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The multimerization state of retroviral RNA is modulated by ammonium ions and affects HIV-1 full-length cDNA synthesis *in vitro*

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ABSTRACT

Genomic human immunodeficiency virus type 1 (HIV-1) RNA fragments containing the dimer linkage structure (DLS) can be dimerized and multimerized in the presence of NH_4^+ and in the absence of any other cation and any viral or cellular protein. This effect strongly supports the notion that dimerization and multimerization of genomic RNA occurs via purine-quartet formation in quadruple helical RNA structures. The efficiency of RNA dimerization and multimerization in the presence of ammonium ions is about 400 fold increased as compared to alkali metal ions such as potassium. Dimerized retroviral RNA representing a pseudodiploid genome could account for genetic recombination within the virion and during reverse transcription. Application of a novel South-Northern-Blotting procedure with biotinylated RNA and digoxigenin-labelled cDNA *in vitro* reveals that efficient human- and bovine $tRNA^{Lys3}$ primed full-length cDNA-synthesis only takes place with a predominantly monomerized RNA template. Dimerization and multimerization of the RNA significantly reduces full-length cDNA-synthesis. This suggests that monomerization of the dimerized RNA, effected by deionization *in vitro*, is essential for efficient retroviral reverse transcription *in vivo*.

INTRODUCTION

Dimerization of retroviral RNA is thought to play a crucial role in several steps in the life cycle of retroviruses. Rous sarcoma virus (RSV) RNA monomer triggers the synthesis of the polyprotein precursor, whereas dimerized RSV RNA fails to allow this process (1). RNA dimerization also seems to be closely related to the encapsidation of the retroviral RNA since *cis*-elements such as the dimer linkage structure (DLS) and the encapsidation site (E)/packaging signal (psi) are located within the same region in the genomes of RSV (1), moloney murine leukemia virus (MMuLV) (2) and HIV-1 (3). It has been suggested that dimerization of retroviral RNA may function as a molecular switch, downregulating translation and positively regulating the encapsidation of non-spliced genomic RNAs.

After budding from the host cell membrane, the progeny virions contain 70 S genomic RNA molecules that most likely consist of dimerized RNA monomers (1, 2, 4).

Several attempts have been made to investigate factors responsible for the dimerization event. It was recently demonstrated that in the absence of any cellular or viral protein, cations can stimulate dimerization of HIV-1 RNA (5). Other reports described a crucial role of a *trans*-acting factor, the HIV-1 nucleocapsid (NC) protein p15 (3, 6) and its aminoterminal cleavage product, NCp7 (7, 8) in the formation of RNA dimers.

The dimerization of the viral RNA does not involve conventional Watson–Crick base-pairing, since viral antisense RNA molecules cannot form dimers and viral RNA dimers are stable in the presence of denaturing agents (5). Most likely the process involves formation of purine tetrads which could be formed by alkali metal ions *in vitro* (5). Recent data suggest that potassium ions strongly stabilize these quartets, leading to quadruple helical structures (9). Here we demonstrate that NH_4^+ mediate RNA dimerization and multimerization *in vitro* much more efficiently than alkali metal ions such as potassium. Vice versa we show that deionization leads to monomerization of the dimerized RNA.

It was recently suggested that the HIV-1 DLS is located between nt positions 315 and 415 (3), whereas we demonstrated that the sequence between 315 and 404 is sufficient for NCp15 mediated dimerization of HIV-1 genomic RNA (6). Moreover, it was demonstrated that deletion of a PuGGAPuA consensus sequence within the HIV-1 DLS, commonly found in many retrovirus genomes (5) abolishes dimer formation completely (9). In contrast, it was demonstrated that dimerization of HIV-2 leader RNA takes place in the absence of the PuGGAPuA motif (10). In addition, it was suggested that in the case of reticuloendotheliosis virus (REV) type A both the 5′ and 3′ halves of the encapsidation site (E) are necessary for RNA dimerization and that the extend of RNA dimerization is influenced by sequences flanking E (11). A close relationship between the HIV-1 DLS and the packaging signal (psi) was suggested previously by others who identified a stem-loop structure of 46

bases around the gag (group-specific antigen) initiation codon (12). Other reports identified HIV-1 genome regions between the 5′ LTR and the gag-initiation codon that were essential for the incorporation of the genomic RNA into virions (13–16).

In addition to the function of the multimerization state of the retroviral RNA as a molecular switch during the assembly and budding events of retroviruses, a further role of dimerized RNA in genetic recombination during the reverse transcription reaction was discussed (17, 18). Although recent contributions suggested that this reaction at least in part could already happen before or during assembly and budding (19, 20), it is still thought that the entire reaction takes place in the cytoplasm of the infected cell (21, 22).

