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John D. Ivanovitch
The University of Montana

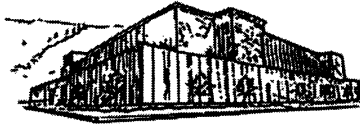
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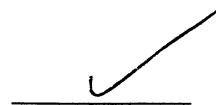
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**Structural analysis of dimerization-competent conformational variants
within the 5'-untranslated region of human immunodeficiency virus
type-2 genomic RNA**

By

John D. Ivanovitch

B.A., University of Montana, Missoula, Montana, 1999

Presented in partial fulfillment of the requirements

For the degree of

Master of Science

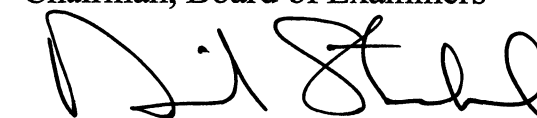
THE UNIVERSITY OF MONTANA

2003

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Ivanovitch, John D., M.S., May 2003 Biochemistry

Structural analysis of dimerization competent conformational variants within the 5'-untranslated region of human immunodeficiency virus type-2 genomic RNA

Director: J. Stephen Lodmell



A central event in the replication cycle of retroviruses is the encapsidation of two homologous strands of genomic RNA. A critical step in this process is the dimerization of the genomic RNA prior to or during encapsidation and budding. In human immunodeficiency virus type-1 (HIV-1) this process has been well documented. HIV-1 initiates dimerization through a stem-loop (SL1) located in the 5'-untranslated region of the viral genome. SL1 contains an autocomplementary hexonucleotide sequence that interacts with its homologous counterpart by a "kissing-loop" mechanism. Mutations or deletions in SL1 have been shown to affect at least three steps of retroviral replication: dimerization, encapsidation, and reverse transcription. Additionally, mutations or deletions in SL1 decreased viral infectivity by one to three orders of magnitude. Although much is known regarding this process in HIV-1, the dimerization mechanism used by human immunodeficiency virus type-2 (HIV-2) has yet to be definitively demonstrated. The research presented for this thesis was designed to analyze the structure of conformational variants of HIV-2 leader region RNA. Using solution structure probing, various HIV-2 RNA constructs were structurally analyzed in order to elucidate conformational changes correlated with dimerization behavior.

Acknowledgements

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Introduction

Background

In 1892, the Russian Scientist Dimitrii Ivanowsky provided the first report of a pathogenic agent smaller than any known bacterium. He observed that upon filtration to remove bacteria from extracts and culture media, the causative agent of the tobacco mosaic virus was not retained. Further work by Beijerinck, Loeffler, Frosch and others verified that not only did pathogens smaller than bacteria exist, they also seemed incapable of replicating outside of the host organism. These infectious agents were labeled ultrafilterable viruses and eventually were simply called viruses, from the Latin term meaning “poison.”

In 1902, yellow fever became the first human virus to be identified. Yellow fever had been known to be widespread in tropical countries since the 15th century, resulting in devastating epidemics. However, the disease was not directly contagious and early searches for an infectious agent proved to be unsuccessful. In 1880, Carlos Juan Finlay, a Cuban physician, postulated that a blood-sucking insect played a role in the transmission of the disease. This paved the way for a study on the etiology of yellow fever commissioned by the U.S. Army in 1889, in part due to the high incidence of the disease among U.S. soldiers who were occupying Cuba at the time. Colonel Walter Reed along with Dr. Jesse Lazear headed the study. Dr. Lazear was the first person to be experimentally infected with blood from a mosquito and his resultant death strongly

suggested that mosquitoes did indeed transmit yellow fever. Ultimately, Reed's studies proved conclusively that mosquitoes are the disease vector for yellow fever.

Additionally, they demonstrated that human blood was the source of the infection. The introduction of mosquito control dramatically reduced the incidence of yellow fever by 1902, and mosquito control remains an important method of control for the disease to this day.

During the early part of the 20th century the discovery of other viruses occurred at a slow pace, due in part to the dangers of working with viruses, as evidenced by yellow fever, and also to the lack of experimental techniques available. However, with the advent of the electron microscope and new molecular biological techniques the list of viruses rapidly expanded. A recent report from the International Committee on Taxonomy of Viruses (ICTV) states that there are approximately 4000 viruses that infect plants, animals and bacteria. All of these viruses have been assigned to one of 71 currently recognized families, 9 subfamilies and 164 genera.

In addition to the ever increasing number of identified viruses, there is now a much more precise definition than that given by early virologists. The definitive properties of viruses are summarized as follows. A virus is a very small, infectious, obligate intracellular parasite. The virus genome is comprised of either DNA or RNA and is replicated and directs the synthesis, by cellular systems, of other virion components. Within an appropriate host cell, de novo assembly from newly synthesized components within the host cell forms progeny virions. A progeny virion assembled

during the infectious cycle is the vehicle for transmission of the viral genome to the next host cell or organism.

Recently there has been an increasing prevalence of emergent viruses resulting in diseases such as West Nile Fever and Severe Acute Respiratory Syndrome or SARS. It seems likely that emergent viruses will become an ever-increasing phenomenon of today's world. With natural geographic barriers to the containment of viruses removed by modern modes of transportation the need to study and understand these viruses becomes critical to developing strategies to overcome the devastating consequences of infection.

One such emergent virus is human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (AIDS) (Hahn et al., 1984). Despite almost 20 years of intensive research, AIDS remains a devastating disease in many parts of the world. With approximately 70 million people infected and more than 20 million dead, the AIDS pandemic is still one of the greatest medical challenges the world faces (UNAIDS. AIDS epidemic update 2002). The research in this thesis focuses on a specific step in the replication cycle of human immunodeficiency virus known as genomic RNA dimerization. However, in order to understand the significance of this research we must first review retroviruses, focusing on a specific retrovirus, HIV.

Retroviridae

The *retroviridae* consist of an expansive family of viruses responsible for a variety of diseases ranging from malignancies to immunodeficiencies. Depending upon their pathogenicity and host range the retroviruses have been divided into three sub-families: the *Oncovirinae* (ASLV, MLV, MMTV, HTLV, BLV), the *Lentivirinae* (HIV, FIV, BIV, Visna-Maedi virus, EIAV, CAEV) and the *Spumavirinae* (SSRV, HSRV).

One unique aspect of retroviruses is that they encapsidate two single-stranded positive RNA molecules (8-11 Kb), making them the only diploid viruses identified to date (Beemon et al., 1974; Duesberg et al., 1973). Electron microscopy of the virion upon gentle lysis revealed that the two RNA strands are non-covalently joined near their 5'-ends in a region termed the dimer linkage structure (DLS) (figure 1) (Bender and Davidson, 1976; Hoglund et al., 1997; Kung et al., 1976).

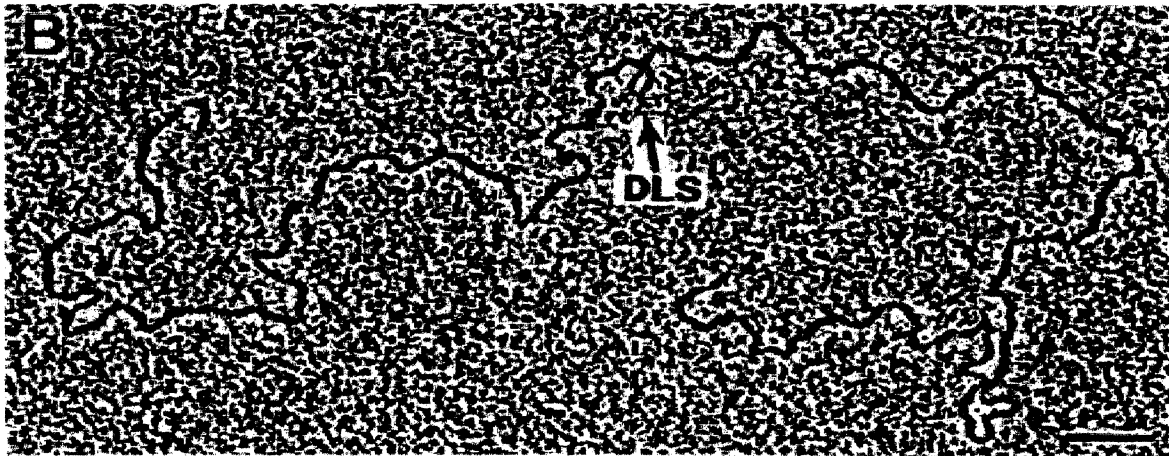


Figure 1. Upon gentle lysis of the virion an electron micrograph shows the 5'-ends of the retroviral RNA joined in a region termed the Dimer Linkage Structure (DLS). Figure taken from Hoglund et al, (1997).

The ssRNAs contain a 5' cap and a 3' poly (A) sequence, as observed in eucaryotic mRNAs (Varmus, 1982). The order of the genes encoding viral proteins is invariably *gag-pol-env*, although for some viruses several open reading frames coding for viral accessory/ regulatory proteins interrupt this sequence (Cullen, 1991).

Additional hallmarks of retroviruses are reverse transcription and integration into the host genome. The first reported evidence for the existence of an RNA-directed DNA polymerase activity in retroviral particles occurred in 1970 with two reports in *Nature* from the laboratories of David Baltimore and Howard Temin (Baltimore, 1970; Temin and Mizutani, 1970). With the discovery of reverse transcription, the central dogma of molecular biology, that the transfer of genetic information is unidirectional, DNA→RNA→protein, needed to be amended. Thus it became apparent that the flow of information could be retrograde, that is RNA could be used as a template for DNA synthesis. Hence, viruses capable of reverse transcription came to be known as retroviruses.

Encapsidating the dimeric retroviral genome is a roughly spherical vessel comprised of viral and cellular proteins as well as lipids derived from the host cell

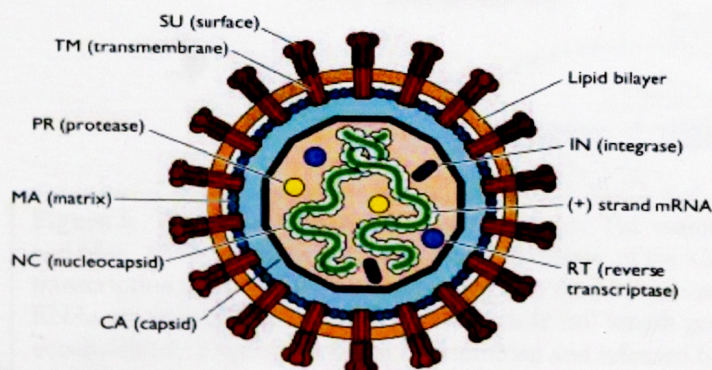


Figure 2. Diagram of a complex retrovirus. Indicated are the names and locations of the component proteins, genomic RNA, and envelope. Figure taken from Flint et al, (2002)

membrane (figure 2). Within the virion, the dimeric RNA is coated with a protein called nucleocapsid (NC), with approximately one molecule for every 10 nucleotides (Negroni and Buc, 1999). Additional components of the virion include a non-random collection of tRNAs necessary for the initiation of reverse transcription, approximately 50 to 100 molecules of reverse transcriptase (RT) and two other viral enzymes, protease and integrase (reviewed in (Frankel and Young, 1998).

As highlighted in figure 3, the major features of the retroviral replication cycle include cell binding/ fusion, reverse transcription, integration into the host genome, transcription, translation of spliced RNAs, dimerization of full length genomic RNA, encapsidation, assembly, and finally release of the viral particle by budding.

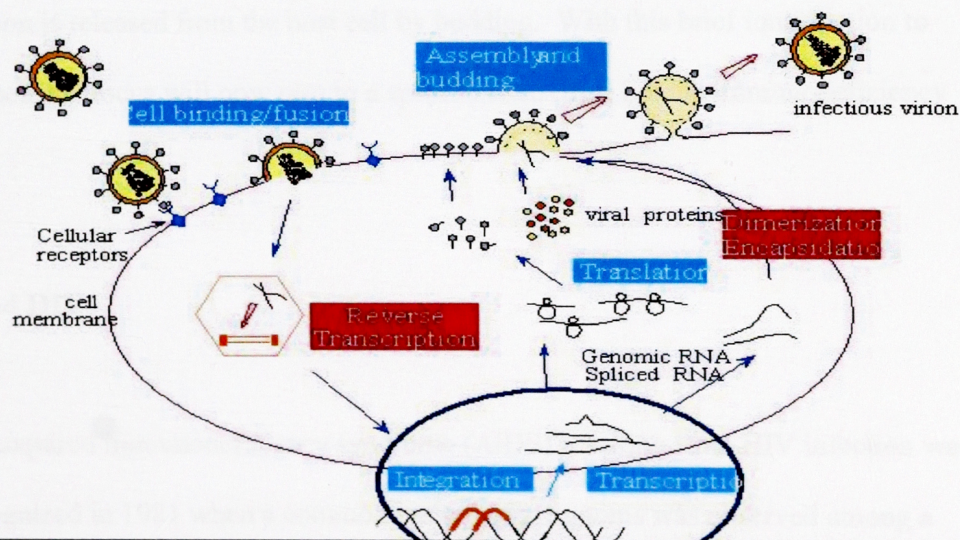


Figure 3. The replication cycle of the retrovirus. The major points are highlighted in red and blue. Cell binding results in fusion and release of the viral components. After reverse transcription the viral DNA is incorporated in the host genome. Upon transcription spliced RNAs are transcribed into viral proteins while full length genomic RNA is dimerized and encapsidated. Finally the virion is assembled and released by budding.

Interaction of the virion's surface proteins with specific host cell receptors results in fusion and release of the virion's contents into the host cell. Nucleocapsid uncoating of the genome, induced by reverse transcriptase, allows reverse transcriptase (RT) to initiate reverse transcription from a specific tRNA annealed to the primer binding site (PBS). Through a complex mechanism involving strand switching, full length genomic DNA is produced. Integration into the host genome is achieved via a virally encapsidated integrase resulting in the proviral stage of the replication cycle.

During the subsequent stages of the replication cycle, the cellular machinery of the host is used to transcribe and translate the various viral proteins used for assembly of the complete virion. Full length genomic RNA is dimerized and encapsidated and the virion is poised for assembly. During assembly, the various enzymes and proteins along with the dimeric genome coated in NC are brought together within the virion. Finally the intact virion is released from the host cell by budding. With this brief introduction to retroviruses, the focus will now turn to a specific retrovirus, human immunodeficiency virus.

AIDS and HIV

Acquired immunodeficiency syndrome (AIDS) resulting from HIV infection was first recognized in 1981 when a common pattern of symptoms was observed among a group of homosexual men in the United States (Brennan and Durack, 1981; Gottlieb et al., 1981). Currently an HIV-infected individual is diagnosed with AIDS when their

immune system is seriously compromised and manifestations of HIV infection are severe. The U.S. Centers for Disease Control and Prevention (CDC) defines AIDS as the presence of one of 26 conditions indicative of severe immunosuppression associated with HIV infection, such as *Pneumocystis carinii* pneumonia, a condition extremely rare in people without HIV. Other AIDS-defining conditions are also opportunistic infections, rarely causing disease in healthy individuals. HIV-infected individuals are also diagnosed with AIDS when their CD4+ T-cell count falls below 200-cells/cubic millimeter of blood (NIH Fact Sheet 2000). Healthy adults have CD4+ T-cell counts of 600-1,500 per cubic millimeter of blood (NIH Fact Sheet 2000).

At the beginning of this century greater than 36 million people worldwide were living with HIV/AIDS (i.e., approximately 35 million adults and 1.5 million children) (UNAIDS. AIDS epidemic update 2002). In the United States an estimated 800,000 to 900,000 people are living with HIV infection (UNAIDS. AIDS epidemic update 2002). Additionally, AIDS is the fifth leading cause of death among all U.S. adults aged 25 to 44, and among African-Americans it is the leading cause of death for men and the second leading cause for women (CDC. HIV/AIDS surveillance report 2001)

While most AIDS cases are due to HIV type 1 (HIV-1), another virus, HIV-2, infects a significant number of people in West Africa, Southeast Asia and Portugal (Reeves and Doms, 2002). Although similar in many ways, there are important differences that distinguish them as separate and distinct viruses. Major differences

include reduced pathogenicity of HIV-2, enhanced immune control of HIV-2 infection and often some degree of CD4-independence (Reeves and Doms, 2002).

HIV is thought to have originated from zoonotic transmission from simian immunodeficiency virus (SIV) infected non-human primates (Hahn et al., 2000; Sharp et al., 1999; Sharp et al., 1995). Indeed, phylogenetic analysis of the pathogenic retroviruses of primates (Figure 4) reveals that HIV-1 is derived from the chimpanzee and HIV-2 from the sooty mangabey monkey (Gao et al., 1994; Yamaguchi et al., 2000).

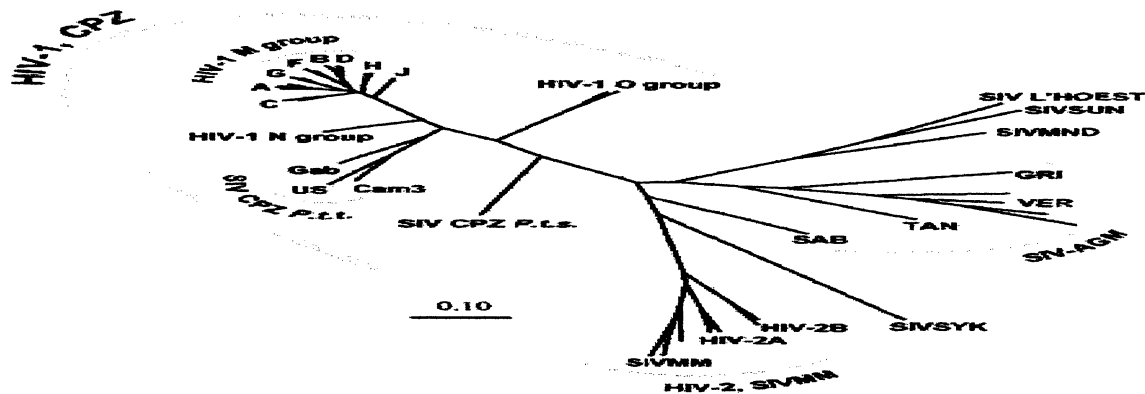


Figure 4. Phylogenetic analysis of primate lentiviruses. SIVs from chimpanzee cluster with HIV-1 while HIV-2 clusters with the sooty mangabey monkey. Figure taken from (Reeves and Doms, 2002).

