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Identification of a High Affinity Nucleocapsid Protein Binding Element from The Bovine Leukemia Virus Genome

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Abstract

Retroviral genome recognition is mediated by interactions between the nucleocapsid (NC) domain of the virally encoded Gag polypeptide and cognate RNA packaging elements that, for most retroviruses, appear to reside primarily within the 5'-untranslated region (5'-UTR) of the genome. Recent studies suggest that a major packaging determinant of Bovine Leukemia Virus (BLV), a member of the human T-cell leukemia virus (HTLV)/BLV family and a non-primate animal model for HTLV-induced leukemogenesis, resides within the *gag* open reading frame. We have prepared and purified the recombinant BLV NC protein and conducted electrophoretic mobility shift and isothermal titration calorimetry studies with RNA fragments corresponding to these proposed packaging elements. The *gag*-derived RNAs did not exhibit significant affinity for NC, suggesting an alternate role in packaging. However, an 83-nucleotide fragment of the 5'-UTR that resides just upstream of the *gag* start codon binds NC stoichiometrically and with high affinity ($K_d = 136 \pm 21$ nM). These nucleotides were predicted to form tandem hairpin structures, and studies with smaller fragments indicate that the NC binding site resides exclusively within the distal hairpin (residues G369- U399, $K_d = 67 \pm 8$ nM at physiological ionic strength). Unlike all other structurally characterized retroviral NC binding RNAs, this fragment is not expected to contain exposed guanines, suggesting that RNA binding may be mediated by a previously uncharacterized mechanism.

Keywords

Bovine Leukemia Virus (BLV); ribonucleic acid (RNA); packaging signal; nucleocapsid protein (NC); isothermal titration calorimetry (ITC)

1. Introduction

During the Late (post-integration) phase of the retroviral replication cycle, two copies of the unspliced, positive-sense RNA genome are selected for packaging from a cellular pool that contains a substantial excess of non-viral and spliced viral mRNAs (Goff, 1990). Extensive genetic and biochemical studies of several retroviruses indicate that genome selection is mediated by interactions between the nucleocapsid (NC) domains of the virally encoded Gag polypeptides and packaging elements near the 5' end of the viral genome (Ψ -site) (Berkowitz et al., 1996; D'Souza and Summers, 2005; Darlix et al., 2011; Garetz et al., 2004;

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Greutorex and Lever, 1998; Jewell and Mansky, 2000; Lu et al., 2011b; Paillart et al., 1996; Paillart et al., 2004; Rein, 1994; Russell et al., 2004). The ψ -sites appear to consist of discrete, sometimes discontinuous elements that overlap with the segments that promote RNA dimerization and splicing, providing potential mechanisms for the discriminate packaging of an unspliced, diploid genome. The packaging signal of the murine leukemia virus (MLV) appears to reside exclusively within the upstream 350-nucleotides of the 5'-Untranslated Region (5'-UTR) of the viral RNA, and a "core encapsidation signal" comprising fewer than 100-nucleotides has been identified that is capable of directing heterologous RNAs into virus-like particles (Adam and Miller, 1988; Mann and Baltimore, 1985; Mann et al., 1983; Miyazaki et al., 2010a; Miyazaki et al., 2010b; Mougel and Barklis, 1997). Similarly, packaging of the human immunodeficiency type-1 virus (HIV-1) RNA appears to be directed mainly by residues within the 5'-UTR and the immediately adjacent region of *gag* (Heng et al., 2012; Lu et al., 2011a; Lu et al., 2011b; McBride and Panganiban, 1996; McBride and Panganiban, 1997; McBride et al., 1997).

Recently, efforts have been made to identify the packaging signals and mechanism of Bovine Leukemia Virus (BLV), a member of the delta retrovirus genus that is closely related to the human T-cell lymphoma viruses (HTLV-I/IV) -- the etiologic agent implicated in human adult T-cell leukemia and T-cell lymphoma (Gillet et al., 2007). HTLV-1 has infected about 20 million people worldwide, of which 2 to 3% have developed acute T-cell leukemia (ATL). An additional 2 to 3% have developed chronic inflammatory syndromes, including HTLV-Associated Myelopathy/Tropical Spastic Paraparesis (HAM/TSP, an inflammatory disease of the central nervous system) (Kabeya et al., 2001). No satisfactory treatments are currently available for HTLV-related diseases. Given its close genetic relationship with HTLV, BLV appears to be a suitable non-primate model for studying disease processes and viral mechanisms associated with this genus of retroviruses (Gillet et al., 2007).