In this communication we have analyzed the influence of the dimerization and multimerization state of a genomic HIV-1 RNA fragment on retroviral cDNA synthesis *in vitro*. This reverse transcriptase (RT) catalyzed process was shown to be initiated by the h (human)-$tRNA^{Lys3}$ and b (bovine)-$tRNA^{Lys3}$ primer molecules *in vitro* (23–25). While it was demonstrated that reverse transcription takes place in the absence of any additional viral or cellular protein (25), it was also shown that NCp15, which promotes the dimerization of retroviral RNA (prior or during the budding event), can enhance retroviral cDNA synthesis (most likely occuring in the newly infected cell; 6, 23). In the latter case, NCp15 most likely supports annealing of the $tRNA^{Lys3}$ primer to the *PBS* (primer-binding site) of retroviral RNA, which was also demonstrated for the aminoterminal cleavage product NCp7 (7, 8). In addition, nucleocapsid proteins can also interact very tightly with the genomic RNA dimer in the capsid (26) and probably with RT (6).

In order to analyze the role of the RNA multimerization state in the process of HIV-1 retroviral cDNA synthesis we employed a novel South-Northern blotting procedure involving the use of a biotinylated RNA and a digoxigenin-labelled minus strand cDNA.

MATERIALS AND METHODS

RNA preparations

h-$tRNA^{Lys3}$ was prepared by *in vitro* transcription from transcription plasmid pBMT7Lys as described (6). Bovine-$tRNA^{Lys3}$ was isolated from calf liver (25). Biotinylated HIV-1 LTR RNA containing three PuGGAPuA consensus sequences, a complete LTR, the DLS (311–404) and the *PBS* was synthesized in an *in vitro* transcription reaction comprised of the following: 50 μg *Asn*I restricted pT3T7LTR DNA (25), 2500 u T7 RNA polymerase (Boehringer Mannheim), 2 mM each of ATP, GTP and CTP, 1.93 mM of UTP, 0.07 mM of Biotin-UTP (Boehringer Mannheim), in the presence of 40 mM Tris.HCl (pH 7.2 at 37°C), 6 mM $MgCl_2$, 5 mM DTT and 4 mM spermidine. After 2 h at 37°C, the DNA was removed by adding 2000 u DNase I for additional 45 min at 37°C. The RNA was phenolized twice and ethanol precipitated in the presence of 2 M NH_4-acetate. The RNA was analyzed for concentration dependency experiments shown in Fig. 1, B and subjected to cDNA synthesis experiments shown in Fig. 3 B. Subsequently, deionization was done by a sephadex G 50 (Pharmacia) spin column chromatography. Deionized RNA preparation were subjected to RNA concentration dependency experiments shown in Fig. 1 A, to salt concentration dependency experiments outlined in Fig. 2 and to cDNA synthesis experiments shown in Fig. 3 A.

Dimerization of the HIV-1 LTR fragment

2 μg of deionized biotinylated HIV-1 LTR fragment were incubated in the presence of different NH_4-acetate and KCl concentrations at 37°C for 30 min. The RNA was subsequently analyzed by Northern blotting.

Retroviral reverse transcription

The reaction was performed in 10 μl mixtures comprising 50 mM Tris.HCl (pH 7.9 at 37°C), 50 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 1mM each of dATP, dGTP and dCTP, with 0.65 mM dTTP and 0.35 mM digoxigenin-dUTP (Boehringer Mannheim) and 2.8 pMol biotinylated HIV-1 LTR RNA (not heat-denatured prior to use) as template. The reaction was done in the absence and presence of 2.8 pmol of a 18-mer oligo complementary to the *PBS*, or 28 pMoles each of h-$tRNA^{Lys3}$ or b-$tRNA^{Lys3}$ as primers and in the absence and presence of 1 μg HIV-1 RT (27). After a 30 min preincubation period (annealing) at 37°C, dNTPs and HIV-1 RT were added for additional 60 min. The reaction mixture was subsequently deproteinized by a phenol/chloroform extraction.

South-Northern blotting/Northern blotting

Samples from the cDNA-synthesis reaction containing RNA and cDNA or RNA samples only were added to 3 Vol of sample buffer containing 7.7% formaldehyde, 40 mM Mops (pH 7.0 at 25°C), 10 mM sodium acetate, 1 mM EDTA, 62.5% deionized formamide in the absence of any dye. After 10 min at 65°C and chilling shortly on ice, the samples were loaded on a 1.0% denaturing agarose gel containing 1% formaldehyde, 40 mM Mops (pH 7.0 at 25°C), 10 mM sodium acetate and 1 mM EDTA. Following electrophoresis (6 V/cm) the gel was rinsed twice for 15 min in 10×SSC (standard sodium citrate) containing 1.5 M sodium chloride and 0.15 M sodium citrate at pH 7.0 (at 25°C). Vacuum blotting of the gel was performed in a PosiBlot™ pressure Blotter (Stratagene) at 75 mm Hg for 60 min. Gels, nylon membranes (Boehringer Mannheim) and 3 MM whatman papers were soaked in destilled water for 5 min and then in transfer buffer containing 1.5 M sodium chloride and 0.15 M sodium citrate for additional 5 min prior to blotting. The transferred RNA was immobilized by crosslinking in a Stratalinker (Stratagene) with 1200 μJ.