Molecular biology of HIV

Although HIV-1 and HIV-2 are less than 50% identical at the nucleotide level, they still share important sequence and genomic organization (Guyader et al., 1987) (figures 5 and 6). The secondary structure models, represented schematically, for the 5'-untranslated regions of HIV-1 and HIV-2 genomic RNA are shown in figure 5. The Secondary structure elements of known function are proposed to attain the same

conformation and are in homologous locations for both viruses. In HIV-1 the trans-activation region stem-loop (TAR), the polyadenylation signal stem-loop, and the domain containing the primer binding site are followed by four small stem-loops necessary for genomic RNA dimerization (SL1/DIS), genomic RNA packaging (psi), splicing of the viral RNAs (SD) and the initiation of gag translation (AUG). Note the similarities between the proposed organization of secondary structure for HIV-1 and HIV-2 genomic RNAs.

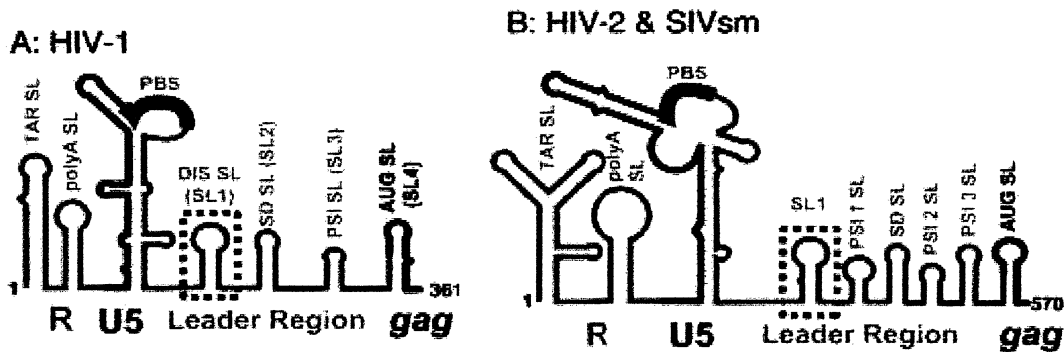


Figure 5. Schematic representation of the secondary structure of the 5'-untranslated region of HIV-1 and HIV-2 genomic RNA. Indicated are the regions with known functions. Figure taken from (Jossinet et al., 2001).

The genome organization for HIV-1 and HIV-2 are also similar (figure 6) with each possessing the open reading frames G-antigen (*gag*), polymerase (*pol*) and envelope (*env*). Additional genes code for the accessory proteins: negative factor protein (*nef*), viral infectivity factor protein (*vif*), viral protein τ (*vpr*), the response elements *rev* and *tat* as well as viral protein U (*vpu*), which is unique to HIV-1.

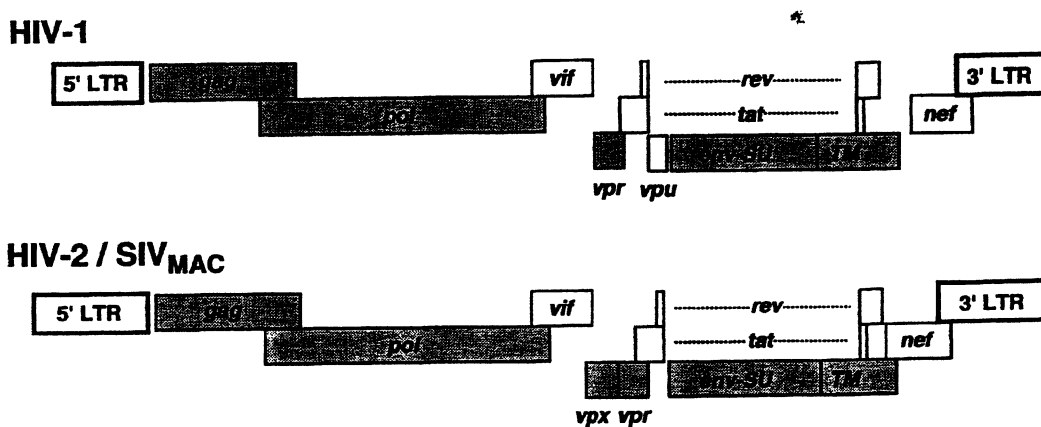


Figure 6. Schematic representation of the RNA genomes of HIV-1 and HIV-2. The major open reading frames for g-antigen (*gag*), polymerase (*pol*) and envelope (*env*) are indicated. Accessory regulatory genes (*tat* and *rev*) as well as nonessential genes (*nef*, *vif*, *vpu*, *vpx*, and *vpr*) are labeled as well. *Vpu* is found exclusively in HIV-1, whereas *vpx* is found only in HIV-2 and certain strains of SIV. Figure taken from Luciw, (1996).

Retroviral Genome Dimerization

It has been definitively established that RNA loop-loop interactions are commonly used to establish initial recognition between two RNA molecules (Brunel et al., 2002; Greathorex and Lever, 1998). Through either Watson-Crick base pairing or non-canonical interactions, hairpin loops can confer either intra- or intermolecular associations (Batey et al., 1999; Brion and Westhof, 1997; Pyle and Green, 1995; van Batenburg et al., 2001; van Batenburg et al., 2000). These interactions facilitate RNA folding by directing local motifs, which can serve as protein recognition signals, active catalytic sites, or for coordinating the assembly of modules in complex RNAs (Brunel et al., 2002). In addition, long-range interactions coordinate the tertiary structure of RNA molecules, which can positively or negatively affect their fates (Dirac et al., 2002; Lanchy et al., 2003b).

As previously alluded to, electron microscopy studies of the retroviral genomic RNA of Rous sarcoma virus (RSV) revealed that it was dimeric, consisting of two homologous ssRNAs associated near their 5'-ends (Mangel et al., 1974; Murti et al., 1981). The dimeric nature of the retroviral genome was further supported by sedimentation analysis and gel electrophoresis (Cheung et al., 1972; Fu and Rein, 1993). However, additional characterization of the DLS progressed slowly, due to the lack of experimental techniques available. However, this changed when Darlix and co-workers were able to demonstrate that *in vitro* transcribed RNAs corresponding to the 5' region of retroviral genomes were able to dimerize in a salt-dependent manner in the absence of proteins or other cellular factors (Darlix et al., 1990). With this advance, the process of genomic dimerization of retroviruses became more amiable to investigation. In particular, the dimerization mechanism for HIV-1 genomic RNA has been extensively characterized.

Numerous studies have linked the dimerization of the retroviral genome to several essential steps of the retroviral replication cycle, including encapsidation, translation and reverse transcription (Berkhout and van Wamel, 1996; Clever and Parslow, 1997; Haddrick et al., 1996; Hirota et al., 1997; Laughrea et al., 1997; Laughrea et al., 1999; Paillart et al., 1996a; St Louis et al., 1998). Thus, understanding the process of dimerization becomes essential to developing antiretroviral therapies designed to disrupt the process.

HIV-1 Genomic RNA Dimerization

Initial *in vitro* studies on HIV-1 genomic RNA mapped the dimer linkage structure of HIV-1 to a region downstream of the splice donor site, containing conserved purine tracts that were thought to mediate dimerization through purine quartets (Awang and Sen, 1993; Marquet et al., 1991; Sundquist and Heaphy, 1993; Weiss et al., 1993). In 1994, the cohort of Skripkin et al. utilized chemical modification interference and site directed mutagenesis to unambiguously identify the nucleotides that were responsible for HIV-1 dimerization *in vitro* (Skripkin et al., 1994). Using chemical modification interference, their results identified a short region located upstream of the splice donor (SD) site, containing a palindromic sequence, as the region necessary for the initiation of genome dimerization (Figure 7).

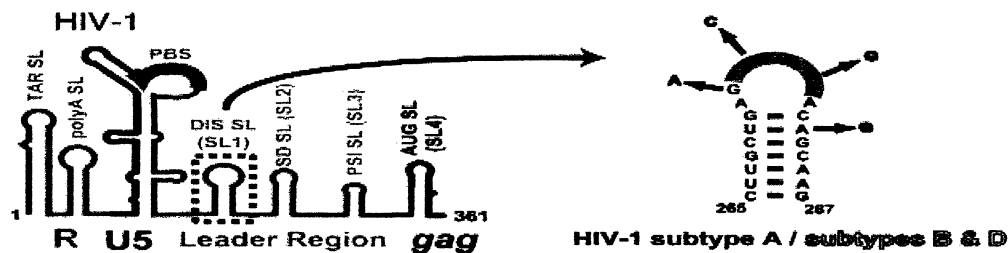


Figure 7. Diagramed is the 5'-untranslated region of HIV-1. To the right the DIS is shown in greater detail, the palindromic sequence used to initiate dimerization is shaded. Figure taken from (Jossinet et al., 2001)

They next demonstrated that mutations in the palindromic sequence resulted in abrogation of dimerization, while deletion of the purine tracts downstream of the SD site did not prevent dimerization, showing that the initiation of dimerization does not require the presence of purine quartets.

The initiation of dimerization was proposed to occur through a kissing loop mechanism in which the exposed palindromic sequences at the tip of the stem loops interacted via Watson-Crick base pairing (figure 8) (Skripkin et al., 1994). Additional research verified the existence of the proposed kissing loop complex (Clever et al., 1996; Fu et al., 1994; Haddrick et al., 1996; Muriaux et al., 1995; Paillart et al., 1994).

Upon release from the cell, the dimeric genome within the virion undergoes a conformational change making it more thermostable (Fu et al., 1994; Laughrea et al., 2001). This process was shown to be coordinated by the viral protein nucleocapsid (NC) *In vivo* (Feng et al., 1996). *In vitro* it was observed that incubation at 37 °C resulted in a population of dimeric RNAs that could be dissociated relatively easily (termed loose dimers), while incubation at 55 °C produced dimers with significantly more stability (termed tight dimers) (Berkhout and van Wamel, 2000). It was proposed that the more stable tight dimer had converted from a kissing loop complex to the more extensively hydrogen bonded extended duplex (Paillart et al., 1996b). The putative mechanism for this conformational switch is shown in figure 8. Additionally, HIV-1 RNA dimerization is modulated through elements located upstream and downstream of the DIS via a long distance interaction (Abbink and Berkhout, 2003; Berkhout et al., 2002; Huthoff and Berkhout, 2001).

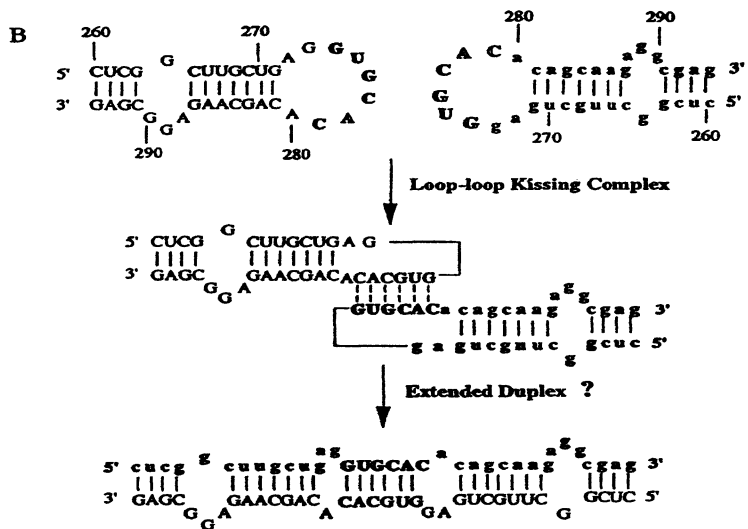


Figure 8. Shown on top are two stem loops poised for the initiation of dimerization. The middle figure represents the interactions occurring during the kissing-loop complex. The bottom figure shows the base pairing involved in an extended duplex. Figure taken from (Paillart et al., 1996b).

Because of the similarities in genome organization between HIV-1 and HIV-2 (see figure 5) it was initially thought that the mechanism for HIV-2 would be similar to that observed for HIV-1. However, as discussed below, this was not to be the case.

HIV-2 Genomic RNA Dimerization

Although it has been proposed that HIV-2 RNA has homologous secondary structure to that of HIV-1 RNA (Berkhout, 1996), the mechanism of HIV-2 RNA dimerization is still a subject of controversy. Initial studies designed to characterize the dimerization initiation site of HIV-2 focused on stem loop 1 (SL1) of the genomic RNA. Due to SL1's location and overall similarity to the dimerization initiation signal (DIS) in HIV-1 RNA it seemed logical that SL1 would promote genome dimerization of HIV-2

genomic RNA (figure 5). However, in 2001 research conducted by Jossinet et al. clearly showed that the SL1 was dispensable for HIV-2 dimerization *in vitro* (Jossinet et al., 2001). Specifically, they demonstrated that HIV-2 RNAs truncated to exclude SL1 were still capable of dimerization. Moreover, the minimal dimerization element of HIV-1 (a 50-mer encompassing HIV-1 SL1) was able to dimerize while a 47-mer encompassing the SL1 of HIV-2 RNA was not. Chemical probing indicated that the HIV-2 SL1 adopted the expected stem-loop structure, both in the 47-mer RNA and in the 1-5561 HIV-2 RNA. Further truncation analysis of the HIV-2 RNA revealed that a palindromic sequence (GGCGCC) located in the primer binding site (PBS) was an *in vitro* dimerization element.

Another group of researchers later submitted a contradictory report, suggesting that the SL1 of HIV-2 RNA mediated *in vitro* dimerization and not the palindromic sequence located in the PBS (Dirac et al., 2001). In their hands, an HIV-2 RNA truncated at the 5'-end of SL1 (1-444) was able to dimerize efficiently while another HIV-2 genomic RNA truncated at the 5'-end of the PBS domain was not. A critical comparison of the *in vitro* dimerization protocols employed in the previous studies revealed significant differences that could account for the observed discrepancies [for a thorough review see (Lanchy and Lodmell, 2002)]. Briefly, these differences included the length of the RNA used, the incubation time and temperatures, the monovalent cations used in the dimerization buffer, as well as the gel electrophoresis conditions used to resolve the dimers.

Also in the Lanchy et al. study, they were able to demonstrate that the 1-444 RNA, used to establish SL1 as the dimerization element of HIV-2 by Dirac et al., was unique among the wild-type constructs tested in its ability to utilize SL1 to form tight dimers; in this instance a tight dimer is referred to as a dimer that is able to resist denaturation during fairly stringent electrophoretic [Tris-Borate EDTA (TBE)] conditions at room temperature. Interestingly, by mutating or deleting regions within the PBS or SL1, they also demonstrated that disruption of the default dimerization element, the PBS, resulted in a switch to the alternative dimerization element, SL1. They further suggested that such a phenomenon could represent a switching mechanism that might be utilized during the viral replication cycle.

Further characterization of HIV-2 genomic dimerization revealed that sequences upstream and downstream of SL1 could influence dimerization, suggesting that HIV-2 genomic RNA, like HIV-1 RNA, can adopt an alternative conformation (Dirac et al., 2002). Additional work by Lanchy et al. precisely mapped the elements upstream and downstream of the major splice donor site that influenced the ability of HIV-2 leader RNA to dimerize *in vitro* (Lanchy et al., 2003b). By using a combination of 5'- and 3'-truncations of the HIV-2 leader RNA they were able to locate two core elements located at nucleotides 189-196 and 543-550 that interfered with the formation of SL1-dependent tight dimers. They suggested that base pairing between these sequences prevented the formation of SL1-dependent tight dimers by sequestering SL1 in a stable intramolecular conformation. As shown in figure 9, a secondary structure model was generated using Mfold and chemical probing data, that incorporates the base pairing of the two identified

interfering elements and the sequestering of SL1. More recent research has revealed that there is another dimerization element for HIV-2 genomic RNA located in a region shown to be important for encapsidation *in vivo*, called the Ψ palindrome (Lanchy et al., 2003a).