Computational and mutagenesis studies identified segments of the BLV genome that are important for packaging (Fig. 1A). Rice and co-workers identified a nucleotide sequence located immediately downstream of the Primer binding site that is conserved among divergent retroviruses (U340-C356 for BLV) (Rice et al., 1985) and residues U321-C400 were predicted to form a tandem stem loop structure (Fig 1B) (Kurg et al., 1995). Mutations designed to disrupt stem-loop V resulted in modest but significant (~3-fold) reductions in genomic RNA packaging, supporting the hypothesis that these residues are important for efficient packaging. Mansky and co-workers showed that deletion of a segment that extends from SL_V to residue 71 of the *gag* open reading frame causes major reductions (~50-fold) in genome packaging and virus production (Mansky et al., 1995) (Fig. 1B,C). Computational and mutagenesis studies indicated that residues A417-U472 form tandem stem loops (SL_{1-2^{gag}}, Fig. 1C), which were proposed to function as the "primary packaging signal" (Mansky and Wisniewski, 1998). Deletions of nucleotides in the *gag* open reading frame that encode for residues of the capsid (CA) domain also modestly reduced packaging levels (~ 7-fold) (Mansky et al., 1995; Mansky and Wisniewski, 1998), but other deletions near the 3' end of *gag* and in the *pol* and *env* genes did not significantly affect genome packaging (Mansky et al., 1995). These findings collectively suggested that the packaging signal of BLV consists of discrete elements that dispersed within the 5'-UTR and *gag* open reading frame.

In an effort to more clearly identify the protein-RNA interactions that may be involved in BLV genome recognition, and to specifically probe for sites that interact with NC, we have prepared the recombinant BLV NC protein (Fig. 1D) and conducted RNA binding studies by gel shift and ITC methods using oligoribonucleotides corresponding to proposed RNA packaging elements.

2. Materials and Methods

2.1 Cloning of recombinant BLV NC protein

The native BLV NC cDNA sequence was optimized by substituting all the less favorable codons with those having the highest usage frequencies by *E. coli* (2001 Novagen catalog). Eight single stranded DNAs, altogether covering the full-length optimized cDNA sequence were purchased from Oligos Etc. Inc. (Wilsonville, OR) in 50 nm scales. Double stranded DNAs, due to sequence complementarity, were obtained by equimolar mixing of two single-stranded DNAs (10 μ M Tris-HCl, pH 7.5; three minutes incubation at 90 °C followed by slow cooling to room temperature). The full-length double stranded DNA was derived from these four pairs of double stranded DNAs by ligation using T4 DNA ligase (New England Biolabs). Two oligonucleotides BAM (5'-GGATCGCGGATCCGCTGTTG-TTAACCGTGA-3') and XHO (5'-GTGGCCGCTCGAGTT-AGGAAACAGCCGGCG-3') (underlined are BamHI and XhoI recognition site, respectively) were used as the primers in the polymerase chain reaction (PCR) to amplify the full-length cDNA and to introduce the BamHI and XhoI recognition sites. The PCR was performed with Ready to Go PCR beads (Amersham Pharmacia Biotech) and consisted of 35 cycles at 94 °C for one minute, 55 °C for one minute, and 72 °C for 1.5 minutes followed by a final extension at 72 °C for ten minutes. The PCR product was gel-purified with the Gel-extraction Kit (Qiagen), digested with the BamHI and XhoI (New England Biolabs), and ligated with likewise digested expression vector Pgex-6p-1 (Amersham Pharmacia Bio-tech) using T4 DNA ligase. The ligation product was then transformed into DH5 α *E. coli* using heat shock. Transformants were screened for the presence of insert by plasmid mini-preps (Quick-Spin DNA extraction kit; Qiagen) and restriction enzyme digestions. Clones containing the NC cDNA insert were verified by sequencing the complete insert by the dideoxynucleotide method (DNA Sequencing Resource Center at the Rockefeller University). The primer used for sequencing was the pGEX 5' sequencing primer (5'-GGGCTGGCAAGCCACGTTTGGTG-3'). The clone used for over expression of the fusion GST-NC was named pBLVNC.

2.2 Expression and Purification of the BLV NC protein

The fusion protein was expressed in *E. coli* BL21 (DE) GOLD (Invitrogen) and purified by affinity chromatography on glutathione-Sepharose (GE Healthcare). GST-NC protein expression was induced with 1 mM isopropyl β -D-thiogalactoside (IPTG) at 37 °C (post induction growth for four hours at 30 °C), bacteria were collected by centrifugation (8,000 rpm for 15 minutes) and were washed once in phosphate-saline buffer (PBS). All protein purification steps were performed at 4 °C. The bacterial pellet was re-suspended in PBS and lysed by Micro fluidizer (Microfluidics, Newton, MA). The nucleic acids were precipitated using polyethyleneimine with a final concentration of 0.45 % (w/v). Debris was pelleted by centrifugation (17,000 rpm for 30 minutes) and the resulting supernatant was filtered through 0.4 μ filter and then allowed for an hour binding to the sepharose beads. The beads were washed with 2 L of PBS and 500 ml cleavage buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5 mM DTT). Cleavage of N-terminal GST protein from the GST-NC fusion was done for 16 hours with the enzyme PreScission protease (GE Healthcare). Completion of digestion was monitored by SDS-PAGE. The purified NC protein was exchanged into buffer (10mM Tris-HCl (pH 7.0), 5–10 mM NaCl, 0.1 mM BME) using Amicon-3 (Millipore) and the concentration was determined by optical absorbance at 280 nM.

