et al., 2007). In full line with its ability to bind more transiently and dynamically to nucleic acids than Gag, the fully processed NCp7 promotes the first strand transfer more efficiently than Gag or Gag-derived proteins at low protein concentrations (Wu et al., 2010). Whereas the annealing reaction was facilitated as the NCp7 concentration increased, the strand transfer was drastically inhibited in the presence of increasing quantities of Gag or Gag-derived proteins. As Gag and partially processed Gag proteins containing the NC domain showed destabilizing and annealing activities almost as effective as NCp7, the inhibition of the strand transfer likely results from a strong restriction of the elongation step, suggesting that Gag reduces the ability of RT to traverse the template ('roadblock' mechanism). Once again, the ability of NCp7 to remain highly flexible and mobile within the nucleoproteic reverse transcription complex appears critical for reverse transcription to proceed (Levin et al., 2005).

Detailed mechanistic investigations of the cTAR/TAR annealing reaction showed that cTAR and TAR anneal in the absence of NCp7 through formation of an unstable loop–loop interaction that further converts into an extended duplex (Vo et al., 2006, 2009; Kanevsky et al., 2011). NCp7 switches the reaction pathway by directing the hybridization of these sequences through the end of their double-stranded stems, as a consequence of NCp7 ability to destabilize the structure of the cTAR stem (Godet et al., 2006; Vo et al., 2009). The destabilizing activity of NCp7 induces complex secondary structure fluctuations of the cTAR ends (Azoulay et al., 2003; Cosa et al., 2004, 2006), leading to the formation of the open reactive species required for the annealing process. The NCp7-induced mechanistic switch shows that the reaction pathway is selected on the basis of the available reactive intermediates and is governed by the intermediates which require the least bp melting prior annealing (Vo et al., 2009). These observations constitute a nice example of how nucleic acid chaperones may remodel annealing reactions to facilitate the formation of the most stable nucleic acid conformations.

The mechanism by which Tat and Tat-derived peptides activate the annealing of the complementary TAR sequences (in the form of DNA) was also thoroughly investigated (Kuciak et al., 2008; Boudier et al., 2010). Like NCp7 or Gag, Tat(1–86) promotes the hybridization of cTAR to TAR DNA. Tat peptides corresponding to the N-terminal acidic domain and to the Cys-rich domain are poorly active in annealing. On the contrary, peptides Tat(44–61) and Tat(48–86) promote TAR DNA/cTAR annealing, Tat(44–61) being the most active of the two. This suggests that the basic RNA binding domain of Tat is critical to promote the annealing reaction. Unlike NCp7, neither Tat nor Tat(44–61) are able to destabilize cTAR. Tat(44–61) is however able to modify the annealing mechanism of cTAR with TAR (in a DNA/DNA annealing context at least) since the analysis of cTAR and TAR DNA mutants clearly evidenced that the reaction is initiated at the bottom of the two reacting species (Boudier et al., 2010). In addition, Tat(1–86) and the mature NC were found to show comparable efficiency in promoting the annealing of cTAR with the DNA equivalent of TAR. Taken together,