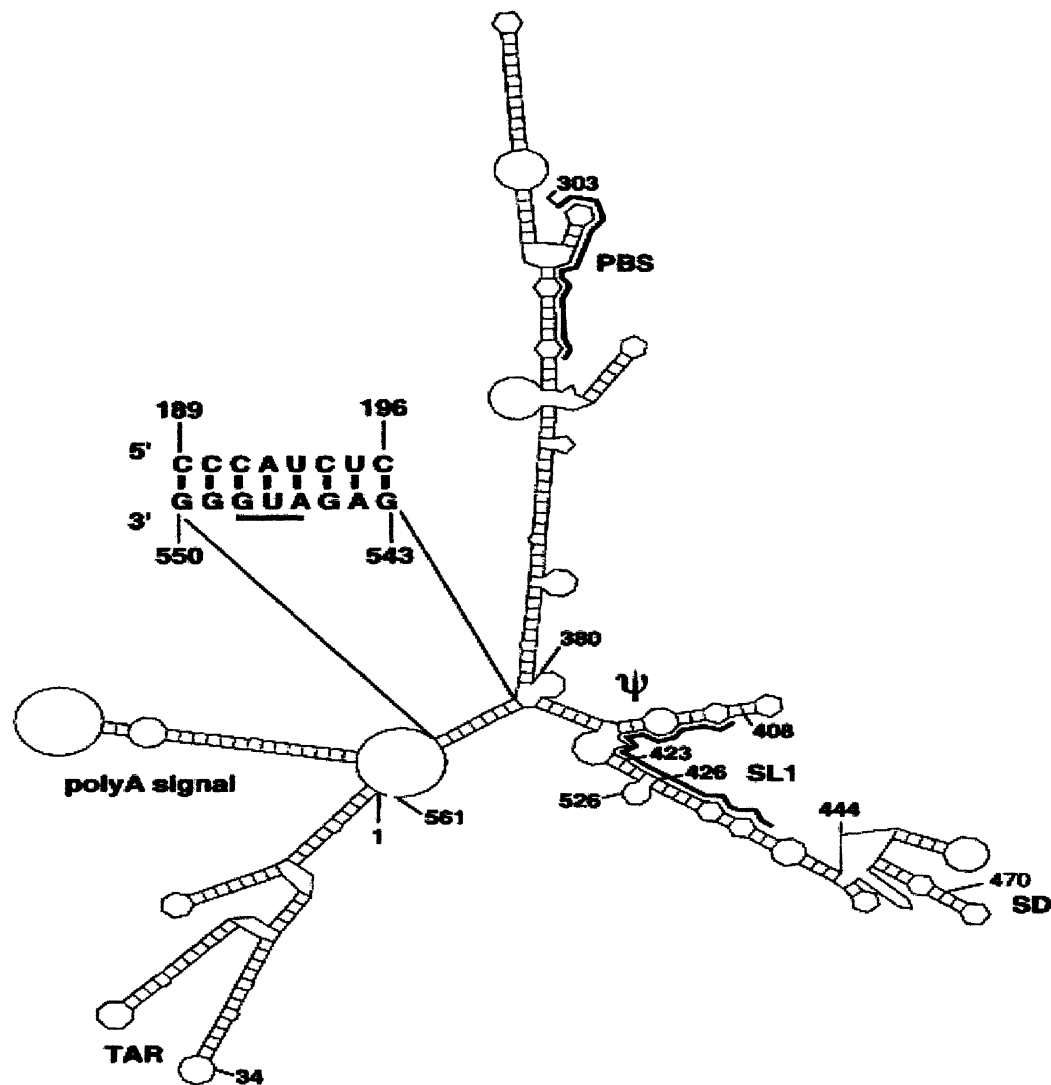


Figure 9. Shown is the secondary structure model of the HIV-2 ROD leader RNA as predicted using M-fold constrained by results obtained from biochemical analysis. The putative long distance interaction is shown to the left. The solid lines indicate the PBS and SL1 domains and the Ψ represents the core encapsidation signal (nts 380-408). Figure taken from (Lanchy et al., 2003b)

Objectives and experimental design

Dimerization is an essential step in the viral replication cycle and therefore represents a potential target for antiretroviral therapy. While the elements necessary for dimerization of the HIV-1 genome have been identified, those for HIV-2 are currently being characterized and debated. The research presented in this thesis was designed to analyze the dimerization-competent conformational variants observed for HIV-2 genomic RNA. Using solution structure probing, various HIV-2 constructs were systematically modified under several *in vitro* dimerization conditions and the consequences of modification analyzed by primer extension as described below.

The initial specific aims as outlined in my research proposal are as follows:

Specific aim one: Identification of the minimal dimerization domain of HIV-2.

I will map the dimerization signal(s) by truncation analysis of RNA transcripts encompassing the 5'-leader region of HIV-2 genomic RNA.

Specific aim two: Chemical structure probing and secondary structure analysis of HIV-2.

Secondary structure maps of monomeric and dimeric RNAs will be constructed using data derived from chemical and enzymatic solution structure probing techniques.

Specific aim three: Chemical modification interference mapping.

Chemically modified RNAs will be subjected to in vitro dimerization assays and the consequences of base modification analyzed by primer extension.

Although some of the experimental design had to be modified to adjust for unforeseen properties of the HIV-2 genome the basic methods remained the same. Briefly, various HIV-2 RNA constructs were subjected to solution structure probing followed by primer extension to reveal the location of the modifications.

Solution Structure Probing

Nucleic acid modifying reagents are valuable tools for analyzing secondary and tertiary structure of RNA molecules. Researchers take advantage of the fact that Watson-Crick faces of nucleotides can be chemically modified if the nucleotide is not base-paired (figures 10 A and B) (Ehresmann et al., 1987). Various modification reagents are available. For instance, dimethyl sulfate (DMS) alkylates the Watson-Crick positions of adenosines (N1) and cytidines (N3) not involved in base pairing or tertiary-structure, while CMCT modifies the N3-position of uridines and, to a lesser extent, the N1-position of guanosines that are not involved in Watson-Crick base-pairing. Additionally, enzymatic modification of ssRNAs is also possible. For example, the enzyme RNase T1 cleaves 3' to guanosines and T2 cleaves 3' to adenosines in ssRNAs.

A

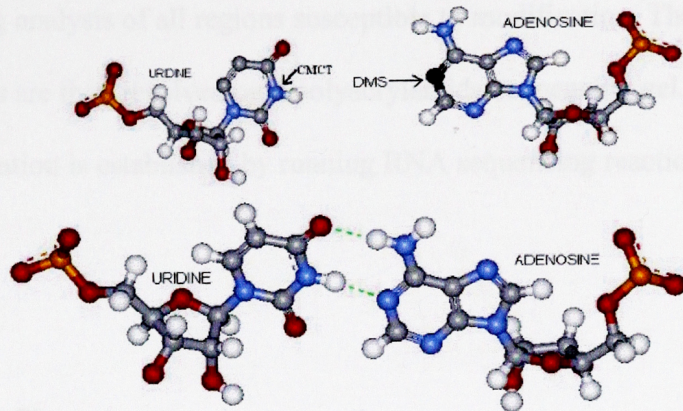


Figure 10 A. Sites of chemical modification for adenosine and uridine. Modification occurs on the Watson-Crick faces at N1 of adenosine and N3 of uridine.

B

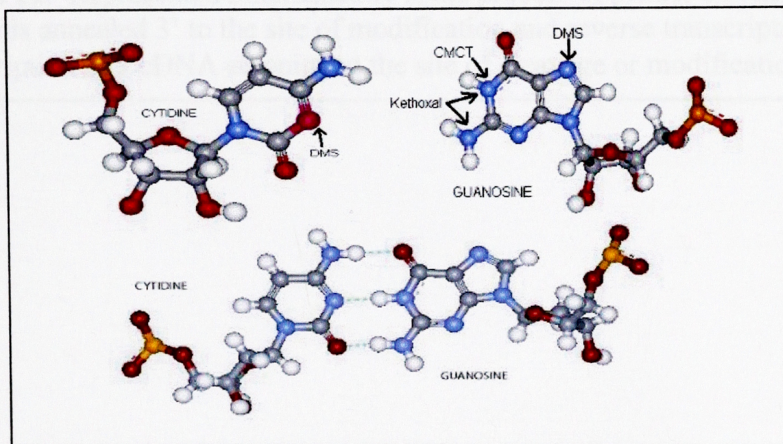


Figure 10 B. Sites of chemical modification for guanosines and cytidines. Modification occurs at the Watson-Crick faces at N1 of guanosine and N3 of cytidines.

In order to visualize the modifications, reverse transcription was used. Reverse transcriptase generates a complementary copy of DNA (cDNA) from an RNA template. When a base in the RNA is chemically modified, or there is a break in the ribose-phosphate backbone, the polymerase will pause 3' to this location (figure 11). Moreover, the RNA is modified or digested in a manner that only allows one 'hit' per molecule, thus

allowing analysis of all regions susceptible to modification. These reverse transcription products are then resolved on a polyacrylamide sequencing gel. The exact site of modification is established by running RNA sequencing reactions in adjacent lanes.



Figure 11. Represented schematically is the process of primer extension. A primer is annealed 3' to the site of modification and reverse transcriptase initiates transcription of a cDNA stopping at the site of cleavage or modification.

Materials and Methods

Template construction for *in vitro* transcription

A sense primer containing a *Bam*HI site and the promoter for the phage T7 RNA polymerase and an antisense primer containing an *Eco*RI site (IDT Integrated DNA Technologies, Inc.) were used to PCR-amplify (Eppendorf) the truncated HIV-2 leader RNAs used for the minimal dimerization study (Table 1, oligonucleotides designated nt).

Table 1: Oligonucleotides Used in This Study

F2-1545	5'-AGTTTCTCGCGCCCATC-3'
F2-1457	5'-ACCTTCACCCGGAGGC-3'
F2-1 343	5'-CACTCAGGCGTGTTCC-3'
F2-1242	5'-GTCCTAACACACCAGGG-3'
F2-1144	5'-GGCAGCTTTATTAAGAGG-3'
asROD242	5'-GTCTTGTTACTCAGGTGAACACC-3'
asROD219	5'-GAATGACCAGGCGGCGACTAGGAG-3'
asECO444BIS	5'-TTGAATTCGCTCCACACGCTG-3'
asECO325	5'-AAGAATTCAAGTCCCTGTTC-3'
asECO381	5'-TAGAATTCGTTCTGCGCCC-3'
nt197	5'-TAGGATCCTAATACGACTCACTATAGGTCCTAGTCGCCGCC-3'
nt233	5'-TAGGATCCTAATACGACTCACTATAGGTAACAAGACCCTGG-3'
nt253	5'-TAGGATCCTAATACGACTCACTATAGGACCCTTCTTGCTTTGG-3'
nt325	5'-AAGAATTCAAGTCCCTGTTC-3'
nt381	5'-TAGAATTCGTTCTGCGCCC-3'
as550	5'-CCCATCTCCCACAATCTTCTACC-3'

The PCR products were fractionated on a 0.8% agarose (FisherBiotech) gel and the DNA extracted using the Ultrafree-DNA extraction columns (Millipore) according to the

manufacturer's directions. After purification the products were digested with *Bam*HI and *Eco*RI restriction enzymes (New England Biolabs) and ligated into the *Bam*HI and *Eco*RI sites of the pUC18 plasmid. Next, DH5 α cells were transformed with the plasmids and plated onto LB-ampicillin plates (100 μ g/mL) and grown overnight at 37° C. Colonies were picked, plasmids purified by a mini-prep kit (Eppendorf), and the DNA analyzed by restriction digestion, gel-electrophoresis and sequencing (Murdock laboratory, University of MT). Once incorporation of the appropriate sequence was established it was amplified by maxi-plasmid prep (Eppendorf) and used to generate RNA transcripts (see below).

Additionally, a sense primer containing a *Bam*HI site and the promoter for the phage T7 RNA polymerase and an antisense primer containing an *Eco*RI site (IDT Integrated DNA Technologies, Inc.) were used to amplify the first 381, 444, 493, 526 or 561 nucleotides of the HIV-2 genomic RNA sequence, ROD isolate. The HIV-2 ROD DNA template (modified plasmid pROD10) was provided by the EU Programme EVA/MRC Centralised Facility for AIDS Reagents, NIBSC, UK (Grants QLK2-CT_1999-00609 and GP828102). The numbering is based on the genomic RNA sequence.

RNA synthesis and purification

The different plasmids were linearized with *Eco*RI (New England Biolabs) and the RNAs were generated by an *in vitro* transcription using the Ampliscribe™ T7 transcription kit (Epicentre) according to the manufacturer's directions. Following completion of transcription the DNA template was degraded with the supplied RNase-

free DNase and the RNA precipitated by the addition of 1 volume of 5 M ammonium acetate. The RNA was pelleted by centrifugation at 16,436 x g (Sorvall Biofuge Primo R, 7597 rotor) at 4 °C, ethanol-washed, resuspended in water, phenol:chloroform (1:1) extracted and ethanol precipitated. Finally, the RNAs were subjected to exclusion chromatography (Bio-Gel® P-4, Bio-Rad). Analysis of the absorption spectrum at 260 nm and 280 nm allowed for quantification of the RNA (Hewlett Packard 8452 A) and gel-electrophoresis (Bio-Rad) was used for qualitative analysis.

***In vitro* dimerization of HIV-2 RNA**

The ability of the RNAs generated in the previous steps to dimerize was analyzed by *in vitro* dimerization assays as described below. The two protocols differ in the incubation temperatures and times as well as the electrophoresis conditions employed. In our hands, the alternate protocol was found to be equivalent to a protocol employed by Dirac et al. to assay for TBE-resistant dimers during electrophoresis (Dirac et al., 2001)

Standard Protocol, adapted from (Jossinet et al., 2001)

Five pmol of HIV-2 RNA were diluted in 4 µL of water, denatured at 92 °C for two min and then quench-cooled on ice. After cooling, 1µL of 5X monomer buffer (final concentrations: 50 mM sodium cacodylate, pH 7.5, 40 mM KCl, 0.1 mM MgCl₂) or dimer buffer (final concentrations: 50 mM sodium cacodylate, pH 7.5, 300 mM KCl, 5 mM MgCl₂) was added. Dimerization was carried out at 37° C for 15 min at which time the reactions were immediately placed on ice and subsequently analyzed by ‘native’ gel-

electrophoresis at 4° C in Tris borate, 45 mM, pH 8.3, 0.1 mM MgCl₂, 0.8% agarose for 90 minutes at 7 V/cm.

Alternate Protocol adapted from (Dirac et al., 2001)

Five pmol of HIV-2 RNA was diluted in 4 µL of water, denatured at 92° C for two minutes and then quench-cooled on ice. After cooling, 1 µL of 5X monomer buffer (final concentrations: 50 mM sodium cacodylate, pH 7.5, 40 mM KCl, 0.1 mM MgCl₂) or dimer buffer (final concentrations: 50 mM sodium cacodylate, pH 7.5, 300 mM KCl, 5 mM MgCl₂) was added. Dimerization was carried out at 55° C for 30 minutes at which time the reactions were immediately placed on ice and subsequently analyzed by agarose gel-electrophoresis at 24 °C in Tris borate, 45 mM, pH 8.3, 0.1 mM MgCl₂, 0.1 mM EDTA 0.8% agarose for 90 minutes at 7 V/cm. For both procedures, ethidium bromide-stained gels were either scanned with a Fluorescent Image Analyzer FLA-3000 (Fujifilm) or photographed directly (Fotodyne).

Enzymatic RNA Probing with RNase T1

The enzyme RNase T1 cleaves 3' to guanosines in ssRNAs. Therefore, this method was used to evaluate the base-pairing of guanosines in HIV-2 genomic RNA. A stop during primer extension would indicate that the guanosine is located in a single-stranded region while lack of cleavage would indicate base-pairing of the guanosine or the enzyme's lack of accessibility.

In a standard experiment, 5 pmol of HIV-2 leader RNA were diluted in 4 μ L of water, heated for 2 min at 92° C and quench-cooled on ice. The samples were incubated in dimer buffer (final concentrations: 50 mM sodium cacodylate , pH 7.5, 5 mM MgCl₂, 300 mM KCl) or monomer buffer (final concentrations: 50 mM sodium cacodylate, pH 7.5, 40 mM KCl, 0.1 mM MgCl₂) for 15 min or 30 min at 37° C or 55° C, respectively. The samples were quench-cooled on ice and 2 μ g of *Escherichia coli* tRNA (Sigma) and 0.11 units of RNase T1 (GibcoBRL) were added. The samples were incubated at 37° C for 5 min and ethanol precipitated. The samples were pelleted by centrifugation at 15,000 rpm (Sorvall Biofuge Primo R, 7597 rotor) at 4° C for 30 minutes, ethanol washed, vacuum dried (Savant Speed Vac SC110) and resuspended in water.

DMS (dimethyl sulfate) chemical probing

Dimethyl sulfate alkylates adenosines at position N1 and to a lesser extent cytidines at position N3 that are accessible and not base-paired at their Watson-Crick faces. In a standard experiment, 5 pmol of HIV-2 leader RNA was diluted in 4 μ L of water, heated for 2 min at 90° C and quench-cooled on ice. The samples were incubated in dimer buffer (final concentrations: 50 mM sodium cacodylate (pH 7.5), 5 mM MgCl₂, 300 mM KCl) or monomer buffer (final concentrations: 50 mM sodium cacodylate, pH 7.5, 40 mM KCl, 0.1 mM MgCl₂) for 15 min or 30 minutes at 37° C or 55° C, respectively. The samples were quench-cooled on ice, 1 μ l of 1:100 DMS (Sigma Aldrich), diluted in 95% ethanol, was added and the samples incubated at 37 ° C for 5 minutes followed by ethanol precipitation. The samples were pelleted by centrifugation

at 16,436 x g (Sorvall Biofuge Primo R, 7597 rotor) at 4 °C for 30 minutes, ethanol washed, vacuum dried (Savant Speed Vac SC110) and resuspended in water.

Oligonucleotide rescue probing

HIV-2 RNA dimers formed at physiological temperatures (37 °C) do not withstand denaturation on Tris-Borate EDTA agarose gels at room temperature (Lanchy and Lodmell, 2002). However, an oligonucleotide directed against the 3'-end of the RNA (as548 see Table 1) restores the ability to withstand denaturation (Lanchy et al., 2003a). In order to evaluate this phenomenon, RNAs were subjected to chemical and enzymatic probing in the presence of as548 as described below.

Chemical and enzymatic probing were as described above with the following changes: 5 pmol of HIV-2 RNA comprising nucleotides 1-561 were diluted in 4 µL of water with or without 10 pmol of antisense oligonucleotide as548 (see table 1), heated for 2 min at 92° C and quench-cooled on ice. Following modification, the samples were resuspended in 1X RQ1 DNase buffer (Promega) with 5 units of RQ1 RNase-free DNase (Promega) and incubated at 37° C for 60 min. The DNase was removed using EZ micropure enzyme removers (Millipore) as per the manufacturer's directions. The samples were ethanol precipitated, pelleted, ethanol washed, vacuum dried (Savant Speed Vac SC110) and resuspended in water.

Primer extension

The primer extension reaction proceeds in a three-step process as described by Moazed and Noller (1986). In the first step, a sequence specific primer (Table 1) is annealed to the HIV-2 leader RNA (modified as described previously). The next step is extension which results in the labeling of the DNA product with and the extension/specific termination from the primer. The chase reaction continues the extension of the primer and insures that all products that are still short, but not yet specifically terminated are chased from the gel.

Primer annealing: The DNA oligonucleotides used for primer extension in this study are listed in Table 1. The oligonucleotides were diluted in 4.5X hybridization buffer (50mM HEPES, pH 7.0, 100mM KCl) to 0.2 pmol/ μ L. 0.4 pmol of primer was added to 0.5 pmol of target RNA, the samples were heated at 92° C for one min and allowed to slow cool to 45° C.