2.3 Preparation of RNA samples

The template for SL ν .VI was prepared and sequenced by Gene Gateway (Hayward, CA) as an insert in the PUC 18 plasmid with a 3' SmaI restriction site for linalization and the T7 promoter sequence directly upstream of the template sequence followed by non-native GG to increase the transcription yield. Template plasmids for SL ν .VI were amplified in *E. coli*

DH5 α . The plasmids were extracted from the cells using Plasmid Mega Kit (Qiagen) and linearized with SmaI enzyme (New England Biolabs) at 1 μ g/unit overnight at room temperature. The cut plasmid was then extracted twice with phenol/chloroform and precipitated with ethanol. The pellet was washed with 70 % ethanol (v/v), and dissolved in sterile distilled water. The DNA template for making SL_V**VI** was ordered from Dharmacon Research Inc. and purified by denaturing gel electrophoresis (PAGE). The template was designed with having the T7 RNA polymerase promoter at the 3' end of the complementary sequence of the transcript required. The template was mixed with 1.5 times the top strand of the T7 promoter (5'-TAATACGACTCACTATA-3'), heated and then cooled to anneal the two strands of the double stranded promoter.

SL_V**VI** and SL_V**VI** oligonucleotides were synthesized using standard *in vitro* transcription methods previously described (Milligan et al., 1987) with T7 polymerase and purified to a single nucleotide resolution by gel electrophoresis (Milligan and Uhlenbeck, 1989; Wyatt et al., 1991). The DNA transcripts were transcribed *in vitro* by T7 RNA polymerase in 30 ml reactions containing the optimized amount of template, 20 mM MgCl₂, 2 mM spermidine, 80 mM Tris-HCl (pH 8.1), 2 mM DTT, 10 % DMSO, 0.3 mg T7 RNA polymerase and optimized amount of each NTP (4 mM ATP, 12 mM GTP, 16 mM CTP, 9.4 mM UTP for SL_V**VI** and 4 mM ATP, 6.5 mM GTP, 8.5 mM CTP, 5.1 mM UTP for SL_V**VI**). Reactions were incubated at 37 °C for three hours and quenched with 25 mM EDTA. The RNA was ethanol precipitated, air dried and re-suspended in water containing 4 M urea. After denaturation at 96 °C for five minutes, the RNA was purified by electrophoresis on urea-containing polyacrylamide denaturing gels. The concentration of each sample was determined by measuring the optical absorbance at 260 nM.

Oligoribonucleotides SL_V, SL₁₋₂^{gag}, SL₁^{gag}, SL₂^{gag} and 10nt linker were purchased from Dharmacon Research Inc (Lafayette, CO, USA). The 2'-protecting groups for minimizing the chance of RNA degradation of these RNAs were removed before use as recommended. Each tube was briefly centrifuged and 400 μ l of 2' deprotection buffer was added into the RNA. The RNA was completely dissolved, vortexed for 10 seconds and centrifuged for 10 seconds. Then the RNA was incubated at 60 °C for 3 hours-to-overnight. This was followed by precipitation of RNA with 100 % ethanol at -20 °C over night. The pelleted RNA was further purified with urea-containing denaturing PAGE (% 16–20) to a single nucleotide resolution and RNA was recovered using an electro elution system.

2.4 Native polyacrylamide gel electrophoresis

All RNA and NC samples were prepared at 0.3 mM stock solution in buffer (10 mM Tris-HCl (pH 7.0), 5–150 mM NaCl, 0–1 mM MgCl₂, 0.1 mM BME). RNAs were heated at 95 °C for two minutes and cooled on ice. Then 1 μ l of a 0.3 mM NC solution in buffer was added to 1 μ l of RNA to give a ratio of 1:1 NC:RNA. Increasing amounts of 0.3 mM NC were added to each of 1 μ l of RNA giving rise to NC:RNA ratios ranging from 0:1 to 1.25:1. Buffer was added (10 μ l final volume), and samples were incubated at room temperature for 30 minutes. Sucrose (40 w/v; 2 μ l) was then added and samples (~1.2 μ g RNA) were loaded onto 12 % (for SL₁₋₂^{gag} and SL_V**VI**) native polyacrylamide gels at 160 V, 4 °C in 1XTris-Borate buffer (89 mM Tris-Base, 89 mM boric acid). After electrophoresis, the gel was stained with Stains-all (Sigma) which stains negatively charged molecules, and photographed with a Kodak digital camera.