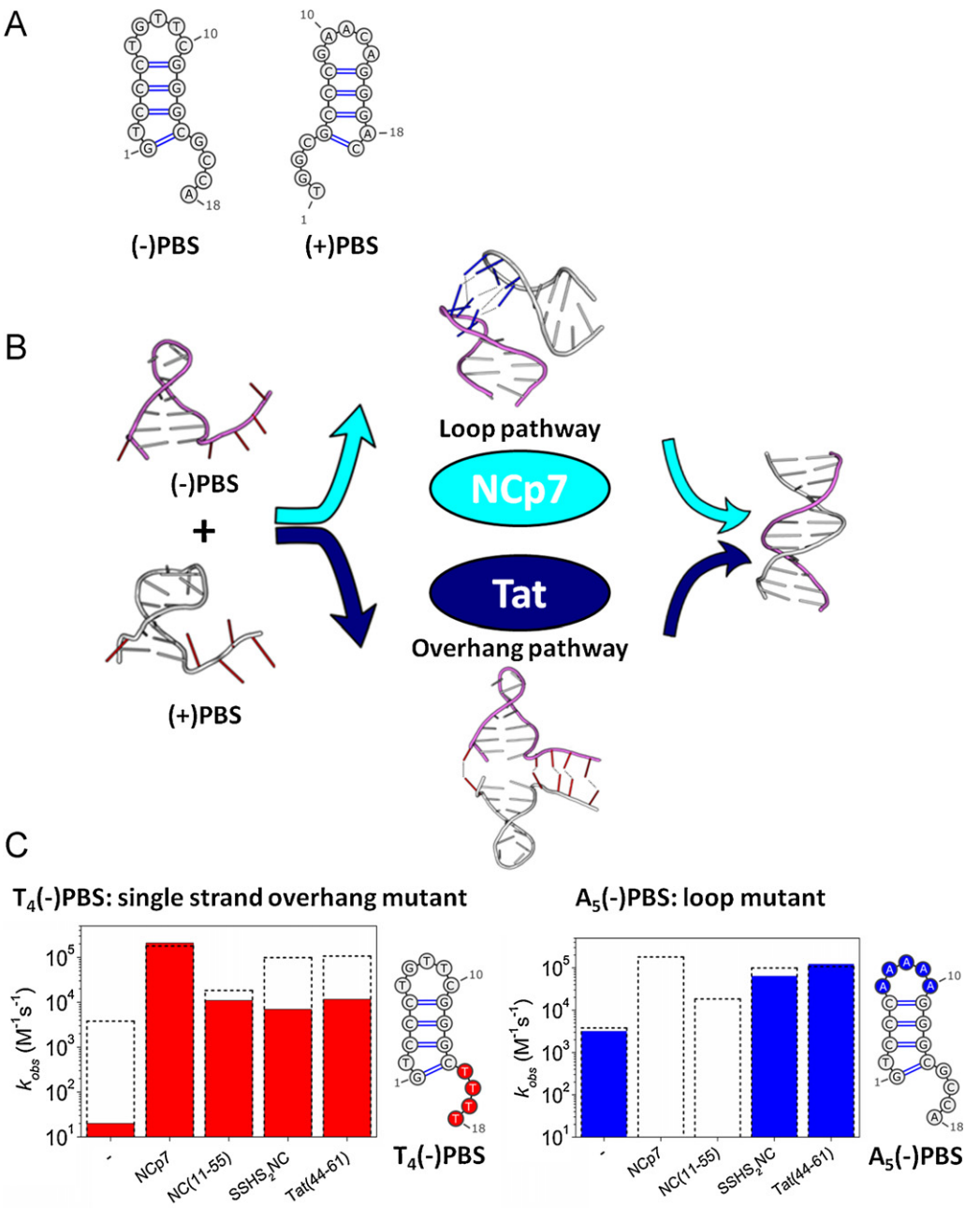

Fig. 5. Detailed analysis of the PBS(+)/PBS(−) annealing mechanism. A. (−) PBS and (+)PBS sequences. B. In the presence of NCp7, the PBS(+)/(−) annealing reaction proceeds mainly through the loop pathway (cyan). In the absence of NCp7, this pathway appears negligible and the PBS(+)/(−) sequences spontaneously anneal through their single-strand overhangs (blue). The single-stranded overhang pathway is also largely dominant when the reaction is promoted by Tat(44–61). C. PBS mutational analysis in the absence and in the presence of NCp7, NC mutants and Tat(44–61). Dotted bars represent the annealing rate in the presence of the native (−) and (+)PBS. NCp7 and NC(11–55) but not $SSHS_2NC$ or Tat(44–61) exhibit destabilizing activity. Mutating the (−)PBS single strand overhang strongly affects the rate of annealing, except if NCp7 or NC(11–55) promotes the reaction. On the contrary, in the presence of intact NC ZFs, the annealing reaction is prevented by mutating the (−)PBS loop while the annealing occurs very efficiently in the absence of NC or in the presence of $SSHS_2NC$ or Tat(44–61). These data clearly evidenced that the destabilizing activity carried by the NC ZFs is responsible for the switch in the annealing pathway.

data on Tat indicates that in the cTAR/TAR DNA reaction, the stimulatory effect of Tat on the first strand transfer resembles that of NCp7, though the hybridization reaction is differently nucleated. Furthermore, Tat and NCp7 were shown to cooperatively activate the annealing of cTAR to TAR DNA, supporting a potent accessory role of Tat in the stimulation of the reverse transcription.

3.3. Chaperoning the plus strand DNA transfer

A second strand transfer reaction is required for reverse transcription to resume. The plus strand transfer involves a sequence of synchronized events (Fig. 4C) consisting in (i) the synthesis of plus strand strong-stop DNA that terminates after copying the 3′ end of the tRNA sequence, (ii) the necessary removal of the tRNA primer and (iii) the subsequent annealing of the minus (−) and plus (+) DNA copies at the level of the 18 nt primer binding site (PBS) sequence (Basu et al., 2008). NCp7 chaperoning assistance is involved in many of the steps above described but we will focus here only on the (+)/(−)PBS annealing reaction. The DNA PBS sequence folds into a bulged stable 4-bp stem-loop hairpin with a partially ordered pentanucleotide loop and a 4-nt single- strand overhang (Johnson et al., 2000). Using fluorescently labelled (+)PBS

