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Single Molecule DNA Interactions of the Nucleic Acid Chaperone Protein from the Line-1 Retrotransposon
Kathy R. Chaurasiya, James Evans, Suresh Peddigari, Sandra L. Martin, Mark C. Williams.
Reverse transcription in retroviruses and retrotransposons requires nucleic acid chaperones, which facilitate the rearrangement of nucleic acid secondary structure. The nucleic acid chaperone properties of the human immunodeficiency virus type-1 (HIV-1) nucleocapsid protein (NC) have been extensively studied, and nucleic acid aggregation, duplex destabilization, and rapid protein binding kinetics have been identified as major components of its activity. However, the properties of other nucleic acid chaperone proteins, such as ORF1p from the retrotransposon LINE-1, are not as well understood. We used single molecule DNA stretching in combination with site-directed mutagenesis of ORF1p as a method for detailed characterization of its chaperone activity. Wild type ORF1p significantly reduces the cooperativity of the force-induced melting transition from double-stranded DNA (dsDNA) to single-stranded DNA (ssDNA), indicating that DNA melting is more easily initiated in its presence. ORF1p also aggregates both dsDNA and ssDNA, and exhibits relatively rapid binding kinetics. Altering certain residues has dramatic effects on chaperone activity. Stretching curves in the presence of mutant R284A, which is inactive in retrotransposition assays, exhibit a cooperative melting transition and minimal DNA aggregation. Retrotransposition is partially restored with mutant R284K, which alters the melting transition and strongly aggregates ssDNA. Similarly, mutant Y318A has minimal retrotransposition activity, and stretching curves reflect only a small change in melting transition cooperativity. The Y318F mutant, which largely restores retrotransposition, alters the melting transition in a way similar to wild type ORF1p. Thus, DNA stretching results indicate that reduced cooperativity of the melting transition is associated with greater nucleic acid chaperone and retrotransposition activity of ORF1p variants.

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Nucleic Acid Binding Properties of HIV-1 Gag Polyprotein and its Proteolytic Processing Intermediates Studied by Single Molecule DNA Stretching
Jialin Li, Mark C. Williams, Chrisopher Jones, Karin Musier-Forsyth, Siddhartha A.K. Datta, Alan Rein, Robert J. Gorelick, Ioulia Rouzina.
The human immunodeficiency virus type 1 Gag protein is essential for retroviral assembly. During viral maturation, Gag is processed to form matrix (MA), capsid (CA), and nucleocapsid (NC). NC is initially cleaved into NCp15, then NCp9, and finally NCp7. NCp7 functions as a nucleic acid chaperone during retroviral replication, in which it rearranges nucleic acids to facilitate reverse transcription and recombination. The role of Gag and its cleavage intermediates in facilitating nucleic acid remodeling is not well understood, although it is likely they also function as chaperones during viral assembly and early reverse transcription steps. We use DNA stretching to probe the DNA interactions of these proteins. In the presence of NCp7 DNA elongates at a lower force almost reversibly, demonstrating that NCp7 facilitates rapid DNA structural transitions. In contrast, the absence of a clear force-induced DNA elongation transition in the presence of NCp9 and NCp15 suggest that these proteins prevent DNA strand separation by forming a filament around duplex DNA via protein-protein interactions. In the presence of Gag the stretching curves exhibit a clear force-induced melting transition, although the overall DNA length and its length change are less than observed in the absence of protein. This result reflects the ability of Gag to compact long regions of the double-stranded DNA molecule, creating small virus-like DNA/Gag particles, thereby preventing DNA from stretching and melting by force, while leaving the rest of the DNA protein-free. Further studies will elucidate the protein concentration dependence and the kinetics of these protein-DNA interactions. *This work was supported in part by Federal Funds from NCI, NIH under contract N01-CO-12400 (RJG)and the Intramural Research Program of the NIH, NCI, Center for Cancer Research (SAKD, AR).*