2.5 Isothermal titration calorimetry

Dissociation constants for BLV NC binding to SL₁₋₂^{gag}, SL_V**VI**, SL_V, SL_V**VI** and 10 nt linker were determined by standard ITC methods using a VP-isothermal titration calorimeter (MicroCal Corp., Northampton, MA) (Wiseman et al., 1989). The RNA and NC samples

containing buffer (10 mM Tris-Base (pH 7.0), 0.1 mM BME and varying NaCl (5–300 mM NaCl) and MgCl_2 (0–1 mM) concentrations were degassed prior to titration. Protein and RNA concentrations were established by UV-Vis absorption measurements. Heats of reaction ($\mu\text{cal/sec}$) were measured at 30 °C for 28 injections of NC protein (75 μM) into 1.41 ml of RNA (5 μM). The heats of dilution were obtained by titrating the identical protein sample into a cell containing sample buffer and subtracted from the raw data prior to analysis. Heats of dilution were typically 0.03 $\mu\text{cal/s}$ and the maximum heats of binding were 0.3 $\mu\text{cal/s}$, giving an apparent signal-to-noise ratio of 10. The syringe mixing speed was 310 rpm as recommended by the vendor. Binding curves were analyzed by non-linear least-square fitting of the baseline-corrected ITC data to a single binding site or two-site binding model (ITC Origin program, V2.8; Microcal, Northampton, MA). The dissociation constant for each RNA is reported as the mean $\pm$ the standard deviation obtained from three independent titration experiments.

3. Results

3.1 BLV NC protein expression and purification

The native BLV NC gene could not be readily expressed due to the presence of several Arg and Pro codons that are used rarely in *E. coli*. A sequence-optimized insert obtained by site-directed mutagenesis was cloned and inserted into the pGEX expression vector as a GST fusion gene, and the sequence of the clone confirmed by nucleotide sequencing (Rockefeller Protein/DNA Technology Center). This optimized clone over-expressed efficiently in *E. coli*. Treatment of the resulting protein with PreScission protease to remove the GST tag afforded a 74- residue protein (referred throughout this paper as NC) that contains five non-native residues at the N-terminus due to cleavage specificity of the PreScission protease enzyme: NH_3^+ - GPLSG-
V¹HTPGPKMPG¹⁰PRQPAPKRPP²⁰PGPCYRCLKE³⁰GHWARDCPTK⁴⁰ATGPPPGPC
P⁵⁰I⁵¹CKDPSHWKR⁶⁰DCPTLKSKN⁶⁹ (the zinc fingers are underlined). About 10 mg-purified protein per liter of culture media was obtained. Coomassie stained SDS-PAGE of the purified protein showed a single band at about 7.9 kDa. The molecular weight of the purified ¹⁵N-labeled apoprotein (7965.07 ± 0.1 Da, determined by ion-spray mass spectrometry) was as expected ($\text{MW}_{\text{calc}} = 7964.97$ Da).

3.2 Preparation of RNA samples

Oligoribonucleotides $\text{SL}_{1-2}^{\text{gag}}$, SL_V and the 10mer linker connecting the SL_V and SL_{VI} were purchased from Dharmacon Research Inc. After deprotection of the 2'-O-bis (acetoxymethoxy)-methyl (ACE) group, these RNAs were purified by ethanol precipitation followed by denaturing PAGE. Oligoribonucleotides SL_V and SL_{VI} were prepared using standard *in vitro* transcription methods (Milligan et al., 1987). Template for SL_V was prepared from cDNA clones, whereas the template for the SL_{VI} constructs was obtained synthetically (KECK Foundation Biotechnology Research Center) and purified by denaturing polyacrylamide gel electrophoresis (PAGE). Reactions were optimized to give maximum yields by incorporating non-native guanosines for SL_V at the 5' terminus (For SL_{VI} , the guanosines were native) (Fig. 1B). RNA samples were purified by preparative scale denaturing PAGE (Milligan and Uhlenbeck, 1989; Wyatt et al., 1991), with yields typically in the range of 1.0–3.0 μmoles of purified RNA per 90 ml reaction solution (see Methods section for details). Denaturing polyacrylamide gels showing relative electrophoretic mobility and sample purity are shown in Fig. 1E.

3.3 BLV NC exhibits weak affinity for $\text{SL}_{1-2}^{\text{gag}}$

$\text{SL}_{1-2}^{\text{gag}}$ forms both monomer (major) and oligomer (minor) species under conditions of 150 mM NaCl and 1 mM MgCl_2 , based on native polyacrylamide gel electrophoresis (PAGE)

mobilities, Fig. 2A. Titration with NC led to smearing of the RNA bands at higher NC concentrations, but not to discrete band shifts observed previously for high-affinity NC binding by other retroviral NC proteins (Amarasinghe et al., 2001; Zhou et al., 2005). Similar results were obtained for smaller RNA constructs corresponding to the isolated SL_1^{gag} and SL_2^{gag} stem loops (data not shown). RNase contamination tests ruled out the possibility of RNA degradation; thus the smearing of RNA bands upon addition of NC, along with the lack of any distinct band shift, was attributed to the formation of weakly interacting RNA-NC species. NC proteins are generally capable of binding single stranded nucleotides non-specifically and destabilizing nucleic acid helices (Guo et al., 1997; Johnson et al., 2000; Khan and Giedroc, 1992; Tsuchihashi and Brown, 1994; Wu et al., 1996), which may explain the smearing observed upon addition of NC to gag-derived RNAs. Isothermal Titration Calorimetry (ITC) experiments confirmed that, under physiological-like conditions ($[NaCl] = 150mM$, $[MgCl_2] = 1mM$, $pH=7.0$), NC interacts weakly with SL_{1-2}^{gag} , Fig. 2B.