with various (−)PBS mutants, the properties of NCp7 and Tat on the (+)/(−)PBS hybridization were comparatively investigated. In vitro, (+)PBS can spontaneously anneal to (−)PBS (Ramalanjaona et al., 2007). The PBS(+)/(−) annealing reaction proceeds mainly through the single-strand overhangs of the PBS sequences while nucleation through loop–loop interaction appears negligible in the absence of peptides (Fig. 5A). This was ascribed from a PBS mutational analysis showing that hybridization rates of loop mutants were poorly affected while mutations that decreased the sequence complementarity in the ss overhangs almost completely impaired the reaction (Fig. 5B). In sharp contrast to the nucleic acid sequences involved in the first strand transfer, NCp7 does not melt the stable (−)PBS stem (Egelé et al., 2004, 2005). Nevertheless, NCp7 strongly promotes the annealing of (+)/(−)PBS stem-loops by increasing the annealing rate by about 60-fold, mainly by accelerating the loop pathway. As a consequence, NCp7 modifies the mechanism of the (+)/(−)PBS annealing reaction by activating the loop–loop kissing pathway that is negligible without NCp7 (Ramalanjaona et al., 2007) (Fig. 5A and B). The ability of NCp7 to switch the annealing reaction from the single strand overhang pathway to the loop–loop kissing pathway strongly correlates with the ability of NCp7 to rearrange the PBS loop and to restrict the dynamics of the PBS loop bases (Bourbigot et al., 2008; Godet et al., 2011). The latter were investigated using 2-Aminopurine, a structural fluorescent probe that minimally affects the folding of the PBS loop and its binding parameters with NCp7. In full line with NMR data (Bourbigot et al., 2008), comparison of the 2Ap fluorescence quantum yields in the absence and in the presence of NCp7 clearly evidenced that NCp7 significantly rearranged the PBS loop, notably by decreasing base stacking. Time-resolved fluorescence anisotropy also revealed that NCp7 restricts the picosecond to nanosecond dynamics of the PBS loops by limiting the overall flexibility of the loops and freezing the local mobility of the bases where NCp7 is bound. Local structural rearrangement and freezing of the local base dynamics of the loop are strictly dependent on the integrity of the zinc finger hydrophobic platform and constitute general features of the destabilizing activity of NCp7 (Avilov et al., 2008, 2009; Godet et al., 2011). Thus, the destabilizing activity of NCp7 is directly responsible for the switch to the loop–loop annealing pathway.

As for the first strand transfer, Tat(44–61) is able to strongly promote the annealing reaction of (+)/(−)PBS. In the presence of only 2 Tat(44–61) molecules per PBS, the annealing rate constant was found six times faster than the one observed for NC(11–55) under saturating concentrations (Fig. 5A) to reach a rate close to that observed in the presence of the full-length NCp7. But in sharp contrast to NCp7, Tat(44–61) was not able to stimulate the annealing pathway through the loop pathway (Fig. 5A and B), likely due to its very limited ability to rearrange the PBS loops (unpublished data). Tat(44–61) shows striking similarities with $SSHS_2NC$ or $SSHS_2NC$(11–55), two NC mutants where the cysteines have been substituted by serines to prevent zinc binding and which consequently do not exhibit destabilizing activity. Like Tat, these two zinc finger mutants do not modify the ODN dynamics and structure (Godet et al., 2011). Thus, Tat(44–61) stimulates the (−)/(+)PBS annealing through already available existing pathways in the absence of peptides (i.e. through the ss overhangs). Taken together, these data show that Tat is able to promote the annealing reaction of the complementary PBS sequences, albeit through a different mechanism from that observed in the presence of NCp7.

4. Conclusion

The activities of Tat-derived peptides and NCp7 in the different in vitro models reviewed here are macroscopically very similar. Both Tat peptides and NCp7 promote the annealing of different complementary sequences, as well as the placement of primer tRNA on the viral RNA. Nonetheless, comparison of the mechanism of action of Tat and NCp7 evidences striking differences in their ability to chaperone nucleic acid rearrangements. The most important differences result from the inability of Tat, unlike NC, to destabilize transiently nucleic acids and modify the local dynamics of the nucleobases. So, even if both proteins are able to aggregate nucleic acids and promote their annealing, the destabilizing activity of NCp7 mediated through its folded zinc fingers is responsible for specific nucleic acid rearrangements and annealing pathways. Taken together, these data evidence that chaperoning mechanisms are multiple and if the RNA-chaperone concept is relatively straightforward, its expression appears quite diverse. A direct consequence is that no simple or consensual assay exists for evidencing and characterizing the RNA chaperone activity. Moreover, characterization of the HIV-1 chaperone activities in vivo appears highly challenging due to the fact that the HIV-1 genome encodes for at least three proteins (NCp7, Tat and Vif) exhibiting redundant NA chaperone activities, highlighting the critical necessity of chaperones for viral replication. Due to the differential abundance of these proteins along the viral life cycle, it is likely that these proteins exhibit their NA chaperoning activities at different steps of viral replication. Therefore, spatio-temporal regulation (Henriet et al., 2007) and possible cooperativity (Boudier et al., 2010) within these different “chaperone solutions” selected to solve the “folding problem” represent very exiting fields to explore.

Conflict of interest

The authors declare no conflict of interest

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