The extension reaction was initiated by the addition of 2 μ L of extension mix to a final concentration of (.13 mM Tris-HCl (pH 8.5), 69.2 mM KCl, 32 mM MgCl₂, 32 mM dithiothreitol, 5.57 μ M dATP, dGTP, dCTP, 0.29 μ M dTTP (Amersham), 5.7 μ Ci [α -³²]-dTTP (PerkinElmer) and 0.11 units/ μ L avian myeloblastosis reverse transcriptase (Seikagaku America). The reaction mixtures were incubated at 42° C for 40 min, then 1 μ L of chase mix, 1 mM dATP, dGTP, dCTP, dTTP(Amersham), was added and the samples were incubated at 42° C for 15 min.

The reactions were terminated by the addition of 75 μ L of precipitation buffer (70% ethanol, 84 mM NaOAc, pH 6.5, and 0.8 mM EDTA, pH 8.0) with a 10 minute incubation at room temperature. The DNA transcripts were pelleted by centrifugation at 14,000 RPM (Eppendorf 5415 C) at room temperature (22 °C average), ethanol washed, vacuum dried (Savant Speed Vac SC110) and resuspended in 10 μ l tracking dye (7M urea, 1X TBE, 0.025% bromophenol blue and 0.025% xylene cyanol FF). 1 μ l of each sample was assayed for 32 P TTP incorporation by scintillation counting (Packard Tri-Carb 1500 liquid scintillation analyzer).

Sequencing was done by the method of Sanger and co-workers (Sanger et al., 1977). The reactions are as described above with the following additions: prior to extension 1.5 μ M of a specific ddNTP (Amersham; lane specific, A, G, C and U) was added to four individual tubes. Additionally, prior to the chase step, 6.7 μ M of a specific ddNTP was added to the four previous tubes. The samples were denatured for 2 min at 92° C and quench-cooled on ice. 1.5 μ L of each sample was loaded on an 8% polyacrylamide gel (600 x 250 mm) and electrophoresed (Gibco BRL model SA) at 80 watts for 3-4 hours. The gel was then transferred to filter paper (Whatman 3MM) and vacuum dried (Bio-Rad 583 gel dryer) for 30 min at 70° C. The dried gel was either exposed on X-ray film (Kodak Biomax MR) for 12-16 hours or an imaging plate (Fuji) was applied and subsequently analyzed on the phosphoimager (Fujifilm FLA-3000).

RESULTS

Statement of the problem

As previously mentioned, the dimerization of HIV-2 genomic RNA appears to be significantly different from that of HIV-1 genomic RNA. Functional studies of HIV-2 genomic RNA have revealed the presence of three *in vitro* dimerization elements as well as the existence of a long distance interaction that negatively affects tight dimer formation. The research conducted for this thesis was done in order to clarify and resolve the RNA structural elements and rearrangements involved in HIV-2 genomic RNA dimerization.

Overall strategy and design

To resolve the RNA structural elements and rearrangements involved in HIV-2 genomic RNA dimerization, several HIV-2 RNA constructs were subjected to solution structure probing under various *in vitro* dimerization conditions (see materials and methods) (figure 11.5). Briefly, either truncated or mutated HIV-2 leader RNAs were dimerized at 37° C for 15 minutes (standard/Jossinet protocol) or 55° C for 30 minutes (alternate/Dirac protocol) with or without oligonucleotide as548 present. Dimerization was carried out at the different temperatures because of the observation that HIV-1 genomic RNA formed tight dimers following thermal treatment, most likely due to a thermally-induced conformational change (Berkhout and van Wamel, 2000).

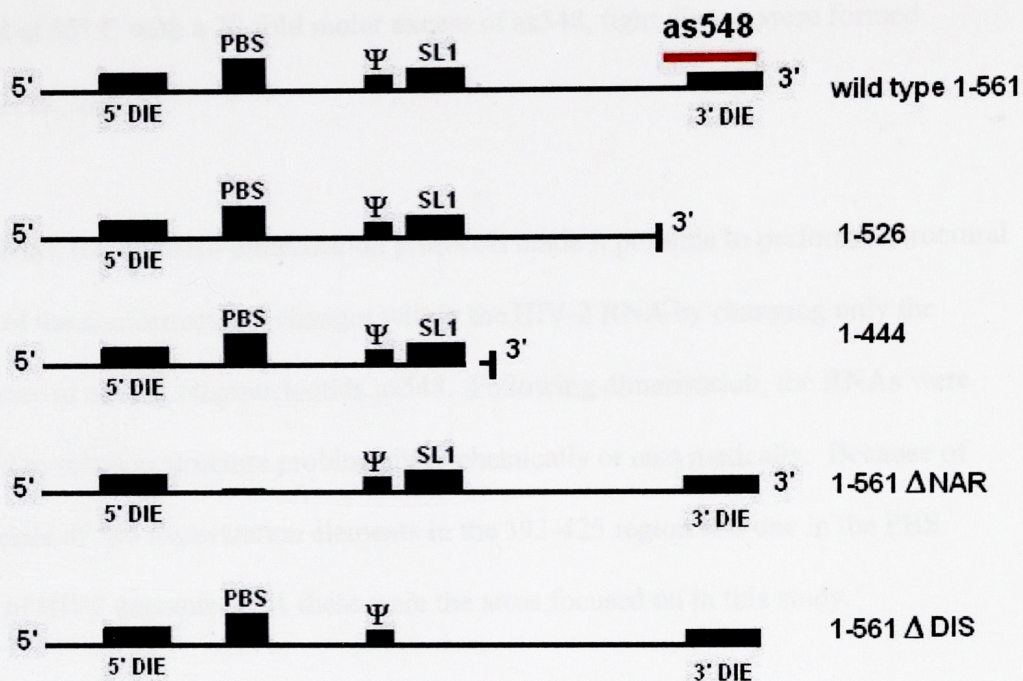


Figure 11.5. Schematic representation of the HIV-2 RNA constructs used in this study. The top figure represents the full-length leader region of wild-type HIV-2 genomic RNA. Shown are the putative dimerization elements of HIV-2. From left to right, the 5' dimer interference element (DIE), the primer binding site palindrome (PBS), the Ψ palindrome (Ψ), the SL1 palindrome (SL1), and the 3' dimer interference element. The line above the 3' DIE represents the region targeted by oligonucleotide as548. The 1-526 construct has the 3' DIE removed and the 1-444 construct is truncated just 3' of SL1. Δ NAR has the PBS palindrome removed and Δ DIS has 4 nucleotides in the SL1 palindrome removed.

Additionally, an HIV-2 RNA construct truncated at the 3' end of SL1 (nucleotides 1-444) formed loose dimers at 37 °C but not tight dimers following incubation in dimer buffer at 55 °C (Dirac et al., 2001; Lanchy and Lodmell, 2002). In this case, a tight dimer is recognized as one that withstands denaturation upon electrophoresis through a TBE agarose gel at approximately room temperature, presumably through more stable intermolecular interactions. Oligonucleotide as548 was used to analyze HIV-2 RNA

dimerization because it was observed that when wild type 1-561 HIV-2 RNA was dimerized at 55° C with a 20-fold molar excess of as548, tight dimers were formed (Lanchy et al., 2003b).

Thus, the different dimerization protocols made it possible to perform a structural analysis of the conformational changes within the HIV-2 RNA by changing only the temperature or adding oligonucleotide as548. Following dimerization, the RNAs were subjected to solution structure probing either chemically or enzymatically. Because of the presence of two dimerization elements in the 393-425 region and one in the PBS domain of HIV2 genomic RNA these were the areas focused on in this study.

Because dimerization represents a critical step in the replication cycle of retroviruses, it represents a potential target for antiretroviral therapies. Thus, understanding of the mechanism employed by viruses to dimerize their genomic RNA becomes essential in developing antiretroviral therapies directed to disrupt the dimerization process.

Functional analysis of HIV-2 RNA dimerization elements

As seen in figure12, 1-561 wild-type HIV-2 RNA was not able to form any significant amount of tight dimers when incubated at 55 °C and assayed on a Tris Borate EDTA (TBE) agarose gel at 28 °C (figure 12, lane 7). However, an HIV-2 RNA truncated at the 3'-end of the RNA (1-444) was able to form a tight dimer (figure 12, lane

3). Moreover, an oligonucleotide directed against nucleotides 397-426 of stem loop 1, asDIM, was able to abrogate tight dimerization of the 1-444 construct (figure 12, lane 4).

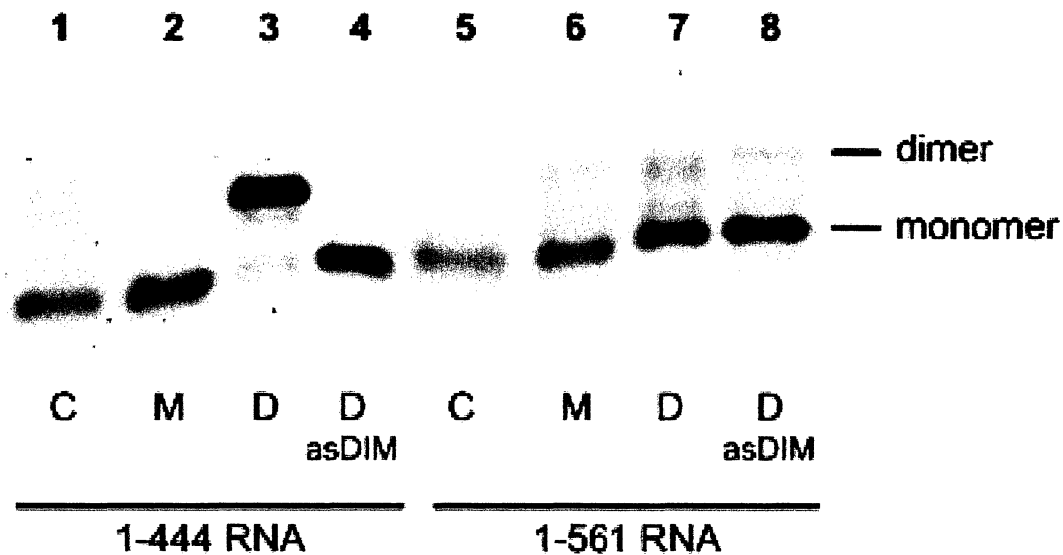


Figure 12. Tight dimer formation of 1-444 and 1-561 HIV-2 RNAs. 1-444 and 1-561 HIV-2 RNAs were incubated at 55 °C in dimer buffer (D), monomer buffer (M), or no buffer (C) for 30 minutes. After incubation the RNAs were subjected to electrophoresis on a TBE agarose gel at 28 °C. Only tight dimers withstand denaturation. The 1-444 RNA dimers were able to withstand denaturation upon electrophoresis (lane 3) while the 1-561 dimers did not (lane 7). The samples were also dimerized in the presence of a 20-fold excess of oligonucleotide asDIM, which is complementary to nucleotides 397-426 of SL1. The oligo asDIM was able to disrupt the ability of 1-444 RNA to form tight dimers (lane 4). Figure taken from (Lanchy et al., 2003a)

The existence of a long range interaction between nucleotides upstream and downstream of SL1 has been reported by our laboratory (see figure 8 in the Introduction) (Lanchy et al., 2003b). Moreover, the interaction of nucleotides 189-196 and 543-550 was shown to modulate dimerization *in vitro* (Lanchy et al., 2003b). Antisense oligonucleotide as202 targets the upstream region of the dimer-interfering element and when present during dimerization was able to restore the ability to form tight dimers

(figure 13, lane 2). Additionally, antisense oligonucleotide as548, which targets the downstream region of the dimer-interfering element, was able to restore tight dimerization (figure 13, lane 3). The addition of both as202 and as548 resulted in an almost complete shift of the RNA to the tight dimer pool (figure 13, lane 8). Moreover, the addition of asDIM inhibited tight dimer formation (figure 13, lanes 5, 6, and 9).

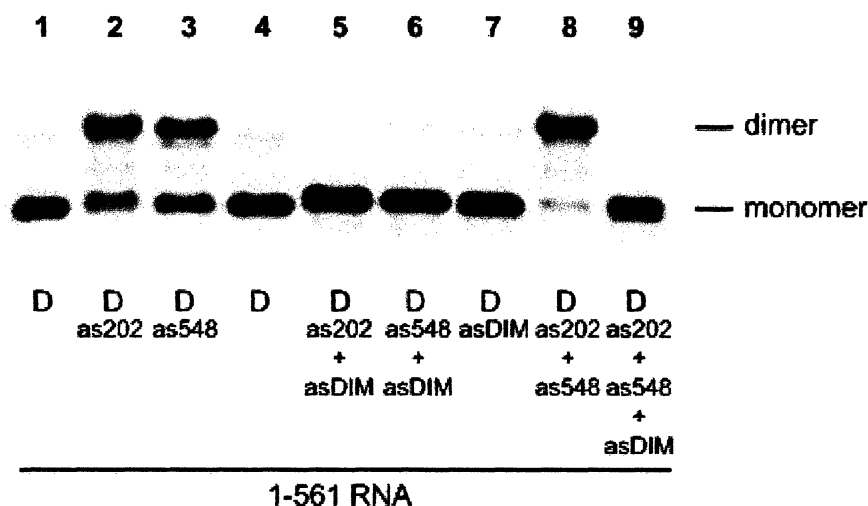


Figure 13. Antisense oligonucleotide-directed restoration and suppression of 1-561 HIV-2 RNA tight dimerization. 1-561 RNA was incubated at 55 °C for 30 minutes in dimer buffer with or without a 20 fold excess of the oligonucleotides as202, as548 and asDIM or a combination of these. Oligonucleotides as202 and as548 target the upstream and downstream dimer-interfering elements, respectively, that were previously identified. Figure taken from (Lanchy et al., 2003a)

Surprisingly, mutations in stem loop 1 (SL1) did not result in a decrease of tight dimer formation (see figure 14.) As expected, when an RNA lacking nucleotides 409-436 of the SL1 structure (1-561 ΔSL1 RNA) was incubated in dimer buffer at 55 °C for 30 minutes, no tight dimer was observed during TBE-agarose gel electrophoresis at 28 °C (figure 14 A, lane 1). However, when the same RNA was incubated in the presence of a

20-fold excess of as548, tight dimers were present during electrophoresis (figure 14, lane 3). Importantly, the addition of a 20-fold excess of asDIM resulted in suppression of tight dimer formation (figure 14, lane 4).

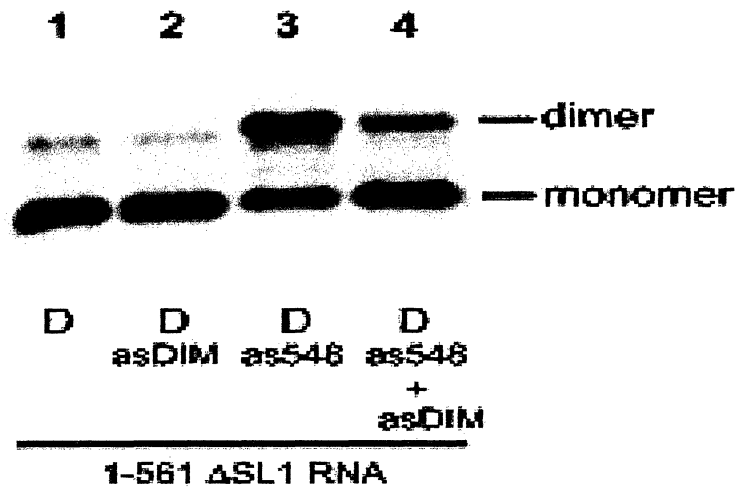


Figure 14. Oligonucleotide as548-directed rescue of tight dimerization in 1-561 RNAs lacking SL1 and PBS dimerization elements. 1-561 Δ SL1 RNA was incubated in dimer buffer at 55 °C for 30 minutes (lane 1) or in the presence of a 20-fold excess of asDIM (lane 2), as548 (lane 3) or both (lane 4). 1-561 Δ SL1 RNA lacks nucleotides 409-436 of the SL1 structure. Figure taken from (Lanchy et al., 2003a)

Analysis of this phenomenon revealed the presence of a palindromic sequence upstream of SL1 in a region previously shown to be involved in *in vivo* encapsidation (Griffin et al., 2001). When 1-561 Δ SL1 RNA was incubated in dimer buffer at 55 °C for 30 minutes in the presence of a 20-fold excess of oligonucleotides as548 and as Ψ , tight dimers were not observed during TBE-agarose gel electrophoresis (figure 15 A, lane 2). Oligonucleotide as Ψ targets nucleotides 392-401 of the core encapsidation signal of HIV-

2 RNA. Additionally, an RNA lacking both the SL1 and PBS dimerization elements displayed the same behavior (Figure 15 B, lane 4).