3.4 BLV NC binds SL_{V-VI} with high affinity

The 85 nucleotide SL_{V-VI} RNA migrates as a single band on native PAGE gels with mobility of a monomer. In contrast to results obtained for SL_{1-2}^{gag} , titration of SL_{V-VI} with NC under conditions of physiological ionic strength led to discrete, stoichiometric band shifts consistent with high affinity binding and formation of a 1:1 NC- SL_{V-VI} adduct, Fig. 2A. Binding was also monitored by ITC (Fig. 2C). Under conditions of near-physiological ionic strength (150 mM NaCl, 1 mM $MgCl_2$), an exothermic binding isotherm was obtained ($K_d = 136 \pm 21$ nM), consistent with results obtained previously for other NC-RNA complexes (D'Souza et al., 2001; Dey et al., 2005; Spriggs et al., 2008; Zhou et al., 2005). Unlike previous findings, binding appeared to be much weaker at low ionic strength (5 mM NaCl, no added $MgCl_2$), and gave rise to a binding isotherm consistent with weaker binding by multiple NC molecules. This was surprising, since BLV NC is highly basic, and electrostatic interactions were expected to contribute favorably to NC-RNA interactions. Tight, stoichiometric binding appears to require Mg^{2+} and the highest affinity was observed for samples containing low concentrations of NaCl (5.0 mM) and physiological concentrations of $MgCl_2$ (1.0 mM) ($K_d = 93 \pm 12$ nM). Mg^{2+} is known to promote the proper folding and structural stabilization of a number of RNAs, including tRNAs (Draper et al., 2005; Romer and Hach, 1975; Serra et al., 2002; Summers et al., 1992), and our findings suggest that the proper folding of SL_{V-VI} may also be Mg^{2+} dependent.

3.5 BLV NC binds specifically to SL_{VI}

SL_{V-VI} is predicted to consist of two stem loops connected by a short linker (SL_V +linker+ SL_{VI}). The 10nt linker connecting the SL_V and the SL_{VI} stem loop contains a UCUG motif, which was shown to be the primary recognition sequence of the MMLV packaging signal, and is exposed upon dimer induced conformational changes in the genome (D'Souza and Summers, 2004). MMLV NC binds to this motif with a dissociation constant of ~95nM when the surrounding sequences are included (Dey et al., 2005). For this reason we investigated whether BLV NC interaction with the UCUG containing BLV linker resembles it in BLV genome recognition. Unlike SL_V , an investigation of the linker, 5'-CAACUCUCUG-3', NC interaction by ITC afforded favorable, negative enthalpic heats that can be fit to a 1:1 binding isotherm with a dissociation constant of $K_d = 92 \pm 16$ nM under low ionic strength (5 mM NaCl) (Fig. 3A). These findings initially suggested to us that BLV and MLV NC proteins behave similarly in that they target unstructured RNA sequences. However, unlike observations for MLV NC, no binding was detected by ITC under conditions of physiological-like ionic strength (150 mM NaCl + 1 mM $MgCl_2$) (Fig. 3A). Thus, the binding of BLV NC to the SL_{V-VI} linker at low ionic strength appears to be

mediated exclusively by electrostatic interactions, and it is unlikely that the SL_V-_{VI} linker participates directly in NC binding under physiological conditions.

Similar results were obtained for the isolated SL_V RNA. Under conditions of low ionic strength, SL_V exhibited weak affinity for NC characterized by positive binding enthalpies at NC:RNA ratios greater than ~1.2:1 (Fig. 3B). Positive NC-RNA binding enthalpies are attributed to NC-induced destabilization and unfolding of RNA structure resulting from the chaperone activity of the protein (Feng et al., 1999; Urbaneja et al., 2002). Under conditions of physiological-like ionic strength (150mM NaCl, 1mM MgCl₂), which should stabilize the SL_V RNA structure and inhibit weak electrostatic NC: RNA interactions, no binding could be detected by ITC (Fig. 3B).

