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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 polyprotein 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 ± 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 ± 8 nM at physiological ionic strength). Unlike all other structurally characterized retroviral NC binding RNAs, this fragment is not expected to contain exposed guanosines, suggesting that RNA binding may be mediated by a previously uncharacterized mechanism.

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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 polyproteins 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; Greatorex, 2004; Greatorex and Lever, 1998; Jewell and Mansky, 2000; Lu et al., 2011b; Paillart et al., 1996, 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,b; 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,b; McBride and Panganiban, 1996, 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 2–3% have developed acute T-cell leukemia (ATL). An additional 2–3% 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

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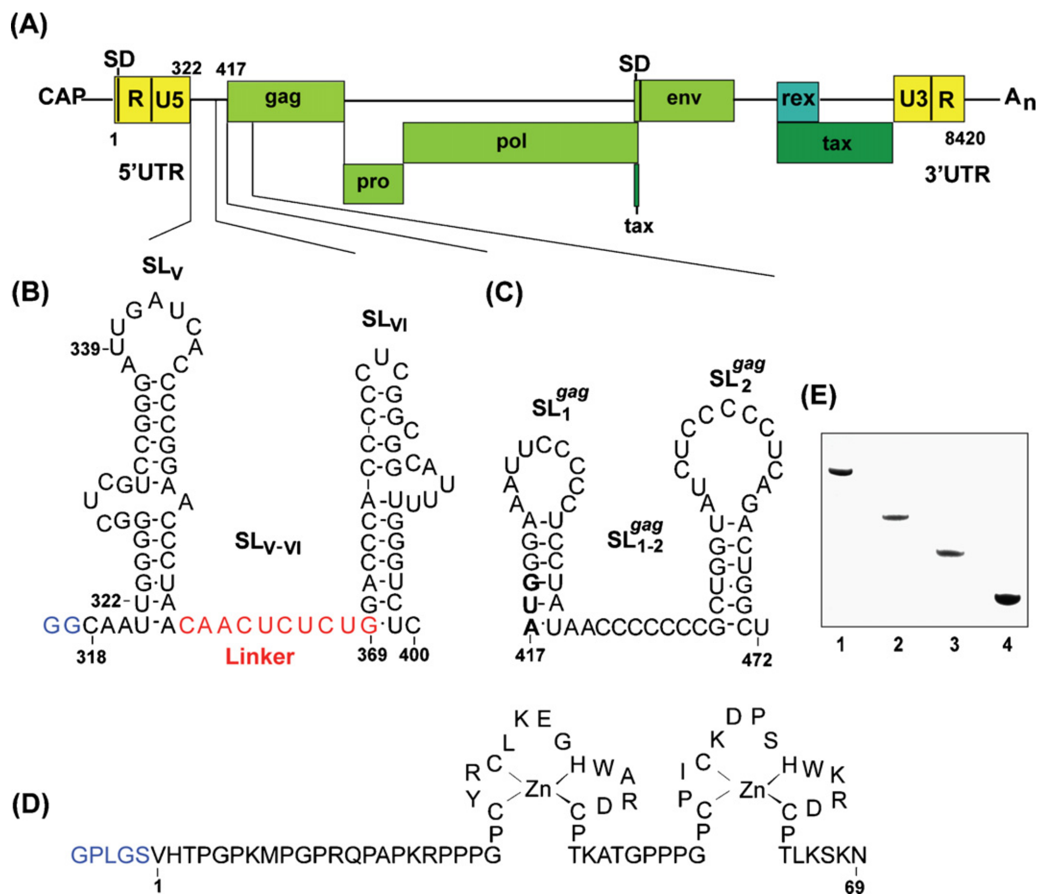

Fig. 1. (A) Representation of the bovine leukemia virus (BLV) RNA genome showing relative locations of the splice donor site (SD) and RNA packaging signal. Nucleotides are numbered using the first residue after the 5′-cap as position number 1. The 18 nucleotide Primer Binding Site was indicated by numbers 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) and (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 and 5 correspond to RNA constructs SL_{V-VI}, $SL_{1-2}{}^{gag}$, SL_V and 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.

et al. 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 et al. (1995) 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 (Fig. 1B and 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. (Wilson-ville, OR) in 50 nm scales. Double stranded DNAs, due to sequence complementarity, were obtained by equimolar mixing of two single-stranded DNAs (10 μM Tris–HCl, pH 7.5; 3 min 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.

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