Multicolor rainbow detection

The detection method was modified from a described protocol (28).

Detection of RNA and DNA from the cDNA Synthesis reaction (South-Northern blotting). 1. Detection (cDNA). The nylon membrane was rinsed briefly in maleic acid solution (0.1 M maleic acid, 0.15 M NaCl (pH 7.5 at 25°C)). After incubating the membrane 30 min in blocking solution (1% (w/v) blocking reagent (Boehringer Mannheim) in maleic acid solution), anti-digoxigenin-AP conjugate was added to a final concentration of 150 mU/ml and the filter incubated for additional 30 min. Unbound conjugate was removed by washing the filter twice for 15 min each with maleic acid solution. The membrane was equilibrated shortly in AP buffer (0.1 M Tris-HCl (pH 9.5 at 25°C), 0.1 M NaCl, 0.05 M $MgCl_2$) before it was incubated in color substrate solution *Blue* (1 tablet containing 2 mg naphtol-AS-phosphate and 3.5 mg Fast Blue BN (Boehringer Mannheim) was dissolved in 10 ml of AP buffer) until blue bands were clearly

detected. The alkaline phosphatase color reaction was stopped by rinsing the membrane shortly in water. The alkaline phosphatase was subsequently inactivated by incubating the membrane for at least 10 min at 85°C in 50 mM EDTA (pH 8.0 at 25°C).

2. Detection (RNA). The membrane was rinsed shortly in maleic acid solution and incubated for 30 min in blocking solution as described above with the exception that 5% (w/v) of blocking reagent were used. After adding streptavidine-AP conjugate (Boehringer Mannheim) to a final concentration of 150 mU/ml for 30 additional min, the membrane was washed twice with maleic acid solution. The membrane was again equilibrated briefly in AP buffer, before it was incubated in color substrate solution *Red* (1 tablet containing 2 mg naphtol-AS-phosphate and 1 mg Fast Red TR was dissolved in 10 ml AP buffer). The reaction was allowed to preceed until red bands became clearly visible. The reaction was stopped with ddH_2O.

Detection of RNA only (Northern-blotting). RNAs from the *in vitro* transcrption reactions and the salt dependency were detected as described above (*2. Detection (RNA)*) with the exception of the following alterations: To detect the digoxigenin labelled RNA marker and the biotinylated RNA samples simultaneously streptavidine-AP conjugate and anti-digoxigenin-AP conjugate were added at the same time to final concentrations of 150 mU/ml each; in addition, color substrate solution *Blue* was used.

RESULTS

Multimerized HIV-1 RNA can be converted to a predominantly monomerized form by deionization

Biotin labelled HIV-1 LTR RNA containing the DLS (positions 311 to 404, containing three PuGGAPuA consensus sequences) was synthesized from pT3T7LTR DNA (25) by *in vitro* transcription. RNA ethanol precipitated in the presence of 2 M NH_4-acetate was analyzed by Northern-Blotting. Amounts of RNA between 25 and 200 ng migrate on a 1% denaturing agarose gel as a monomer (Fig.1 B, lanes 1−4). At amounts of 500 and 1000 ng, a dimerized form of the RNA becomes visible (Fig.1 B, lanes 5 and 6). When 3000 and 5000 ng of RNA were loaded (Fig. 1 B, lanes 7 and 8), the RNA appears in addition to the monomerized and dimerized forms in multimer conditions which are trimers and tetramers. After deionization of the RNA by a sephadex G 50 spin column chromatography, the RNA appears in a predominantly monomerized form (Fig. 1 A) even at higher amounts (5000 ng; Fig.1 A, lane 6). These results demonstrate the *in vitro* conversion of a multimerized HIV-1 LTR RNA into a predominantly monomerized form by deionization.