In contrast, a 31 nt RNA corresponding to the SL_{VI} stem loop exhibited high affinity for BLV NC. Under conditions of low ionic strength (5 mM NaCl) a well-defined, stoichiometric band shift was observed by native PAGE for SL_{VI} upon titration with NC, Fig. 4A. Beyond NC: SL_{VI} ratios of 1:1, an additional band was observed indicative of the formation of a NC₂:SL_{VI} complex. The fact that the second band shift did not appear at NC:RNA ratio below 1:1 indicates that SL_{VI} binds the second NC molecule with affinity that is significantly (> 10 fold) reduced compared to the initial NC binding. Similar sequential binding behavior was observed by ITC (Fig. 4B). The fitted equilibrium dissociation constant for the first site ($K_d = 0.15$ nM) was too low to be considered reliable under the experimental conditions employed (low ionic strength). The fitted dissociation constant for the second NC binding site (93 ± 37 nM) is at least ~100 fold weaker than that of the first site. Binding to the second site was almost completely inhibited in the presence of 1 mM MgCl₂ (Fig. 4B), even at low overall ionic strength (5 mM NaCl), and the best fits for the data obtained under these conditions were achieved with a 1:1 NC:RNA binding mode ($K_d = 13 \pm 4$ nM, Fig. 4B). However, as observed in experiments performed without added salts, the ITC data exhibited steep transitions and saturation behavior near NC:RNA stoichiometries of 1:1, indicating that NC binding to SL_{VI} is still too tight for accurate measurement under these conditions. At physiological ionic strength (150 mM NaCl, 1 mM MgCl₂), SL_{VI} consisted primarily of a monomer species, based on native PAGE behavior, and addition of NC afforded a well-defined, stoichiometric band shift at and above 1:1 NC: RNA ratios. ITC experiments performed under these conditions confirmed that NC interacts with SL_{VI} stoichiometrically and with affinity ($K_d = 67 \pm 8$ nM; Fig. 4C) that is approximately 2.5 times greater than that observed for HIV-1 NC binding to its cognate SL3 stem loop packaging element under similar conditions. Replacement of Na⁺ with the physiological cation, K⁺, in the binding buffer resulted in a similar NC-RNA binding affinity ($K_d = 69 \pm 16$ nM, 150mM KCl, 1mM MgCl₂) (Fig. 4D).

Because several previous NC-RNA binding studies were conducted using low ionic strength sample conditions (5–10 mM NaCl), we conducted ITC experiments over a range of NaCl concentrations (with [MgCl₂] = 1 mM) (Fig. 4C), which allowed us to estimate the affinity of NC for SL_{VI} at lower ionic strength where binding was too tight for accurate measurement. Extrapolation of a log [K_d] versus -log [NaCl] plot (Fig. 4C) afforded an estimated NC dissociation constant at low salt ([NaCl] = 10 mM; [MgCl₂] = 1.0 mM) of (extrapolated dissociation constant $K_d = 2 \pm 0.6$ pM), which is five orders of magnitude tighter than the value measured for HIV-1 NC binding to the ψ -RNA stem loop SL3 (Amarasinghe et al., 2000), and three orders of magnitude tighter than the affinity measured for the Rous sarcoma virus NC protein binding to its minimal, 75 nt packaging element (Zhou et al., 2005).

4. Discussion

Retroviral genome packaging is initiated by the formation of a complex between a small number of Gag proteins (perhaps a dozen or fewer (Jouvenet et al., 2008; Jouvenet et al., 2009; Jouvenet et al., 2011; Kutluay and Bieniasz, 2010)) and RNA elements located near the 5' end of the unspliced genome (Berkhout, 1996; Berkowitz et al., 1996; Jewell and Mansky, 2000; Rein, 1994). Non-viral vectors that contain the 5'-most 734 nucleotides of the BLV genome can be efficiently packaged into BLV virus-like particles, indicating that the packaging signal residues exclusively reside within this region (Jewell and Mansky, 2005). Deletion experiments identified discrete elements within the 5'-UTR (Kurg et al., 1995) and gag ORF (Mansky et al., 1995) that are critical for efficient packaging. For example, deletion of 5'-UTR residues (U340-A417) led to a 5-fold reduction in packaging, and mutation of six bases (C346-G351) in the conserved consensus sequence immediately adjacent to the primer binding-site (U340-C356) (Rice et al., 1985) resulted in a 3-fold decrease in packaging (Kurg et al., 1995). These studies indicate that residues U340-A417 within the 5'UTR are critical for packaging. Deletion analysis indicate that BLV packaging signal was contained within a 147nt-region spanning the residues, U340-G487 (Mansky et al., 1995). Secondary structure predictions, coupled with site directed mutagenesis experiments, have indicated that tandem stem loops in the *gag* ORF, SL₁₋₂^{gag} (A417-U472) (Mansky and Gajary, 2002; Mansky et al., 1995; Mansky and Wisniewski, 1998) is the critical element. The finding that critical elements of the packaging signal reside within the *gag* open reading frame was unusual (although the most efficient packaging has been found to depend on residues within *gag* (Bender et al., 1987; Berkowitz et al., 1995)), and suggested that the mechanism of BLV genome packaging may differ, to some extent, from that employed by other retroviruses.