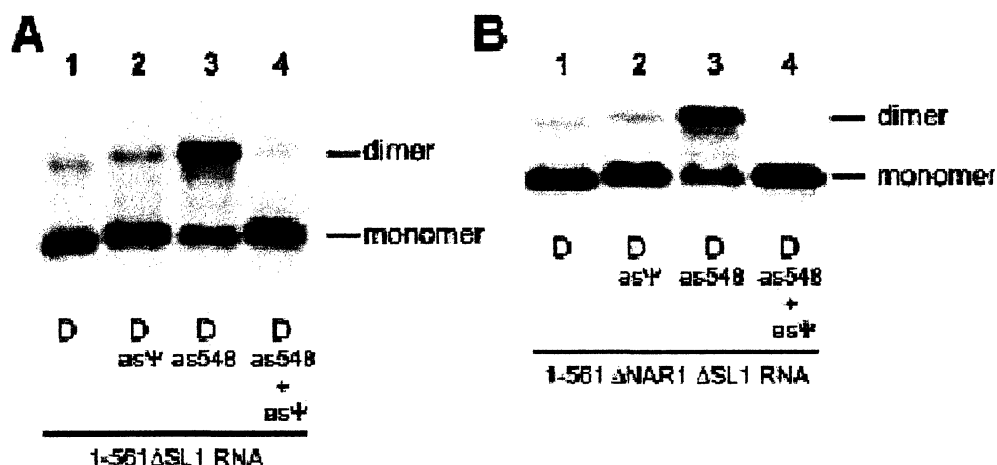


Figure 15. Oligonucleotide inhibition of HIV-2 RNA tight dimer formation. **(A)** 1-561 ΔSL1 RNA was incubated in dimer buffer at 55° C for 30 minutes (15 A, lane 1) or in the presence of a 20-fold molar excess of oligonucleotide asΨ (16 A, lane2), as548 (15 A, lane3) or both (lane 4). 1-561 ΔSL1 RNA lacks nucleotides 409-436 of the SL1 structure. Oligonucleotide asΨ targets nucleotides 392-401 of the core encapsidation signal of HIV-2 RNA. **(B)** The conditions are the same as in (A), but an HIV-2 construct lacking both the SL1 region and the PBS palindrome was used. Figure taken from from (Lanchy et al., 2003a)

However, Tris buffer was used in the above experiments while sodium cacodylate was used in the solution structure probing assays. Sodium cacodylate is used for solution structure probing because of its neutrality toward the modification reagents used. Therefore, the oligonucleotide-rescue experiments were repeated to compare the results between the two protocols. As can be seen in figure 13, with sodium cacodylate replacing Tris, the RNAs still lacked the ability to form significant tight dimers when resolved on a TBE agarose gel at approximately room temperature (figure 16, lanes 3, 4, 8, 9). However, the addition of a 20-fold excess of oligonucleotide as548 restored the

RNAs ability to form tight dimers (figure 16, lanes 5 and 10). Additionally, the 1-444 RNA displayed the same ability to form tight dimers as shown in the previous experiments (figure 16, lanes 14 and 15).

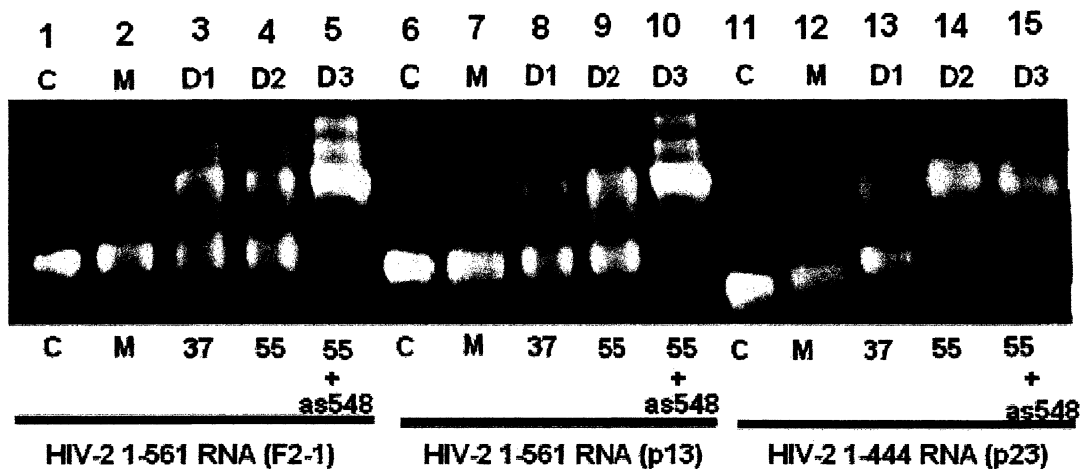


Figure 16. Antisense oligonucleotide-mediated restoration of wild type 1-561 HIV-2 RNA tight dimerization. 1-561 RNAs were incubated for 30 minutes in dimer buffer at 55 °C with or without oligonucleotide as548 present. The samples were then resolved on a TBE agarose gel at 28 °C. The lanes are as indicated; C= control, 1-561 RNA incubated with no buffer, M= 1-561 RNA incubated in monomer buffer at 37 °C, D1= 1-561 RNA incubated in dimer buffer at 37 °C, D2= 1-561 RNA incubated in dimer buffer at 55 °C, D3= 1-561 RNA incubated in dimer buffer with a 20-fold molar excess of oligonucleotide as548. Lanes 1-5 represent wild type 1-561 HIV-2 RNA used in previous experiments. Lanes 6-10 represent wild type 1-561 HIV-2 RNA used in the above experiments. Lanes 11-15 represent a wild type 1-561 RNA that was truncated at position 444.

The removal of putative dimerization inducing elements did not diminish the ability of oligonucleotide as548 to restore tight dimerization. As shown in figure 17, the PBS dimer element (lanes 6-10 and), The PBS domain, and the SL1 dimer element (lanes 16-20) have been removed. Additionally, replacing Tris with sodium cacodylate did not diminish the ability of as548 to restore dimerization in the mutated RNAs (figure 16, lanes 10, 15, 20).

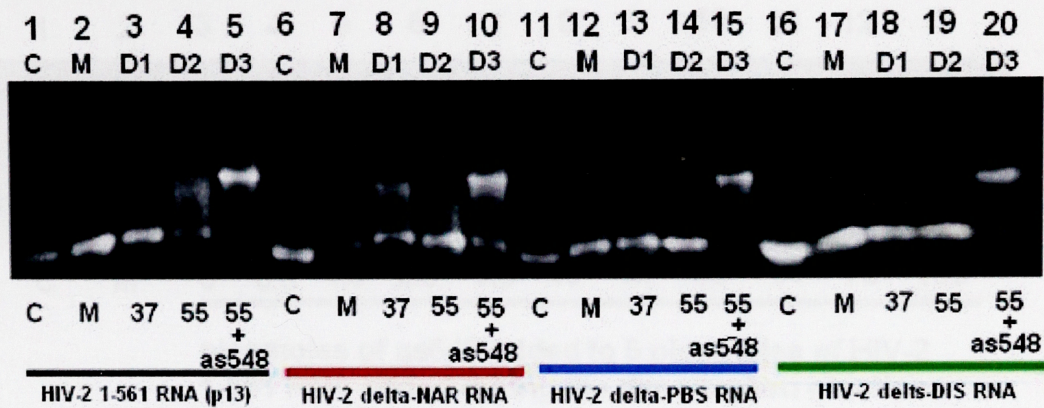


Figure 17. Oligonucleotide as548 rescue of HIV-2 RNAs. 1-561 RNAs (p13, ΔDIS, ΔNAR and ΔPBS) were incubated for 30 minutes in dimer buffer at 55 °C with or without oligonucleotide as548 present. The samples were then resolved on a TBE agarose gel at 28 °C. The lanes are as indicated: C= control RNA with no buffer, M= RNA in monomer buffer incubated at 37 °C, D1= RNA with dimer buffer incubated at 37 °C, D2= RNA with dimer buffer incubated at 55 °C, D3= RNA incubated with dimer buffer at 55 °C with a 20-fold excess of oligonucleotide as548.

Finally, the minimum amount of oligonucleotide as548 that would rescue tight dimerization was determined. This was done because it was observed that as548 interfered with the primer extension reaction used to visualize modifications after solution structure probing (data not shown). As can be seen in figure 18, a 1:1 molar ratio of oligonucleotide as548 and HIV-2 RNA shifts a significant proportion of the population to the tight dimer pool (compare lane 3 to lane 7). A 2:1 ratio of oligonucleotide as548 and HIV-2 RNA resulted in a complete shift of the RNAs to the dimer pool (compare lanes 3 and 8). Therefore a 2:1 ratio of oligonucleotide as548 to HIV-2 RNA was used for all oligonucleotide-rescue assays described herein.

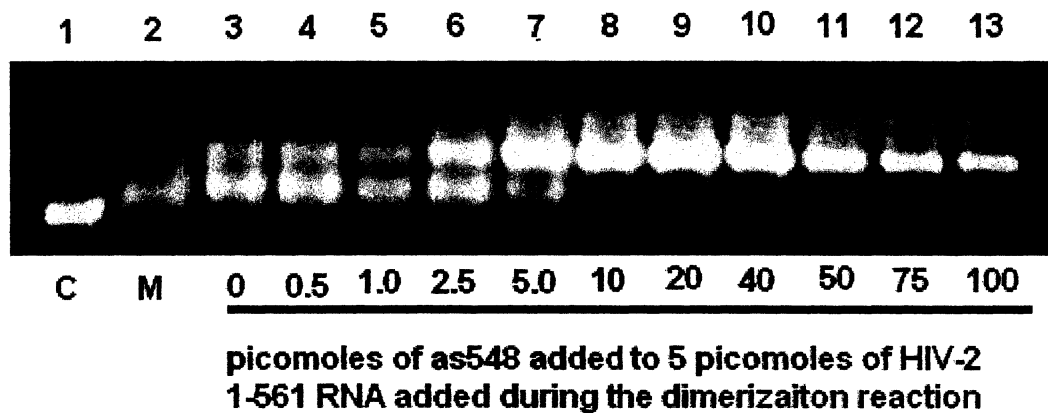


Figure 17. Oligonucleotide as548 titration. 5 picomoles of 1-561 HIV-2 RNA was incubated for 30 minutes at 50 °C in dimer buffer with an increasing concentration of as548 (in picomoles) as indicated. The samples were resolved on a TBE agarose gel at 28 °C. M= 1-561 RNA incubated in monomer buffer at 37 °C for 15 minutes, C= control 1-561 RNA incubated with no buffer.

Structural analysis of dimerization competent conformational variants within the leader region of the HIV-2 genomic RNA.

The data presented above would suggest that HIV-2 RNAs undergo conformational rearrangements when using the various *in vitro* dimerization protocols. In order to resolve the secondary structures involved in these rearrangements, solution structure probing of various HIV-2 constructs was undertaken. Solution structure probing provides a way to map the structure of RNAs in solution at nucleotide resolution. In this method, enzymatic cleavages or chemical modifications are introduced at a statistical and low level at single stranded regions, depending on the probe used (one cut or modification per molecule) (Ehresmann et al., 1987). Primer extension is then used to detect stops of transcription at the modified or cleaved nucleotides.

Structural analysis of stem loop 1 (SL1)

HIV-2 RNA dimers can be functionally divided into two classes, loose and tight dimers, based upon their ability to withstand denaturation during electrophoresis in a TBE agarose gel at 28 °C. In order to identify the nucleotides involved in conformational rearrangements, solution structure probing was employed. To investigate the secondary structure map of HIV-2 RNA, wild-type 1-561 HIV-2 leader RNA was treated with chemical and enzymatic probes. The results obtained agreed overall with a published secondary structure model of HIV-2 genomic RNA (Berkhout, 1996) (figure 18).

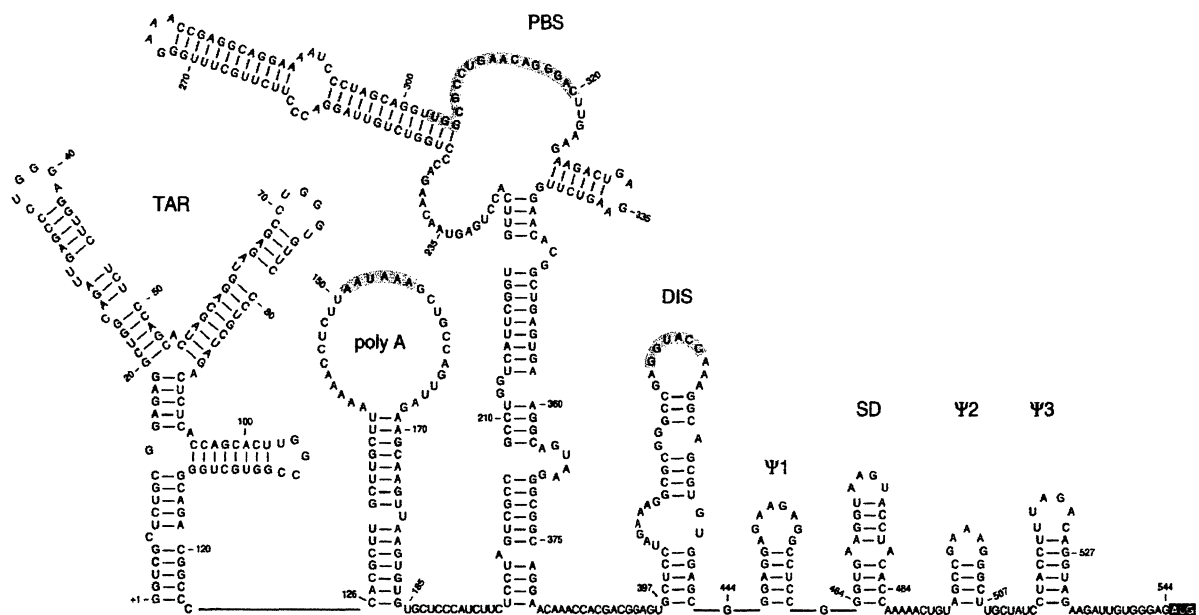


Figure 18. Proposed secondary structure map of the 5'-untranslated region of HIV-2 RNA. The regions of known function are indicated. The authors of this map designated SL1 as the dimerization initiation site (DIS). Figure taken from (Berkhout, 1996).

In a published model, SL1 was shown with nucleotides G442 and G440 base paired with C398 and C400 (figure 20 B (McCann and Lever, 1997)). However, as can be seen in figure 19 A, when probed with the enzyme RNase T1, which cleaves 3' of

unpaired guanosines. nucleotides 442 and 440 are clearly reactive under the conditions used in this experiment. The 392-401 region was shown previously to be involved in both inter- and intramolecular associations in the HIV-2 RNA (Lanchy et al., 2003a).

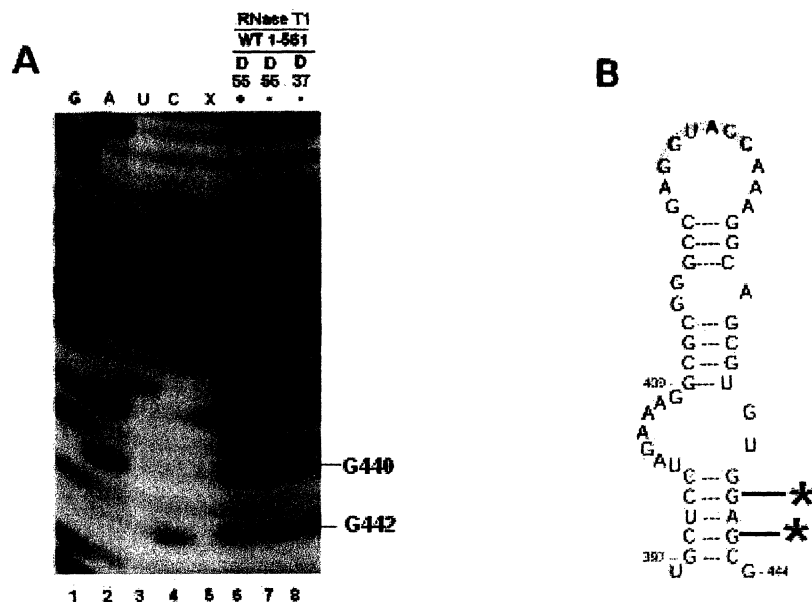


Figure 19. RNase T1 probing of wild-type 1-561 RNA. **(A)** Lanes 1-4 represent sequencing lanes, lane 5 is the RNA primer extended without RNase T1 modification. Lanes 6-8 show the RNA after treatment with RNase T1. In lane 5 the RNA was subjected to dimerization at 55 °C with as548 present prior to RNase T1 treatment. Lane 6 is the same as lane 5 without as548 present during dimerization. In lane 8 the RNA was dimerized at 37 °C without as548. The samples were resolved on an 8% polyacrylamide gel. The reactive nucleotides, G430 and G432, are indicated. RNase T1 cleaves 3' of single stranded guanosines. **(B)** Secondary structure model of HIV-2 SL1 RNA, as proposed by McCann and lever (Abbink and Berkhout, 2003; McCann and Lever, 1997). Nucleotides G430 and G432, cleaved by RNase T1, are indicated by asterisks.

As shown in figure 20, there is no change in reactivity of nucleotides G420 or G421, located in the SL1 palindrome, when subjected to RNase T1 digestion after incubation in monomer buffer at 37 °C (figure 20 A, lane 6), dimer buffer at 37 °C (figure 20 A, lane 7) or dimer buffer at 55 °C (figure 20 A, lane 8). Nucleotides G420 and G421 are located at the 3'-end of the palindrome **GGUACC** located at the apex of

SL1. These results would suggest that under these conditions nucleotides 420 and 421 are in a single stranded region that would exclude the use of the SL1 palindrome as a dimerization element.