NH_4^+ mediates the conversion of a monomerized HIV-1 LTR RNA into the multimerization state much more efficiently than K^+

In order to investigate the role of NH_4^+ in the multimerization of the retroviral RNA we analyzed 2 μg of RNA in the absence and presence of different NH_4 acetate and KCl concentrations (Fig.2 A/B). In the absence of any salt predominantly monomerized RNA is evident (Fig.2 A/B, lanes 1). At 0.5 mM NH_4-acetate some dimerized RNA appears (Fig. 2 A, lane 3). In the presence of 5 mM NH_4 acetate the RNA is visible in a monomerized and dimerized form at equal amounts (Fig. 2 A, lane 5). At NH_4 acetate concentrations of 100 mM and 1000 mM additionally multimerized forms (trimers and tetramers) of the RNA are present (Fig. 2 A, lanes 7 and 8). In addition, RNA from an *in vitro* transcription reaction which was precipitated by 2 M NH_4 acetate was analyzed. As demonstrated in Fig.2 A, lane 9, this RNA appears in a monomerized and dimerized form, which corresponds to the RNA preincubated with 5 mM NH_4 acetate. In comparison, we investigated RNA dimerization and multimerization in the presence of different KCl concentrations. As demonstrated in Fig. 2 B, an increase of the dimer form of the RNA could first be achieved at KCl concentrations of 500 mM (Fig.2 B, lane 5) and 1000 mM (Fig.2 B, lane 6). Efficient dimerization and multimerization takes place at KCl concentrations of 2000 mM (Fig.2 B, lane 7) and 3000 mM (Fig.2 B, lane 8). To compare the multimerization efficiency of K^+ and NH_4^+ directly, we added 5 mM NH_4 acetate to this RNA preparation (Fig.2 B, lane 9). The multimerization state of this RNA corresponds to that of the RNA in the presence of 2000 mM KCl (Fig. 2B, lane 7). These data demonstrate that the multimerization efficiency of NH_4^+ is about 400 fold increased in comparison to K^+.

Influence of the multimerization state of HIV-1 LTR RNA on full-length cDNA synthesis *in vitro*

We wanted to analyze, if the multimerization state of the viral RNA plays a role in the reverse transcription reaction of HIV-1 *in vitro*. For that purpose, we used predominantly monomerized and partially dimerized and multimerized HIV-1 LTR RNA containing the *PBS* as templates in an *in vitro* reverse transcription reaction. In the absence of reverse transcriptase (RT) and any primer the predominantly monomerized (Fig. 3 A, lane 1) and multimerized RNA (Fig. 3 B, lane 1) is visible analyzed by a novel south-northern blotting procedure where the RNA is marked red and the cDNA marked blue. As expected, no cDNA was synthesized in the absence of any primer and RT with monomerized (Fig.3 A, lane 1) and multimerized (Fig.3 B, lane 1) RNA. After addition of RT to the reaction truncated cDNA products marked blue are evident over a wide size range with the monomerized RNA template (Fig.3 A, lane 2). In the case of multimerized RNA the truncated cDNA products are significantly weaker (Fig.3 B, lane 2). In the presence of an 18-mer oligo complementary to the *PBS*, the b-$tRNA^{Lys3}$ and h-$tRNA^{Lys3}$ primer molecules, the full-length cDNA product is only visible with a predominantly monomerized RNA template (Fig.3 A, lanes 3, 4 and 5). Using the multimerized RNA template, no full-length cDNA product is visible in all three cases (Fig. 3 B, lanes 3, 4, and 5). In addition, the truncated cDNA products are also much more weaker, and a red colored smear, representing the degraded RNA, is visible, which is missing in the case of monomerized RNA. Moreover, a distinct band representing cDNA corresponding to full-length RNA is visible in the absence of any primer (Fig. 3 A, lane 2) and—dependent on the intensities of the full-length cDNA product—also in the presence of the oligo (Fig. 3A, lane 3), b-$tRNA^{Lys3}$ (Fig. 3 A, lane 4) and h-$tRNA^{Lys3}$ (Fig. 3 A, lane 5) primer molecules using the monomerized RNA template. These data indicate that HIV-1 RT can initiate cDNA synthesis primer independent leading to a minus strand cDNA product of the exact size of the monomerized RNA template. If the cognate primer is present, cDNA synthesis starts at the correct site at the *PBS* where the primer is annealt to. A dimerized/multimerized RNA template does not allow either primer independent cDNA synthesis of the complete length of the template (lack of the 1080 bp cDNA band;