We have now shown that BLV NC does not exhibit significant affinity for SL₁₋₂^{gag}. This does not rule out the possibility that this element serves some other role in packaging, such as stabilizing a critical RNA structure or interacting with cellular or other viral constituents. Interestingly, gel-shift mobility assays revealed that the BLV matrix protein (MA) also binds with specificity to *gag*-derived RNAs that contain SL₁₋₂^{gag} (Katoh et al., 1991; Katoh et al., 1993), which led to suggestions that MA, in addition to NC, may function in packaging by binding to specific regions of the viral genome (Wang et al., 2003). It is thus conceivable that the SL₁₋₂^{gag} element promotes packaging by binding to the BLV MA domain, rather than the NC domain, and further studies along these lines are warranted.

Our studies indicate that BLV NC binds with high affinity to SL_{V.VI}, consistent with the *in vivo* packaging experiments of Kurg and colleagues (Kurg et al., 1995). Additionally, under physiological-like sample conditions, the binding site residues reside exclusively within the SL_{V.I} fragment. Binding under these conditions ($K_d = 67 \pm 8$ nM) is significantly tighter than that observed previously for HIV-1 NC binding to RNA tetra loops in low-salt buffers ($K_d \sim 150$ nM; [NaCl] = 10 mM; [MgCl₂] = 0 mM) (Amarasinghe et al., 2000) or MLV NC binding to single stranded RNAs containing Py-Py-Py-G (Py = pyrimidine) sequences in similar low-salt buffers (~100–300 nM) (D'Souza et al., 2001; Dey et al., 2005). The affinities of HIV-1 and MLV NC for their cognate RNAs decrease with increasing ionic strength, and it is likely that, under similar, native-like conditions, BLV NC-SL_{V.I} interactions may be more than an order of magnitude tighter than those of HIV-1 and MLV. Surprisingly, the extrapolated BLV NC:SL_{V.I} dissociation constant at low ionic strength (10 mM NaCl, 1 mM MgCl₂) is more than four orders of magnitude tighter than the values measured for HIV-1 NC:SL3 and NC:SL2 binding under similar conditions (Amarasinghe et al., 2000). It is not immediately obvious why different retroviral NC proteins exhibit such different affinities for their RNA packaging signals. One possibility is that the affinity of the packaging signals for NC is context-dependent, and highest affinity (and perhaps specificity)

requires the intact 5'-UTR. It is also conceivable that genome recognition by different retroviruses is mediated by different NC: RNA stoichiometries. For example, BLV genome packaging might be mediated by as few as one or two NC proteins that bind with very high affinity, whereas HIV-1 genome packaging might require several NC domains that bind with reduced affinities.

In all previously characterized retroviral NC-RNA structures, binding is mediated, at least partly, by insertion of one or more exposed guanosines to hydrophobic pockets on the surfaces of the CCHC-type zinc fingers. Binding is promoted by the formation of hydrogen bonds between the guanosine O6, N¹H and N²H²¹ groups and backbone NH and O atoms of peptide residues located at the bottom of the pocket (Amarasinghe et al., 2000; De Guzman et al., 1998; South and Summers, 1993). The N- and C-terminal HIV-1 NC zinc fingers bind in this manner to the second and last exposed guanosines of the SL2 and SL3 GG (A/U) G tetra loops (Amarasinghe et al., 2000; De Guzman et al., 1998). The single MLV NC zinc finger binds in an analogous manner to exposed guanosines in unstructured UAUCUG elements (D'Souza and Summers, 2004; Dey et al., 2005), and the N-terminal zinc finger of the RSV NC protein binds to the single exposed guanosine of a U(217)GCG tetraloop (Zhou et al., 2007). It is noteworthy that the C-terminal RSV NC zinc finger does not bind to exposed guanosines, but instead interacts with exposed adenosine bases in unstructured strands that link adjacent stem loops (Zhou et al., 2007). Interestingly, the predicted secondary structure of SL_{VI} does not contain a single exposed guanosine, but instead contains an apical CUC loop, a bulged C, and a larger CAUUU bulge. Although NC molecules can have potent chaperone activity (Darlix et al., 2011), preliminary NMR studies indicate that NC does not induce unwinding of the SL_{VI} RNA (Yildiz and Summers, unpublished). The RNA is also unusual relative to other NC targets in that it only binds tightly to NC in the presence of Mg⁺², which can sometimes inhibit NC binding and chaperone activity (Summers et al., 1992). Together, these observations suggest that the NC-RNA structures responsible for directing BLV genome recognition and packaging may be significantly different from those of other retroviruses. High-resolution structural studies of the BLV NC:SL_{VI} designed to test this hypothesis are underway.

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Highlights

Previously proposed packaging signals tested.

Only one of several proposed packaging elements binds NC with high-affinity.

NC binding element unexpectedly lacks features common to other retroviral NC binding sites.