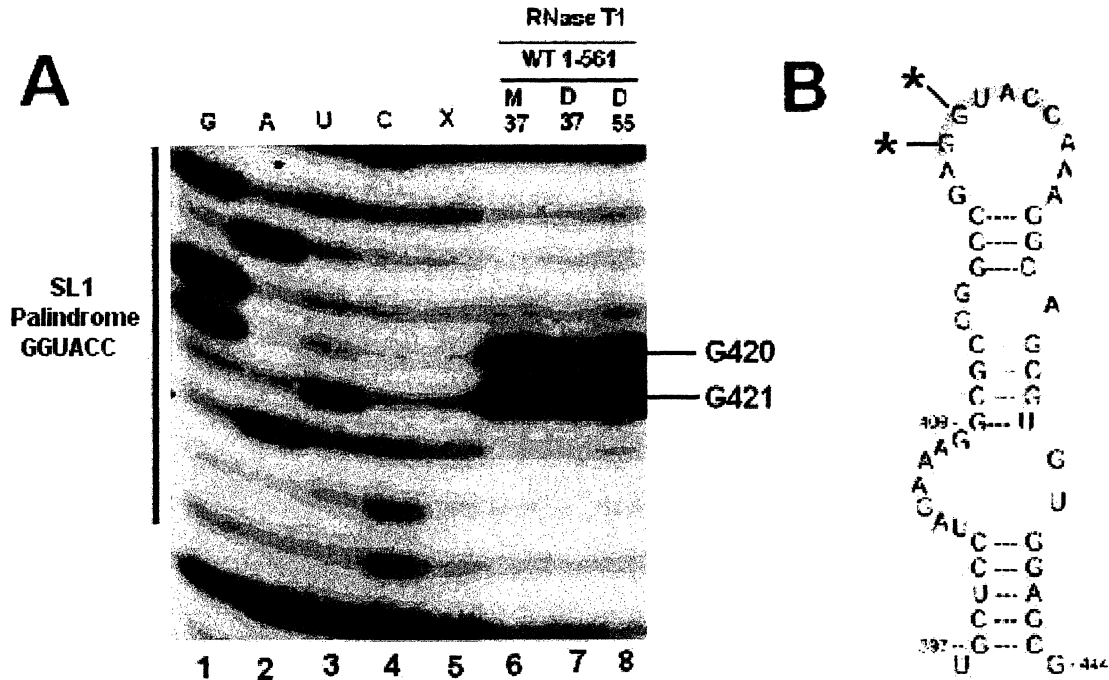


Figure 20. RNase T1 probing of 1-561 RNA in monomer and dimer buffers. **(A)** Lanes 1-4 are sequencing lanes. Lane 5 is 1-561 RNA primer extended without RNase T1 treatment. Lanes 6-8 show 1-561 RNAs subjected to RNase T1 treatment as indicated. Lane 6 shows 1-561 RNA incubated in monomer buffer at 37 °, in lane 7 was 1-561 RNA incubated in dimer buffer at 37 °, and lane 8 is 1-561 RNA incubated in dimer buffer at 55 °C. The samples were resolved on an 8% polyacrylamide gel. **(B)** Secondary structure map of HIV-2 SL1. Indicated are nucleotides G420 and G421 that are susceptible to RNase T1 cleavage under the conditions described. RNase T1 cleaves 3' to guanosines in single stranded regions.

oligonucleotide as548, nucleotides G420 and especially G421 show a diminished reactivity towards cleavage from RNase T1. As shown in figure 21 when wild type 1-561 RNA was incubated in dimer buffer at 55° C with a 2-fold molar excess of as548 and then subjected to RNase T1 digestion, nucleotides G420 and G421 showed a decrease in susceptibility to cleavage (figure 21, lane 6). But, when wild type 1-561 RNA was

incubated in dimer buffer at 55° C or 37° C without as548 the RNA was susceptible to cleavage (figure 21, lanes 7 and 8).

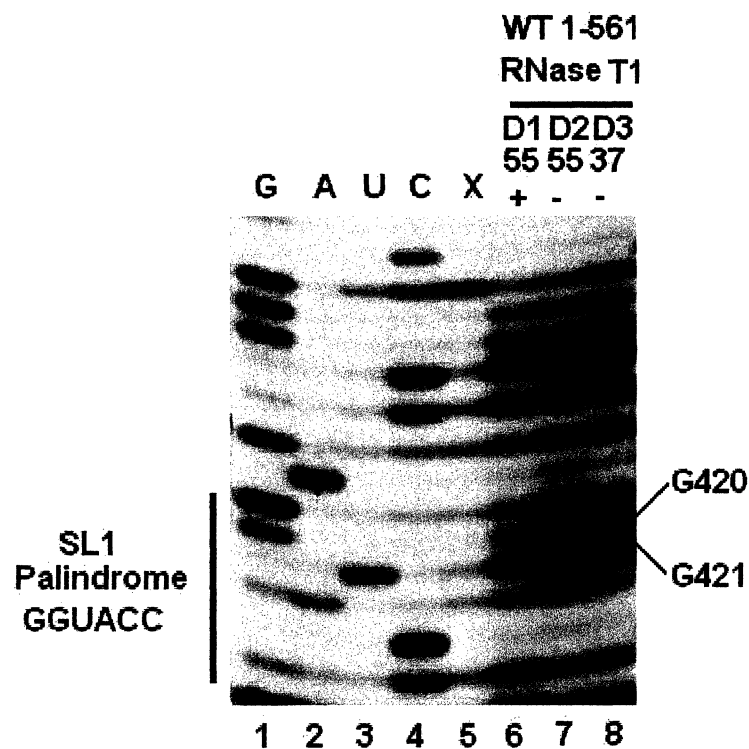


Figure 21. RNase T1 probing of wild type 1-561 RNA dimerized with or without oligonucleotide as548. Lanes 1-4 are sequencing lanes. Lane 5 shows 1-561 RNA primer extended without RNase T1 treatment. Lanes 6-8 show 1-561 RNAs subjected to RNase T1 treatment as indicated. Lane 6 shows 1-561 RNA incubated in dimer buffer at 55 °C with a 2-fold excess of as548, in lane 7 is 1-561 RNA incubated in dimer buffer at 55 °C with no as548, and lane 8 is 1-561 RNA incubated in dimer buffer at 37 °C. The samples were resolved on an 8% polyacrylamide gel.

Additionally, an HIV-2 RNA construct with the PBS palindrome (304-GGCGCC-309) deleted (1-561 ΔNAR RNA) was subjected to RNase T1 digestion following dimerization as indicated in figure 22. When 1-561 ΔNAR RNA was incubated in dimer buffer at 55° C with as548 the nucleotides G420 and G421 showed a decrease in susceptibility to cleavage (figure 22, lane 16). But, when 1-561 ΔNAR RNA was

incubated in dimer buffer at 55° C or 37° C without as548 the RNA was susceptible to cleavage (figure 22, lanes 17 and 18).

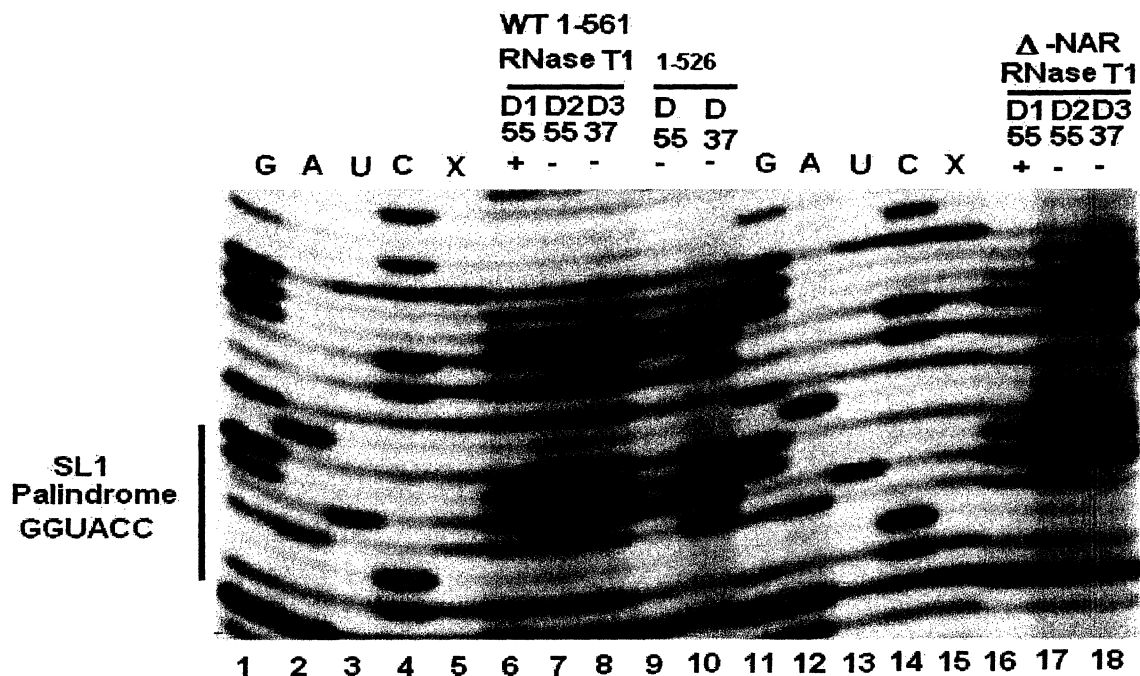


Figure 22. RNase T1 probing of wild type 1-561 RNA, 1-526 RNA and 1-561 ΔNAR RNAs dimerized with or without oligonucleotide as548. Lanes 1-4 and 11-14 are sequencing lanes. Lane 5 is 1-561 RNA primer extended without RNase T1 treatment. Lanes 6-8 show 1-561 RNAs subjected to RNase T1 treatment as indicated. Lane 6 shows 1-561 RNA incubated in dimer buffer at 55 °C with a 2-fold excess of as548, in lane 7 is 1-561 RNA incubated in dimer buffer at 55 °C without as548, and lane 8 is 1-561 RNA incubated in dimer buffer at 37 °C. Lanes 9 and 10 show 1-526 RNA incubated in dimer buffer at 55 °C and 37 °C, respectively. Lanes 11-14 are sequencing lanes. Lanes 15-18 show ΔNAR RNA subjected to RNase T1 treatment as indicated. Lane 15 shows ΔNAR RNA incubated in dimer buffer at 55 °C with a 2 fold excess of as548, in lane 16 is ΔNAR RNA incubated in dimer buffer at 55 °C with no as548, and lane 17 is ΔNAR RNA incubated in dimer buffer at 55 °C with no as548, and lane 18 is ΔNAR RNA incubated in dimer buffer at 37 °C. Lane 9 and 10 show 1-526 RNA incubated in dimer buffer at 55 °C and 37 °C, respectively. The samples were resolved on an 8% polyacrylamide gel.

Further, a construct truncated at the 3'-end of the HIV-2 leader RNA (1-526 RNA) was analyzed and the results were comparable to those seen for the wild type 1-561 RNA and 1-561 ΔNAR RNA (figure 22, lanes 9 and 10). Oligonucleotide as548 is directed against

nucleotides 527-548 of the HIV-2 leader RNA. The 1-526 RNA was used because it should mimic the effects of adding as548 to full-length wild-type 1-561 RNA.

Solution structure probing of the SL1 region with DMS showed that a conformational change occurs between the monomeric and dimeric forms of HIV-2 leader RNA.

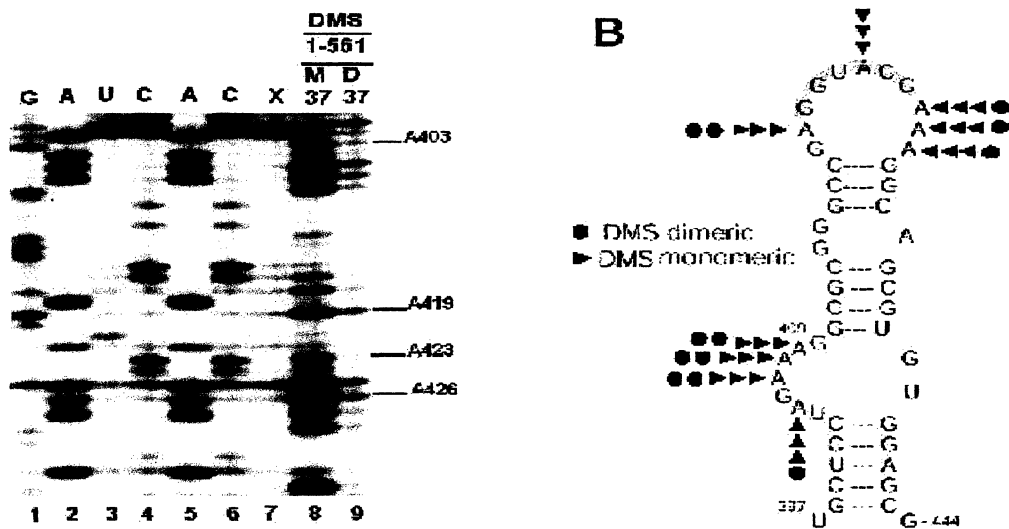


Figure 23. DMS structure probing of 1-561 monomeric and dimeric RNAs. (A) Lanes 1-6 are sequencing lanes as indicated above. Lane 7 is a control in which 1-561 RNA is primer extended without DMS modification. Lane 8 is 1-561 incubated in monomer buffer at 37 °C and then DMS modified. Lane 9 is 1-561 incubated in dimer buffer at 37 °C and the DMS modified. The samples were resolved on an 8% polyacrylamide gel. DMS alkylates the N1 positions of adenosines and, to a lesser extent, the N3 position of cytidines not involved in Watson-Crick base pairing. (B) Secondary structure map of HIV-2 SL1 (McCann and Lever, 1997). Indicated as dark circles are adenosine reactivities when the RNA is incubated in dimer buffer, dark triangles represent adenosine reactivities when the RNA is incubated in monomer buffer. An increase in the symbol present indicates an increase in reactivity to DMS for that nucleotide.

Solution structure probing of the ψ palindrome

Because the ψ palindrome was shown to be involved in HIV-2 leader RNA dimerization, an attempt was made to determine the specific secondary structures corresponding to the silenced or dimerization-competent forms of the RNA. The ψ palindromic region following RNase T1 digestion is shown in figure 24. Note the lack of cleavage seen for nucleotides G392, G393, G395 and G397 in the palindromic region (compare to G404) (figure 24, lanes 6, 7, and 8). This lack of reactivity was seen whether the RNA was dimerized at 55 °C with as548 (figure 24, lane 6), without as548 (figure 24, lane 7) or dimerized at 37 °C (figure 24, lane 8).

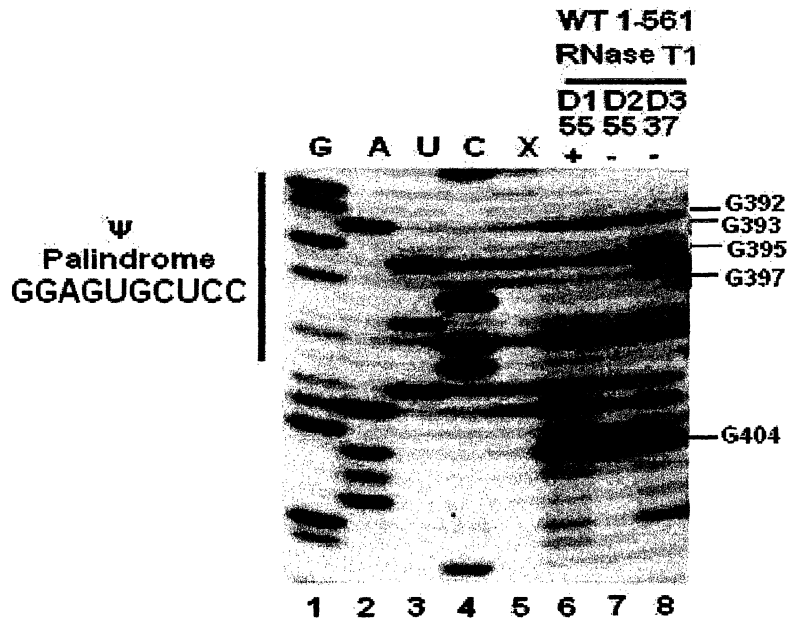


Figure 24. RNase T1 digestion of the Ψ palindromic region. Lanes 1-4 are sequencing lanes. Lane 5 is 1-561 RNA primer extended without RNase T1 digestion. Lanes 6-8 represent 1-561 RNA subjected to RNase T1 digestion following incubation in dimer buffer as indicated. In lane 6, 1-561 RNA was incubate in dimer buffer at 55 °C for 30 minutes with a 2-fold excess of as548. In lane 7, 1-561 RNA was incubated at 55 °C for 30 minutes without as548. In lane 8, 1-561 RNA was incubated in dimer buffer for 15 minutes at 37 °C. The samples were resolved on an 8% polyacrylamide gel

When HIV-2 RNA constructs 1-526 (lacking nucleotides 528-561) and 1-561 Δ NAR RNA (lacking nucleotides 304-309) were subjected to RNase T1 digestion, similar results were obtained as for the wild type 1-561 RNA (figure 25); specifically, the lack of cleavage seen for nucleotides G392, G393, G395 and G397 in the palindromic region.

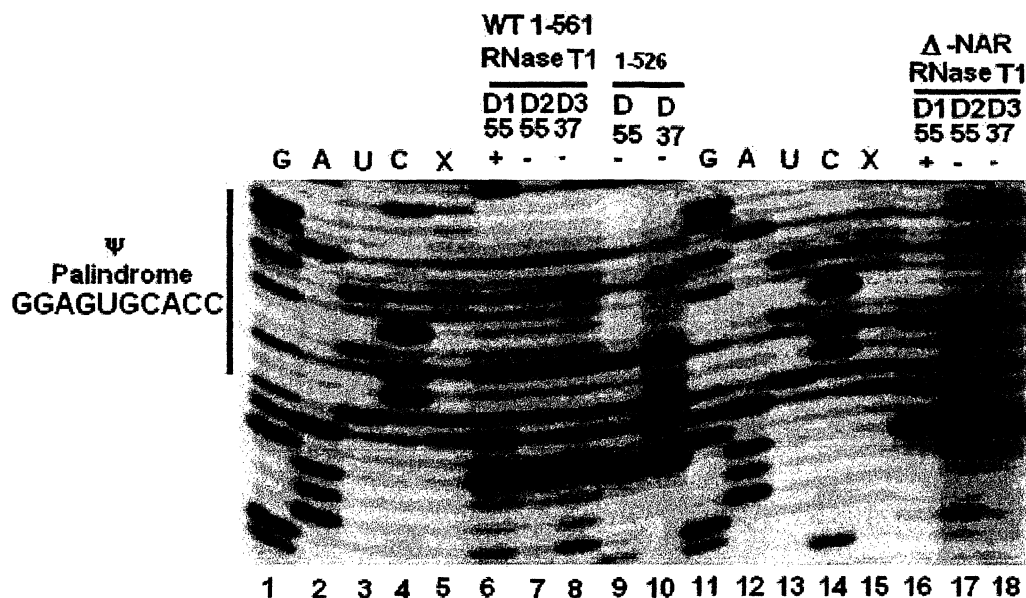


Figure 25. RNase T1 probing of 1-561 RNA, 1-526 RNA and Δ NAR RNAs dimerized with or without oligonucleotide as548. Lanes 1-4 and 11-14 are sequencing lanes. Lane 5 is 1-561 RNA primer extended without RNase T1 treatment. Lanes 6-8 show 1-561 RNAs subjected to RNase T1 treatment as indicated. Lane 6 shows 1-561 RNA incubated in dimer buffer at 55° C with a 2-fold excess of as548, in lane 7 is 1-561 RNA incubated in dimer buffer at 55° C with no as548, and lane 8 is 1-561 RNA incubated in dimer buffer at 37° C. Lanes 16-18 show Δ NAR RNA subjected to RNase T1 treatment as indicated. Lane 16 shows Δ NAR RNA incubated in dimer buffer at 55° C with a 2 fold excess of as548, in lane 17 is Δ NAR RNA incubated in dimer buffer at 55° C with no as548, and lane 18 is Δ NAR RNA incubated in dimer buffer at 37° C. Lane 9 and 10 show 1-526 RNA incubated in dimer buffer at 55° C and 37° C, respectively. The samples were resolved on an 8% polyacrylamide gel.