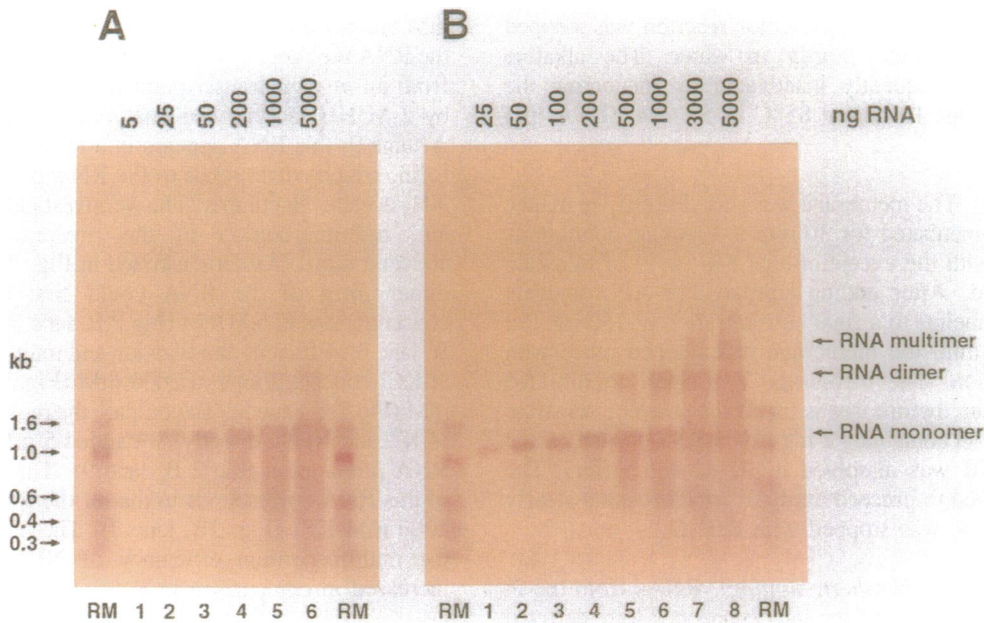

Figure 1. Concentration dependency of monomerized and dimerized viral RNA. **(A)** 5 ng (lane 1), 25 ng (lane 2), 50 ng (lane 3), 200 ng (lane 4), 1000 ng (lane 5) and 5000 ng (lane 6) of Bio-UTP labelled HIV-1 LTR RNA deionized by a sephadex G 50 spin column chromatography and **(B)** 25 ng (lane 1), 50 ng (lane 2), 100 ng (lane 3), 200 ng (lane 4), 500 ng (lane 5), 1000 ng (lane 6), 3000 ng (lane 7) and 5000 ng (lane 8) of Bio-UTP labelled HIV-1 LTR RNA ethanol precipitated in the presence of 2 M NH_4 acetate were analyzed in a constant volume of 25.5 μl each by Northern blotting and detected with color substrate solution blue. 200 ng each of Dig (digoxigenin)-UTP labelled RNA size standard III (Boehringer Mannheim) were loaded (lanes RM).