Figure 1.

(A) Representation of the Bovine Leukemia Virus (BLV) RNA genome showing relative locations of the splice donor site (SD) and RNA packaging signal. Nucleotides are numbered using the first residue after the 5'-cap as position number 1. The 18 nucleotide Primer Binding Site was indicated by numbers nt322 and nt339. Secondary structure of a segment of the BLV (B) 5'UTR (shown in red is the 10-nucleotide linker connecting the SL_V and SL_{VI} stem loops) (C) 5' gag. (D) Denaturing PAGE results (12 % (w/v) polyacrylamide) showing relative electrophoretic migration and sample purity for the RNA constructs used in these studies. Lane numbers 1, 2, 3, 4, 5 correspond to RNA constructs SL_{V-VI}, SL₁₋₂^{gag}, SL_V, SL_{VI} respectively. To increase the transcription yields, non-native GG residues shown in blue were added at the 5' termini of SL_{V-VI}. 5' termini GG of SL_{VI} are native (E) Amino acid sequence and the zinc-binding mode of the BLV NC protein. Non-native residues derived from the PreScission protease cleavage site (used to cleave GST during purification) are shown in blue.

Figure 2.

NATIVE-PAGE (12%) data showing titration of BLV NC with RNA segments (A) from 5' *gag*, SL_{1-2}^{gag} (lanes 1–4) and from 5' UTR, SL_{V-VI} (lanes 5–8) ([RNA]=30 μM ; lanes 1–4 and 5–8 correspond to addition of 0.0, 0.5, 1.0, 1.25 equivalents of NC to SL_{1-2}^{gag} and SL_{V-VI} , respectively). The presence of smearing RNA bands with NC increments for NC- SL_{1-2}^{gag} titration indicates that NC binds SL_{1-2}^{gag} via nonspecific-weak interactions, whereas formation of a well defined, electrophoretic band shift upon addition of NC into SL_{V-VI} indicates that high affinity NC binding site resides within the 5' UTR. Representative ITC data obtained for NC binding to (B) SL_{1-2}^{gag} and (C) SL_{V-VI} under different ionic conditions (shown in the inset). Top panel: raw data with each peak corresponding to the heat produced upon addition of NC ([NC] = 75 μM , 10 μl per injection) to RNA, ([RNA]=5 μM , 1.41 ml). Bottom panel: binding isotherms obtained after peak integration.

Figure 3.

Representative ITC data obtained upon titration of BLV NC with (A) 10nt SL_V.v_I Linker RNA (5'-CAACUCUCUG-3') (B) SL_V hairpin RNA. Different colors denote the different conditions employed (shown in the inset). Under low ionic strength (magenta panels) NC binds to SL_V via very weak interactions whereas to Linker via tight interactions (Equilibrium dissociation constant for NC-Linker complex, $K_d = 92 \pm 16$ nM). However, under conditions of physiological-like ionic strength (green panels), NC did not bind to either the Linker or SL_V RNAs.

Figure 4.

(A) Native-PAGE (14%) data showing results of titrating SL_{VI} with NC (5mM NaCl, 1 mM MgCl₂). Under the conditions employed, the SL_{VI} RNA migrates as monomeric (major, M) and dimeric (minor, D) species. Addition of NC leads to formation of a specific 1:1 NC-RNA complex that migrates faster than the dimeric RNA and 2:1 NC: SL_{VI} complex that migrates slower than the dimeric RNA. (B) Representative ITC data obtained for titration of SL_{VI} (5 μ M, 1.41ml) with NC (75 μ M, 10 μ l per injection) under conditions of 5mM NaCl (magenta panel) and 5mM NaCl+1mM MgCl₂ (cyan panel). Under low ionic strength (5mM NaCl), SL_{VI} exhibits two NC binding sites (Equilibrium dissociation constants for the first site and the second site are $K_{d1} < 1$ nM and $K_{d2} = 93 \pm 37$ nM respectively). Weaker binding site is inhibited upon addition of 1mM MgCl₂. (C) Representative ITC data obtained for titration of SL_{VI} in the presence of varying concentrations of NaCl. Different colors denote the different NaCl concentrations employed (shown in the inset). The equilibrium dissociation constants were measured as 67 ± 8 nM ([NaCl] = 150 mM), 181 ± 49 nM ([NaCl] = 200 mM) and 932 ± 245 nM ([NaCl] = 300 mM). The Plot of the $\log(K_d, \text{nM})$ versus $-\log([NaCl], \text{M})$ (correlation coefficient=0.9982) (see the inset), which afforded an extrapolated dissociation constant of 2 ± 0.6 pM at [NaCl] = 10mM. (D) ITC data obtained upon titration of SL_{VI} with NC under physiological-like ionic conditions (140 mM KCl, 1

mM MgCl₂) showing that replacement of Na⁺ with K⁺ does not affect the binding affinity ($K_d = 69 \pm 16$ nM).