Additionally, when an HIV-2 construct lacking nucleotides 421-424 (1-561 Δ DIS RNA) was subjected to RNase T1 digestion, only mild cleavage of the guanosines in the Ψ palindromic region was observed (figure 26, lanes 14-16 as indicated)

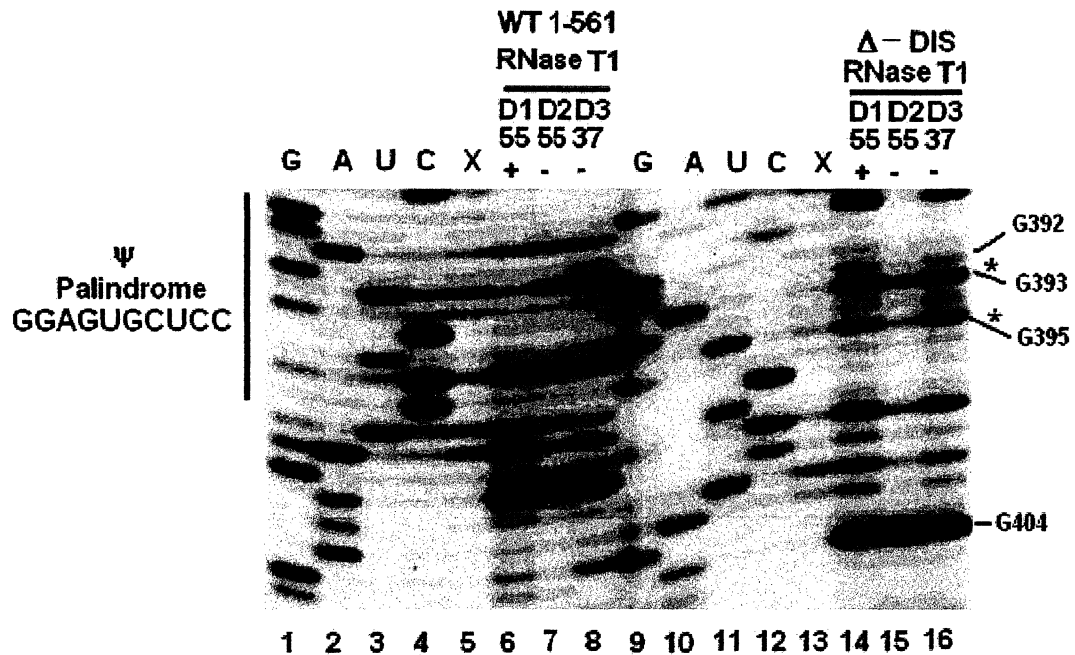


Figure 26. RNase T1 probing of 1-561 RNA, 1-526 RNA and Δ DIS RNAs dimerized with or without oligonucleotide as548. Lanes 1-4 and 9-12 are sequencing lanes. Lanes 5 and 13 are 1-561 RNA primer extended without RNase T1 treatment. Lanes 6-8 show 1-561 RNAs subjected to RNase T1 treatment as indicated. Lane 6 shows 1-561 RNA incubated in dimer buffer at 55° C with a 2-fold excess of as548, in lane 7 is 1-561 RNA incubated in dimer buffer at 55° C with no as548, and lane 8 is 1-561 RNA incubated in dimer buffer at 37° C. Lanes 14-16 show Δ DIS RNA subjected to RNase T1 treatment as indicated. Lane 14 shows Δ DIS RNA incubated in dimer buffer at 55° C with a 2 fold excess of as548, in lane 15 is Δ DIS RNA incubated in dimer buffer at 55° C with no as548, and lane 16 is Δ DIS RNA incubated in dimer buffer at 37° C. The samples were resolved on an 8% polyacrylamide gel.

Solution structure probing of the primer binding site domain

The palindrome GGCGCC located at the 5'-end of the primer binding site (PBS) was shown to be a default dimerization element for all HIV-2 RNAs incubated at 37° C . Additionally, through truncation analysis (Jossinet et al., 2001), site directed mutagenesis and oligonucleotide-directed gel shift assays (Lanchy and Lodmell, 2002; Lanchy et al., 2003b) only one HIV-2 RNA construct (1-444) was shown to utilize the SL1 palindrome for dimerization when incubated at 55° C (Dirac et al., 2001; Lanchy and Lodmell, 2002). To analyze the structural elements involved in this differential use of dimerization signals, the PBS domain of HIV-2 RNA was subjected to solution structure probing using the various dimerization protocols.

As seen in figure 27, G304, G305 and G307 of the PBS palindrome are only mildly susceptible to RNase T1 cleavage with only slight variability between RNA constructs (figure 27 A). Some of the residual cleavage observed could be due to a small population of the RNAs in a conformation that makes these nucleotides accessible to cleavage. However, it has been shown that the PBS does not form tight dimers. Thus, the PBS dimers could be dissociating enough to allow a small amount of cleavage.

Located 5' of the PBS palindrome, nucleotides G281 and G282 showed variable susceptibility to RNase T1 cleavage with different dimer conditions. Nucleotides G281 and G282 were strongly cleaved when incubated in dimer buffer at 55 °C (figure 27 A,

lanes 7 and 14). However, they were only moderately cleaved when the RNA was incubated in dimer buffer at 55° C with as548 present, or the 1-526 construct was used (figure 27 A, lanes 6, 9, and 13). Total protection of the nucleotides was observed when the RNA was incubated in dimer buffer at 37° C (figure 27 A, lanes 8, 10, 12, 15).

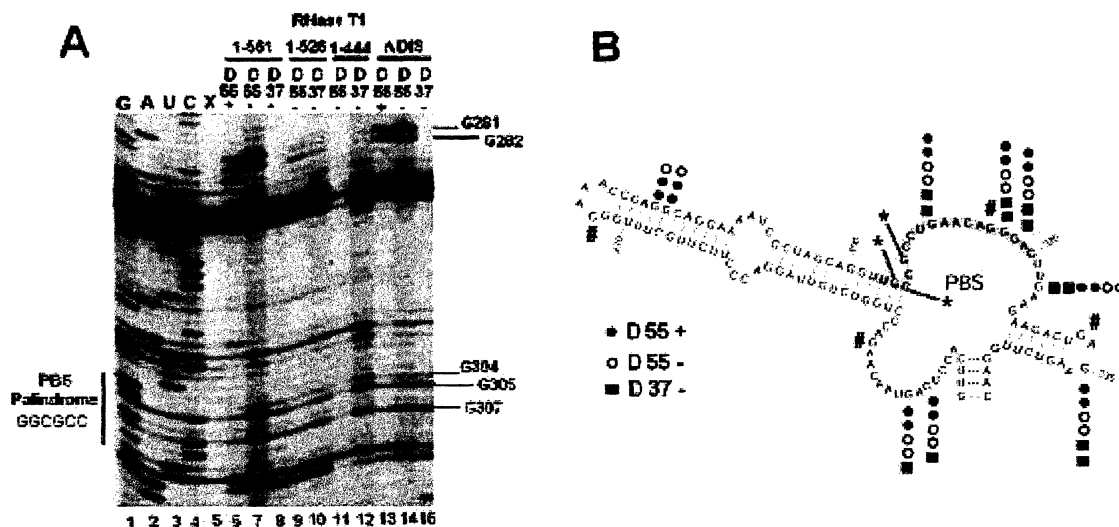
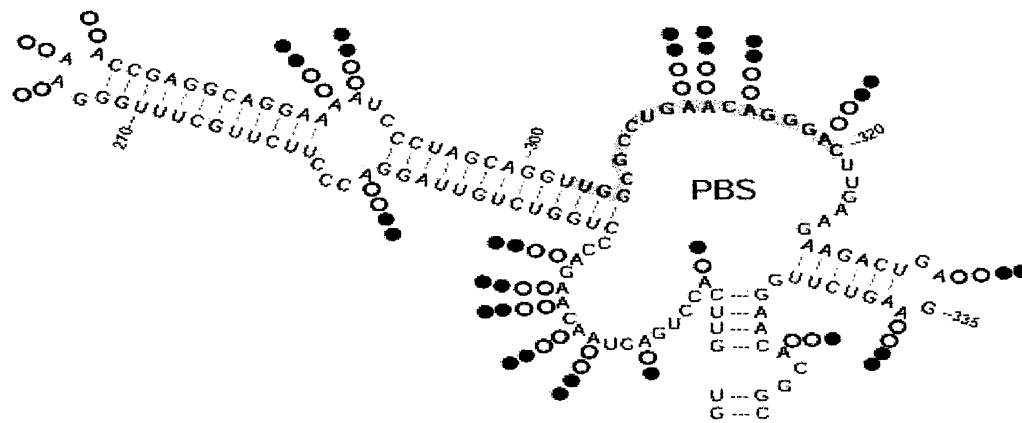


Figure 27. RNase T1 digestion of HIV-2 RNA constructs 1-561, 1-526, 1-444 and ΔDIS. **(A)** Lanes 1-4 are sequencing lanes. Lane 5 is 1-561 RNA primer extended without RNase T1 digestion. Lanes 6-15 represent the RNA constructs subjected to RNase T1 digestion following incubation in dimer buffer as indicated. In lane 6 1-561 RNA was incubate in dimer buffer at 55° C for 30 minutes with a 2-fold excess of as548. In lane 7, 1-561 RNA was incubated at 55° C for 30 minutes without as548. In lane 8, 1-561 RNA was incubated in dimer buffer for 15 minutes at 37° C. In lanes 9 and 10 1-526 RNA was incubated in dimer buffer at 55° C and 37° C, respectively. Lanes 11 and 12 show 1-444 RNA incubated in dimer buffer at 55° C and 37° C, respectively. In lane 13, ΔDIS RNA was incubated in dimer buffer at 55° C for 30 minutes with a 2-fold excess of as548. In lane 14 ΔDIS RNA was incubated at 55° C for 30 minutes without as548 and in lane15 ΔDIS RNA was incubated in dimer buffer for 15 minutes at 37° C. The samples were resolved on an 8% polyacrylamide gel. **(B)** Secondary structure map of HIV-2 SL1. Indicated as dark circles are guanosine reactivities when the RNA is incubated in dimer buffer at 55° C for 30 minutes with a 2-fold excess of as548, clear circles represent guansoine reactivities when the RNA is incubated in dimer buffer at 55° C without as548. Dark squares indicate guanosine reactivities when the RNA is incubated at 37° C for 15 minutes. # signifies a non reactive nucleotide in an area of predicted accessibility. * signifies a nucleotide with only slight reactivity when dimerized using different protocols An increase in the symbol present indicates an observed increase in reactivity to RNase T1 cleavage for that nucleotide.

Finally, the PBS domains of HIV-2 RNAs wild type 1-561 and 1-444 were subjected to solution structure probing with DMS. This was done because it was observed that incubation in dimer buffer at 55 °C for 30 minutes resulted in tight dimer formation for the 1-444 RNA but not the wild type 1-561 RNA. The DMS reactivities observed for the adenosines in the PBS domain are summarized in figure 28. The solid circles represent DMS reactivities of the 1-444 RNA, and the clear circles are those observed for the wild type 1-561 RNA. The only distinct difference between the two constructs was a lack of reactivity of adenosines A274-A276 in the wild type 1-561 RNA construct compared to the 1-444 construct. This region is a GNRA tetraloop and it is possible that the 1-444 RNA is in such a conformation as to allow the tetraloop to interact with a tetraloop receptor. This interaction would protect these nucleotides from DMS modification.



- 1-561 DMS dimeric conditions @ 55 C
- 1-444 DMS dimeric conditions @ 55 C

Figure 28. Summary of adenosine reactivities to DMS for HIV-2 RNA constructs 1-444 and 1-561 when dimerized at 55 °C for 30 minutes. Shown is the secondary structure map of the PBS domain of HIV-2, Berkhout, (1996). The solid circles represent DMS reactivities for the 1-444 RNA and the clear circles are those observed for the 1-561 RNA.

Discussion

The characterization of the mechanism of HIV-2 genomic RNA dimerization has revealed significant differences with the reported mechanism for HIV-1 RNA. Of these differences, the most intriguing is the multiple dimerization elements shown to be used by HIV-2 leader region RNA, *in vitro*. In this study, a structural analysis of the dimerization-competent conformational variants of HIV-2 genomic leader RNA was undertaken. The results indicate that a rearrangement of secondary structure within the HIV-2 leader RNA can be observed when treated using various *in vitro* dimerization protocols. Secondly, the data presented here corroborate and support previous experiments demonstrating the use of multiple dimerization elements by HIV-2 RNA.

Stem loop 1 secondary structure

It has been proposed that nucleotides 397-401 of the HIV-2 leader RNA base pair with nucleotides 443-439 to form the base of stem loop 1 (SL1) in HIV-2 RNA (McCann and Lever, 1997) (see figure 19 B). However, the data presented herein showed that nucleotides G440 and G442 remained susceptible to RNase T1 digestion. Cleavage by RNase T1 is indicative of guanosines located in single stranded regions. Additionally, it has been shown that nucleotides 392-401 can form an intermolecular interaction with a homologous region in another HIV-2 RNA (Lanchy et al., 2003a). Further, it has been suggested that this region can form an intramolecular interaction with the 5' region of stem loop 1 (Lanchy et al., 2003a). Thus, nucleotides 398 and 400 would not be base

paired with nucleotides 440 and 442, leaving them potentially accessible to RNase T1 cleavage, as observed (see figure 19). The cleavage of G440 and G442 was observed whether the RNA was dimerized at 37° C or 55° C, suggesting a thermostable conformation of the HIV-2 RNA that keeps these two nucleotides accessible. Shown in figure 29 are two proposed secondary structure models of HIV-2 RNA SL1. Neither model fits with the structure probing data obtained in this study.

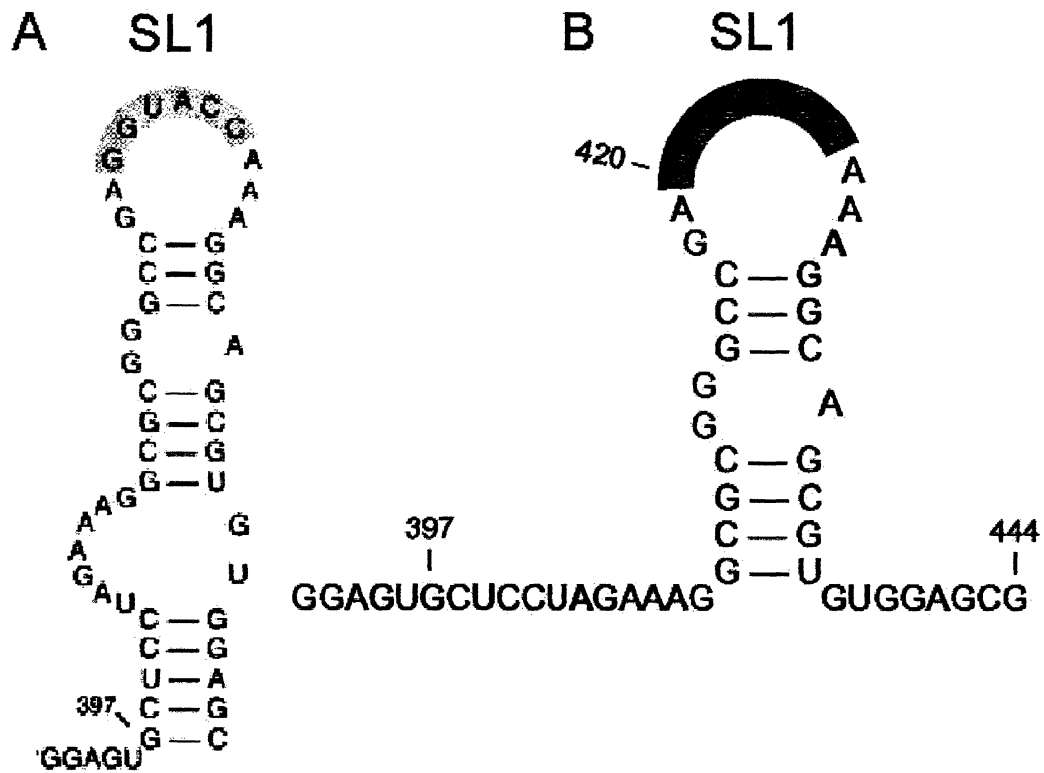


Figure 29. Proposed secondary structures of the SL1 region of HIV-2 leader RNA. (A) SL1 secondary structure proposed by (McCann and Lever, 1997). In this conformation the stem is extended by base pairing of nucleotides 397-401 with nucleotides 443-439. (B) SL1 secondary structure proposed by (Berkhout, 1996). In this model the Ψ palindrome (nucleotides 392-401) was not base paired.

Solution structure probing of stem loop 1

Solution structure probing of stem loop 1 (SL1) revealed that the palindromic sequence (GGUACC) located at the apex of the stem is not used as an *in vitro* dimerization initiation element for full-length wild-type HIV-2 leader RNAs. When wild-type 1-561 HIV-2 RNA was incubated in dimer buffer at 37° C or 55° C, nucleotides G420 and G421 of the SL1 palindrome were both accessible to cleavage by RNase T1 (see figure 20 A). Because RNase T1 cleaves 3' to unpaired guanosines, the observed cleavages would imply that nucleotides G420 and G421 are in a single stranded region and not involved in inter- or intramolecular interactions under these conditions. This is in agreement with other data from our laboratory that show that SL1 is not used under most conditions as a dimerization initiation element.