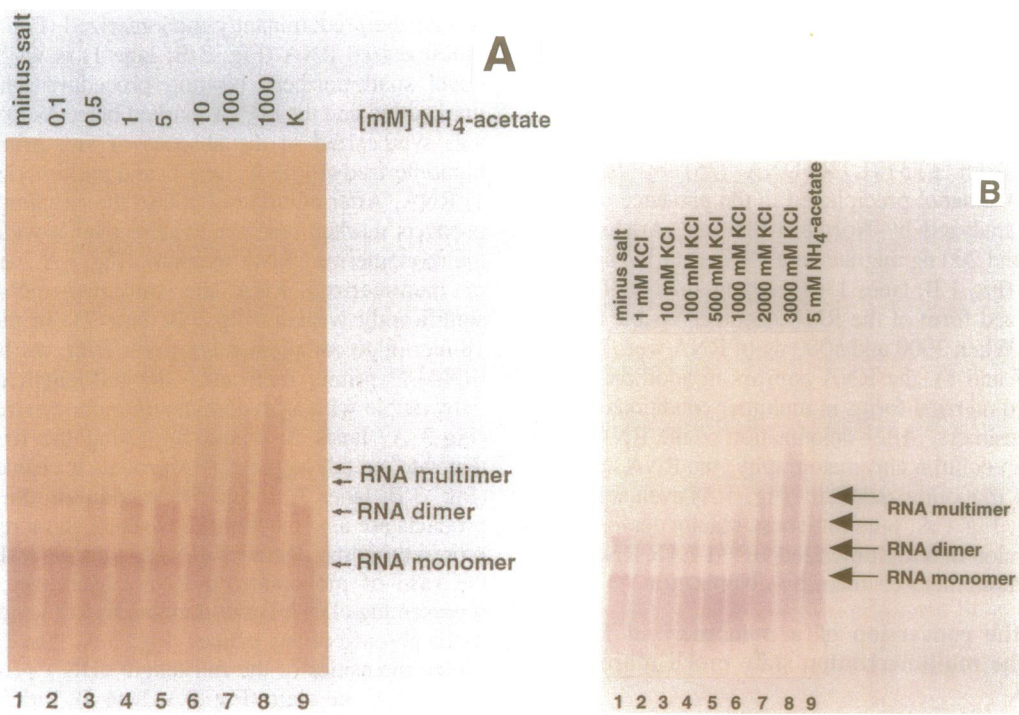

Figure 2. Influence of the NH_4 acetate and KCl concentrations on HIV-1 LTR RNA multimerization. **(A)** 2 μg each of Bio-UTP labelled HIV-1 LTR RNA deionized by sephadex G 50 spin column chromatography in the absence of salt (lane 1) and in the presence of 0.1 mM (lane 2), 0.5 mM (lane 3), 1 mM (lane 4), 5 mM (lane 5), 10 mM (lane 6), 100 mM (lane 7) and 1000 mM (lane 8) NH_4 acetate were analyzed in a constant volume of 25.5 μl each by Northern blotting and detected with color substrate solution blue. As a reference (K) 2 μg of Bio-UTP labelled HIV-1 LTR RNA precipitated in the presence of 2 M NH_4 acetate were analyzed (lane 9). **(B)** 2 μg each of Bio-UTP labelled HIV-1 LTR RNA purified also by sephadex G 50 spin column chromatography in the absence of salt (lane 1) and in the presence of 1 mM (lane 2), 10 mM (lane 3), 100 mM (lane 4), 500 mM (lane 5), 1000 mM (lane 6), 2000 mM (lane 7), 3000 mM (lane 8) KCl and 5 mM NH_4 acetate (lane 9) were analyzed and detected as described under (A).

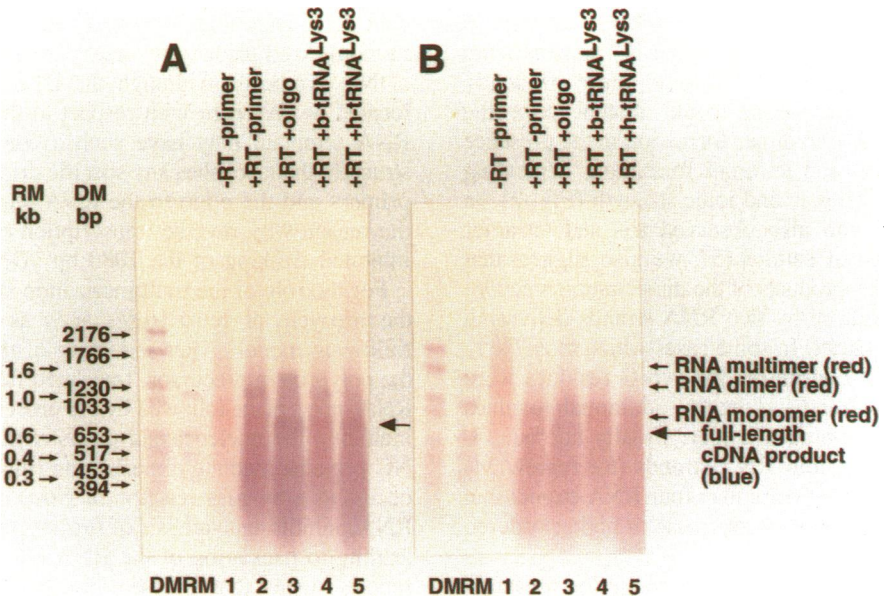

Figure 3. Influence of monomeric and multimeric HIV-1 LTR RNA on HIV-1 cDNA synthesis. 2.8 pMol of monomerized HIV-1 LTR RNA deionized by a sephadex G 50 spin column **(A)** or 2.8 pMol of multimerized HIV-1 LTR RNA ethanol precipitated in the presence of 2 M NH_4 acetate **(B)** were subjected to a reverse transcription reaction and analyzed by South-/Northern blotting. Reactions were performed in the absence of primer and RT (27; lanes 1), in the presence of 1 μg (7.7 pMol) RT (lanes 2) and in the presence of 7.7 pMol RT and the following primers: 2.8 pMol 18-mer oligo complementary to the *PBS* (lanes 3), 28 pMol of b-$tRNA^{Lys3}$ (lanes 4) and 28 pMol of h-$tRNA^{Lys3}$ (lanes 5). 200 ng each of Dig-dUTP labelled DNA molecular size marker VI (Boehringer Mannheim) (lanes DM, DNA marker) and 200 ng each of Dig-UTP labelled RNA size marker III (Boehringer Mannheim) (lanes RM, RNA marker) were loaded.

Fig. 3 B, lanes 2−5) nor the synthesis of *PBS* primed full-length cDNA synthesis (lack of the 700 bp cDNA band; Fig. 3 B, lanes 2−5). Only truncated cDNA products (blue) are synthesized which are superimposed by red colored degraded RNA (red). These data demonstrate that RNA monomerization is a prerequisite for full-length cDNA synthesis primed by h-$tRNA^{Lys3}$, b-$tRNA^{Lys3}$ or the 18-mer oligo *in vitro*.