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Biochemical and Biophysical Investigation of the Molecular Determinants of HIV-1 Vif Packaging
Kalyan Giri, Klaus Strebel, Ernest L. Maynard.
HIV-1 virion infectivity factor (Vif) is an accessory protein that is packaged into virions and is essential for viral replication of HIV. In the absence of Vif, a host cytidine deaminase, APOBEC3G, is incorporated into virions and delivered to target cells where it mutates viral cDNA. Vif recruits a cullin 5-based ubiquitin ligase that targets APOBEC3G for proteosomal degradation. It has been demonstrated that Vif is packaged into the viral core through interactions with the viral genomic RNA. The mechanistic role of packaged Vif has not been determined, but mutations that disrupt Vif packaging, abolish HIV-1 infectivity.
We have made several Vif mutations in a basic region spanning residues 75-115 that inhibit packaging. Deletion mutagenesis further confirms that residues beyond 115 are dispensable for RNA binding. An RNA binding assay was developed to look at the correlation between packaging and RNA binding. Stem-loop RNAs derived from the 5'-untranslated region of the HIV-1 genome were chemically synthesized and labeled with fluorescein. The binding of Vif to labeled RNA was measured by changes in fluorescence anisotropy. Binding data showed that some packaging mutants had a reduced affinity for RNA while for other mutants, binding affinity was unaffected, suggesting that other factors may contribute to the specificity of packaging. Evidence suggests HIV-1 Nucleocapsid (NC) may assist in the proper packaging of Vif. Our results demonstrate a RNA dependent interaction between Vif and NC that may be relevant to intravirion packaging of these proteins. Since in the absence of functional Vif, APOBEC3G is packaged into the viral core in an RNA dependent manner, we propose that Vif packaging may help to exclude APOBEC3G from the viral core by competing or interfering with APOBEC3G packaging.

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Activation of PKR by Stem Loop RNAs Requires Flanking ssRNA Arms
C. Jason Wong, James L. Cole.
Protein kinase R (PKR) is a key component of the interferon-induced viral response pathway. PKR contains an N-terminal double-stranded RNA (dsRNA) binding domain and a C-terminal kinase domain connected by a ~90-residue linker. Upon binding dsRNA, PKR undergoes autophosphorylation and kinase activation. Activated PKR then phosphorylates the alpha subunit of the eukaryotic initiation growth factor 2 and thus inhibits protein synthesis in virally infected cells. A minimum of 30 bp of dsRNA is required to bind two PKRs, leading to dimerization and activation. Stem-loops are common RNA structural motifs. Recently, it was reported that short stem-loops containing 16 bp and ssRNA flanking arms are also able to activate PKR and that activation requires a 5'-triphosphate. However, the activation mechanism is not known. We have prepared RNAs containing a 15 bp stem-loop and various ssRNA flanking arms and characterized activation and binding of PKR. Activation by these RNAs requires ssRNA arms on both the 5' and 3' sides but it is independent of the presence of a 5'-triphosphate. Sedimentation velocity measurements indicate that a single PKR binds to the activating stem-loop RNA at 200 mM NaCl with Kd = 309 nM. PKR binding affinity is not strongly affected by removal of either of the ssRNA arms or the 5'-triphosphate. At lower [NaCl] (75 mM), each of the stem-loop RNAs can bind two monomers of PKR. However, only the activating RNA exhibits a positive binding cooperativity. We propose that activation by this RNA is due to enhanced population of the species containing two bound PKRs as a result of cooperative binding of the second PKR monomer.

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Inhibition of PKR by Adenovirus-Associated RNA I
Katherine Launer-Felty, C. Jason Wong, Ahmed M. Wahid, Graeme L. Conn, James L. Cole.
Protein kinase R (PKR) is a component of the innnate immunity pathway that is activated by dsRNA to undergo dimerization and autophosphorylation. Adenovirus virus-associated RNA I (VA I) is a short, non-coding transcript that functions to inhibit the activity of PKR in the host cell by acting as an RNA decoy. VA I contains three domains: an apical stem-loop, a central domain, and a terminal stem. Previous work suggests that PKR binding is localized to the apical stem and central domain regions. We have characterized the PKR binding stoichiometry and affinity using sedimentation velocity analytical ultracentrifugation and isothermal titration calorimetry. Although two PKR molecules clearly bind to VA I in the absence of divalent ion, only one PKR binds in the presence of Mg2+ and the binding affinity is reduced by about 20-fold. In contrast, PKR binding to regular dsRNAs is not strongly affected by divalent ion. Thus, Mg2+ may be required for VA I to fold. Interestingly, we do not detect large structural changes in the RNA by small angle X-ray scattering upon addition of Mg2+. Removal of the VA I terminal stem does not affect PKR binding affinity or inhibition. PKR binds more strongly to the highly- structured,