DISCUSSION

In virions of retroviruses like HIV-1 two RNA molecules are linked together in sense orientation at the DLS located at the 5′ ends of both molecules. This dimerization event does not occur via Watson−Crick basepairing but most likely through the formation of purine quartets. Elimination of the purine rich consensus sequence PuGGAPuA, common in many retroviruses (5), leads to the loss of dimerization of HIV-1 RNA *in vitro* (9), whereas other sequences within or flanking the DLS seem to be important for the dimerization event in the case of HIV-2 (10) and REV (11). Dimer formation can be demonstrated in the absence of any viral or cellular proteins in the presence of monovalent cations. Dimer formation was achieved in the presence of Li^+, Na^+, K^+ and Cs^+ (5); K^+ (in the presence of 5 mM Mg^{2+}) can specifically stabilize the dimer (9). These data account for the formation of G-quartets which can be stacked upon each other to form quadruple helical structures (9) also suggested for telomeric DNA sequences found in species such as *Tetrahymina* (29, 30; for review 31). Guanine rich sequences able to form G-quartets were also found in immunoglobulin switch regions, in gene promoters and in chromosomal telomers which are thought to bring the four homologous chromatids together during meiosis and prevent the DNA from degradation (32). G-tetrads are held together by Hoogsteen base pairing through hydrogen bonds between nitrogens or oxygens and hydrogens (32). Two G-quartets stacked upon each other form an eight-coordinate chelation cage with an alkali-cation located within the axial channel, complexing four oxygens of the upper and four oxygens of the lower G-quartet. Here we demonstrate that RNA dimer and multimer formation also occurs in the presence of NH_4 ions in the absence of any viral or cellular protein and any other mono- or bivalent cation. We show that at 5 mM NH_4 acetate (in the absence of any other cations) the RNA which contains three PuGGAPuA motifs reveals the same multimerization state compared to KCl concentrations of 2000 mM which demonstrates that the multimerization efficiency of NH_4^+ is about 400 fold increased compared to K^+. These data confirm the existence of very stable purine quartet structure which can be achieved with ammonium ions (33). Two protons of one NH_4 ion can be hydrogen-bonded to two oxygens in the upper plane and two to the oxygens in the lower plane of the cage which is built up of two purine-quartets stacked upon each other. This situation was proven for the homopurine polymer poly (I) (33). Consequently, each NH_4^+ could interact specifically with only four carbonyl oxygens, whereas alkali ions such as potassium could interact with eight oxygens. In the case of NH_4^+, each tetrameric array is rotated by the helix twist of 31° with respect to the next (33). Alkali ions such as K^+ (1.33 Å), sharing a comparable ionic radius with NH_4^+ (1.44 Å) but unable to form hydrogen bonds, may not support helix formation to such an extend (33). In conclusion, NH_4^+-mediated retroviral RNA dimerization is very efficient *in vitro*. Recently, it was described that 64% association of the RNA could be achieved in the presence of 1000 mM KCl (5). These data are in harmony with our findings that 2000 mM KCl are necessary for about 50%

dimerization of the RNA. These values seem to be to high to be physiologically relevant for the intracellular K^+ concentration is about 150 mM. The fact that NH_4^+ is much more efficiently with respect to RNA dimerization could match more the intracellular pool. In addition to dimer formation in the presence of NH_4^+ we observed tri- and tetramer formation, depending upon RNA concentration (Fig.1) and ionic strength (Fig. 2). In agreement with others, who also observed tri- and tetramer formation in the presence of cations (5), we also suggest that these structures could be by-products of the dimerization reaction. One G-quartet will be built up by two RNA strands delivering two guanins each to the quartet. In some cases which most likely depends on RNA and salt concentrations two G residues were delivered from one RNA strand and the two additional guanines from two other strands forming a trimer. Finally all four G-residues can be served from four RNA strands (for review 34) forming a tetrameric structure. More than four RNA strands can not be formed, explaining why no pentameric or higher ordered structures were detected (Fig.1 and 2).

The multimerized RNA forms are also stable in the presence of denaturants such as formaldehyde and formamide (Fig.1, 2 and 3) supporting that parallel dimer formation occurs via purine-tetrade formation involving Hoogsteen base-pairing leading to quadruple helical structures. The assumption that interstrand purine-tetramers mediate RNA dimerization also *in vivo* could reveal the first biological function for a quadruple helical structure.

HIV-1 cannot form infectious virions if the zinc finger of the nucleocapsid protein p15 or its aminoterminal cleavage product p7 is disrupted. The fact that these non-infectious virions contain no or only minor amounts of genomic RNA (13−16) which is thought to be not dimerized, in addition with the observation that NC p15 (3, 6) and NCp7 (7, 8) could mediate RNA dimerization *in vitro* suggests that NCp15/p7 are essentiell for RNA dimer formation *in vivo*.

Dimerized viral RNA is thought to play a further role in the process of retroviral reverse transcription where genetic recombination takes place during the intermolecular template switch between two RNA molecules (17, 18). Recent reports demonstrate that HIV-1 RT (including the RNase H activity) is sufficient for this process and no other viral or cellular proteins are involved (35, 36). To investigate the influence of the multimerization state of retroviral RNA on retroviral cDNA synthesis we used dimerized and monomerized RNA in the absence and presence of the cognate h-$tRNA^{Lys3}$ and b-$tRNA^{Lys3}$ primer molecules and detected the cDNA product by a novel South/Northern Blotting procedure. Full-length cDNA synthesis with both $tRNA^{Lys3}$ primer molecules as well as with a 18-mer oligo complementary to the *PBS* of the RNA was achieved only with monomerized RNA. The cDNA products synthesized in the presence of the three different primer molecules share the same length because the 5′ ends of both tRNA primer molecules are removed by the RNase H activity of HIV-1 RT during or after initiation of reverse transcription as demonstrated previously (25).

RNA which was dimerized to 50% exclusively yields truncated cDNA products. In addition, cDNA complementary to full-length RNA representing primer independent reverse transcription could also be detected only with monomerized RNA. Degradation of the RNA by the RNAse H activity of HIV-1 RT during reverse transcription was also incomplete in the presence of partially dimerized template RNA. Monomerized RNA, however, was completely degraded during retroviral cDNA synthesis. These data demonstate that previously dimerized RNA must become monomerized prior to reverse transcription to allow full-length cDNA synthesis. Although the DLS of the RNA template is located downstream with respect to the *PBS*, the multimerized RNA template may have such a complex tertiary/quartanary structure that abolishes any specific priming of the cognate tRNA primers and the oligo to the RNA template. This may also be the reason why reverse transcription of the full-length RNA is inhibited (lacking of the 1080 bp cDNA, Fig. 3 B).

For the role of the multimerization state of retroviral RNA in the lifecycle of retroviruses such as HIV-1 we propose the following model. After induction of the provirus, synthesis of the polyprotein precursor, translated from unspliced monomeric RNA, takes place followed by the processing into mature proteins including the nucleocapsid proteins p15/p7. Subsequently, the NC proteins mediate dimerization (multimerization most likely occurs as a by-pass reaction *in vitro*) of the genomic unspliced RNA, inhibiting synthesis of further polyprotein precursors and leading to packaging of the RNA into virions. Although some reports suggest that reverse transcription of the RNA can already take place before or during the assembly process (19, 20), it is most likely that this reaction occurs first in the cytoplasm of newly infected cells. For full-length cDNA synthesis the two dimerized RNA molecules must dissociate. At which stage this process takes place and which conditions are necessary *in vivo* remains speculative. One possibility would be local deionization either in the virion or in the cytoplasm of the infected cell which could probably be regulated by ionic channels leading to monomerization of the RNA as could be demonstrated *in vitro* (Fig.1). It is also conceivable that an unknown viral or cellular factor may facilitate the monomerization event. Dimerization of the viral RNA guarantees that two RNA molecules necessary for the reverse transcription reaction are transported to the newly infected cell. Moreover, different genetic information located on both RNA strands (pseudodiploidy), ensure genetic recombination within the virion (17) and following the HIV-1 RT-mediated intermolecular template switch during retroviral cDNA synthesis (35−37).

Blocking of either the nucleocapsid proteins p15/p7 which mediates RNA dimerization *in vivo* by specific therapeutics disrupting the zinc finger structure or inhibition of the purine quartet formation which leads to quadruple helical structures by specific RNA dimerization inhibitors would interrupt the life cycle of HIV-1 and reveal new targets for the intervention against AIDS.

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ABBREVIATIONS

AIDS, acquired immune deficiency syndrome; AP, alkaline phosphatase; b (prefix), bovine; bp, base pair(s); DIG, digoxigenin; DM, DNA Marker; dNTP, deoxyribonucleoside triphosphate(s); DLS, dimer linkage structure; DTT, dithiothreitol; E, encapsidation site; Fast Blue BN, tetrazotized 3,3′-dimethoxybenzidine; Fast Red TR, 4-chloro-2-methyl-

benzenediazonium; Gag, group-specific antigen; h (prefix), human; HIV, human immunodeficiency virus; I, inosine; kb, kilobase(s) or 1000 bp; LTR, long terminal repeat; MA, matrix protein; MMuLV, Moloney murine leukemia virus; naphtol-AS, 3-hydroxy-2-naphtoic acid anilide; NC, nucleocapsid protein; nt, nucleotide(s); oligo, oligodeoxyribonucleotide; *PBS*, primer-binding site(s); pol, polymerase; psi, packaging signal; Pu, purine(s); REV, reticuloendotheliosis virus; RSV, Rous sarcoma virus; RM, RNA Marker; RT, reverse transcriptase; SSC, standard sodium citrate.

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