To study this further, an assay in which the addition of an oligonucleotide resulted in tight dimer formation, reportedly through SL1, was employed (see figures 15-17). Oligonucleotide as548 targets the 3' dimer interference element of HIV-2 RNA, disrupting a long distance interaction that abrogates the use of SL1 as a dimerization element. The benefit of using oligonucleotide as548 is that two dimers that behave differently when resolved on agarose gels can be probed in solution without changing the incubation conditions.

When wild type 1-561 HIV-2 RNA was dimerized at 55 °C for 30 minutes with a 2-fold excess of oligonucleotide as548 and then digested with RNase T1, nucleotides

G421 and G420 showed a decrease in cleavage intensity (see figure 21). It has been demonstrated that oligonucleotide as548 restored tight dimerization via SL1 when assayed on agarose gels. Thus, the lack of cleavage observed for nucleotides G420 and G421 could be due to an intermolecular interaction between RNA molecules resulting in the protection of these nucleotides

One HIV-2 RNA construct analyzed, HIV-2 1-526 RNA, lacked the 5' element shown to be involved in the long distance interaction that normally sequesters SL1, making it a nonfunctional dimerization element. Additionally oligonucleotide as548 is directed against nucleotides 527-548, where the 1-526 RNA ends. Thus, RNase T1 digestion of 1-526 RNA, following dimerization at 55 °C for 30 minutes, should show the same cleavage pattern as the 1-561 RNA dimerized at 55 °C for 30 minutes with a 2-fold excess of as548. Indeed this was the case (see figure 22, lanes 9 and 10). Another RNA construct, 1-561 Δ NAR RNA, showed the same cleavage pattern (see figure 22, lanes 16-18). 1-561 Δ NAR RNA lacked nucleotides 304-309 comprising the primer binding site palindrome.

These results demonstrate that when nucleotides 527-548 are missing, or targeted by an oligonucleotide, there is a conformational switch that involves nucleotides G420 and G421. Combined with previous data from our lab the results would suggest that HIV-2 RNA tight dimerization proceeds through an intermolecular interaction involving the SL1 palindrome.

Solution structure probing of the SL1 region of HIV-2 RNA with DMS revealed a structural rearrangement between monomeric and dimeric RNAs (see figure 22 A, lanes 8 and 9). Interestingly, nucleotide A423, located in the middle of the SL1 palindrome, is reactive under monomeric conditions but protected when incubated in dimer buffer. The data presented previously would argue against an intermolecular interaction involving A423 under these conditions. Additionally, previous work by our laboratory strongly suggests that SL1 is not used as the primary dimerization element of HIV-2 wild type 1-561 RNA. However, the protection of A423 in dimeric form could be due to an intramolecular interaction.

Indeed, a secondary structure map of wild type 1-561 RNA, generated using Mfold and structure probing data, showed a conformation of the RNA that would protect nucleotide A423 (figure 30). Moreover, the RNA is in a conformation that would exclude the use of SL1 as a dimerization element.

Solution structure probing of the Ψ palindromic region

Previous results in our lab showed the use of an additional dimerization element in HIV-2 RNAs lacking functional SL1 and PBS palindromes (Lanchy et al., 2003a). This additional element was shown to be located upstream of SL1, in a region shown to be important for encapsidation *in vivo* (Griffin et al., 2001). The dimerization element was mapped to a palindromic sequence, GGAGUGCUC, comprising nucleotides 392-401 of HIV-2 leader RNA, now called the Ψ palindrome. RNase T1 digestion of

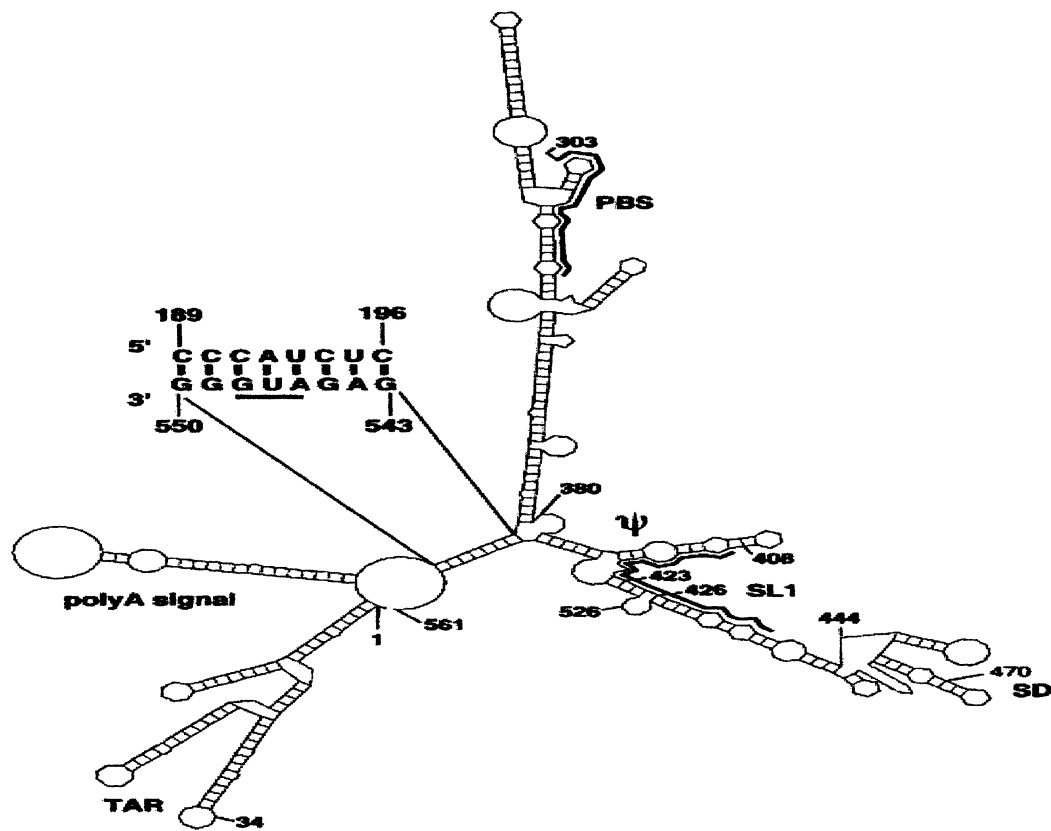


Figure 30. Secondary structure model of the HIV-2 ROD leader RNA as predicted using M-fold constrained by results obtained from structure probing. The putative long distance interaction is shown to the left. The solid lines indicate the PBS and SL1 domains and the Ψ represents the core encapsidation signal (nts 380-408). Figure taken from (Lanchy et al., 2003b). Nucleotide A423 would be protected by an intramolecular interaction as indicated.

dimerized 1-561 RNA showed that the guanosines (G392, G393, G395 and G397) in the palindrome were protected from cleavage under all conditions tested (see figure 24). This result is not unexpected as it has been suggested that when the Ψ palindrome is not being used as an intermolecular dimer element it is involved in intramolecular interactions with the 5' region of SL1 (Lanchy et al., 2003a; Lanchy et al., 2003b).

When other HIV-2 RNA constructs were dimerized and digested with RNase T1, similar cleavage patterns were observed for the guanosines in the Ψ palindrome as was seen for wild type 1-561 RNA (see figures 25 and 26). Specifically, 1-526, Δ NAR and Δ DIS RNAs were analyzed. Δ DIS RNA has nucleotides 421-424 of the SL1 palindrome deleted. An RNA with deletions in SL1 was shown previously to use the Ψ palindrome as an intermolecular dimerization element (Lanchy et al., 2003a). Surprisingly, Δ DIS RNA showed a slight increase in cleavage intensity of guanosines in the Ψ palindrome when compared to the other constructs (see figure 25, lanes 14-16). This could be due to the use of another dimerization element in the 1-561 Δ DIS RNA. However, the cleavages observed are not very strong, and could be due to a subpopulation of RNAs in a conformation in which the guanosines of the Ψ palindrome are more accessible.

Solution structure probing of the primer binding site domain

As previously discussed, studies on HIV-2 genomic RNA dimerization have shown that the PBS palindrome is the default dimerization element for full length wild type HIV-2 leader RNA (1-561 RNA), *in vitro*. Therefore, wild type 1-561 RNA was dimerized at 37° C, 55° C and at 55° C with a 2-fold excess of as548, followed by RNase T1 digestion. As expected the guanosines in the PBS palindrome were only mildly susceptible to RNase T1 cleavage when dimerized at 37° C or 55° C (figure 15 lanes 7 and 8). Guanosines surrounding the PBS palindrome remained susceptible to RNase T1 cleavage (G311, G317, G318 and G323). These data and previous results suggest that

the protection observed for the guanosines in the PBS palindrome are due to an intermolecular interaction involving the dimerization of RNA molecules.

When 1-561 RNA was dimerized at 55° C for 30 minutes with a 2-fold excess of as548 the guanosines in the PBS palindrome showed a decrease in RNase T1 cleavage intensity compared to dimerization without as548 (figure 26, compare lane 6, 7 and 8). As shown previously, the 1-561 RNA uses SL1 as a dimerization element when incubated at 55° C with as548. Thus, the protection of the guanosines in the PBS palindrome are most likely not due to an intermolecular interaction of the 1-561 RNAs. However, the protections observed could be due to a stable intramolecular interaction of these nucleotides, induced by oligonucleotide as548 binding. Additional HIV-2 RNA constructs tested (1-526, 1-444 and Δ DIS) showed similar cleavage patterns to those observed for the 1-561 RNA (see figure 26, lanes 9-15).

It has been shown that 1-444 RNA incubated in dimer buffer at 55° C for 30 minutes utilizes SL1 for tight dimer formation (Lanchy and Lodmell, 2002). However, wild type 1-561 RNA, comprising the entire leader region, subjected to the same dimerization conditions is unable to form tight dimers. It has been further demonstrated that the 1-561 RNA forms a loose dimer, probably a kissing loop complex via the PBS palindrome, when dimerized at 55° C for 30 minutes. Therefore, solution structure probing of the PBS domains of 1-561 and 1-444 RNAs dimerized at 55° C for 30 minutes was performed (see figure 27). The reactivities of adenosines to DMS in the PBS domain are summarized in figure 27.

The only major difference between the two constructs is seen in the 5'-GNRA tetraloop (273-GAAA-276). Tetraloops are recognized structural motifs found in RNA that can assist in coordinating and stabilizing the folding of a RNA molecule (Costa and Michel, 1997). Once formed a tetraloop can form a tertiary interaction by interacting with the minor groove of another helix, termed the tetraloop receptor (Michel and Westhof, 1990).

The adenosines (A274, A275 and A276) are strongly reactive to DMS in the wild type 1-561 RNA construct, but protected in the 1-444 RNA construct. It is possible that removal of nucleotides 445-561 allows for a conformational change in the RNA that forms a tetraloop receptor used by the GNRA tetraloop in the 1-444 construct. This interaction of the tetraloop with its receptor would explain the protection observed for the adenosines in the 1-444 construct. Moreover, this tertiary interaction, induced by a secondary structure rearrangement, could help to stabilize the overall conformation of the RNA. Alternatively, the GNRA tetraloop could be the result of a unique conformation of the wild type 1-561 RNA not found in the truncated 1-444 RNA. Either way, the data suggest a conformational change between an RNA that can form tight dimers (1-444 RNA) and one that can not (wild type 1-561 RNA).

The biological significance

The dimerization elements used by HIV-2 RNA have been linked to important functions *in vivo*. The PBS palindrome is located in a region where a host-encoded tRNA

anneals to initialize reverse transcription. The Ψ palindrome is located in a region shown to be important for encapsidation. The use of SL1 to form tight dimers in HIV-2 RNA has been demonstrated *in vitro* and for HIV-1 the use of SL1 as a dimerization element *in vivo* is thoroughly documented. Finally, the 3' dimer interfering element 3'-GGGUAGAG-5' comprising nucleotides 550-543 overlaps the AUG (shown in bold) translational start codon. Thus it appears that these regions serve the dual purpose of fulfilling their role in the replication cycle of the virus and coordinating HIV-2 RNA dimerization.

The results of this study demonstrate that thermal treatment of HIV-2 RNA is necessary to induce a switch from a loose dimer to a tight dimer. Solution structure probing of the RNA in a tight dimer conformation suggests that SL1 is involved in the formation of these dimers. *In vivo*, the dimeric RNA within the virion has been shown to mature into a more thermostable form, possibly from the formation of an extended duplex. Moreover, the nucleotide annealing activity of nucleocapsid protein has been shown to coordinate this conformational switch.

Because nucleocapsid protein can anneal any nucleotides with complementary sequences (Dib-Hajj et al., 1993; Paillart et al., 1994), it would be important for the two RNAs of the viral genome to be aligned before a stable interaction is introduced. This possibility exists *In vivo*, as the GAG precursor is cleaved to form nucleocapsid after the dimerized RNA has been encapsidated. Thus, sequences other than the SL1 palindrome may be used to correctly align the RNA molecules before nucleocapsid commits the

molecules to a thermostable tight dimer. The use of the PBS palindrome for the initial alignment is interesting because of the inability of this region to form tight dimers *in vitro*.

There is additional evidence that suggests that HIV-2 genomic RNA is selected for encapsidation co-translationally (Griffin et al., 2001; Kaye and Lever, 1999). Moreover, it was shown that nucleotides 380-408 of the genomic RNA are important in this process. This is interesting because the Ψ palindrome, located in this region, was shown to be a dimerization element in HIV-2 RNA when the 5' end of SL1 was deleted. Furthermore, the 3' dimer interfering element contains the AUG translational start codon. Thus, it seems possible that the processes of translation, dimerization and encapsidation could be coordinated by conformational changes in the RNA.

The data presented here provide structural support for the model of *in vivo* dimerization first presented by Jossinet et al. (Jossinet et al., 2001) and furthered by Lanchy et al. (Lanchy et al., 2003a). In this model, the newly transcribed RNAs would interact in the cytoplasm of the cell through the PBS palindrome due to a conformation induced by the long distance interaction that abrogates the use of SL1 (figure 31 B and C).

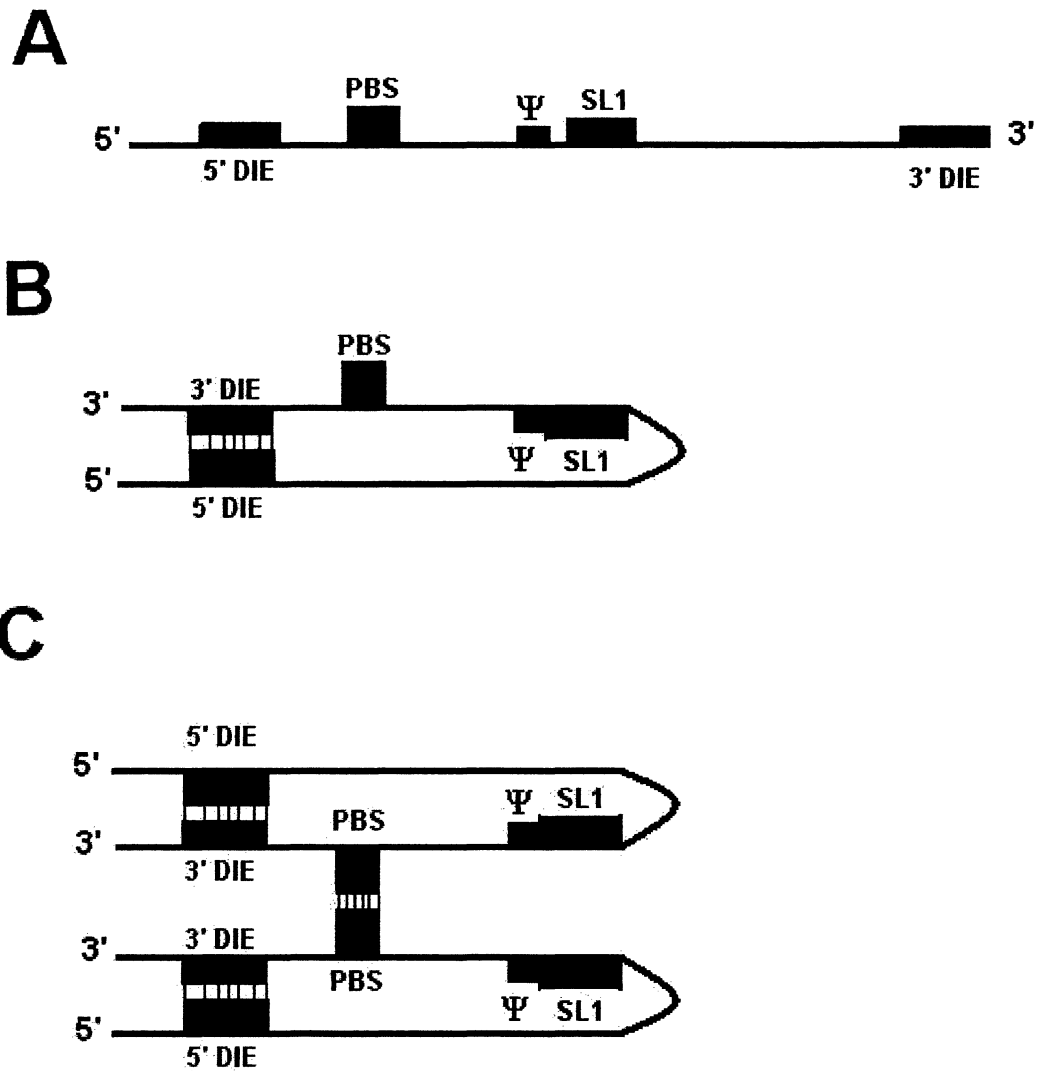


Figure 32. Schematic Representation of conformational changes in the leader region of HIV-2 RNA. (A) Shown are structural elements involved in HIV-2 genomic RNA dimerization. From left to right, the 5' dimer interfering element (DIE), the primer binding site (PBS), the Ψ palindrome (Ψ), stem loop 1 (SL1) and the 3' dimer interfering element (DIE). (B) when the long distance interaction of the dimer interfering elements occurs, SL1 and Ψ become sequestered. (C) with the change in conformation the PBS directs the dimerization of the two RNA molecules.

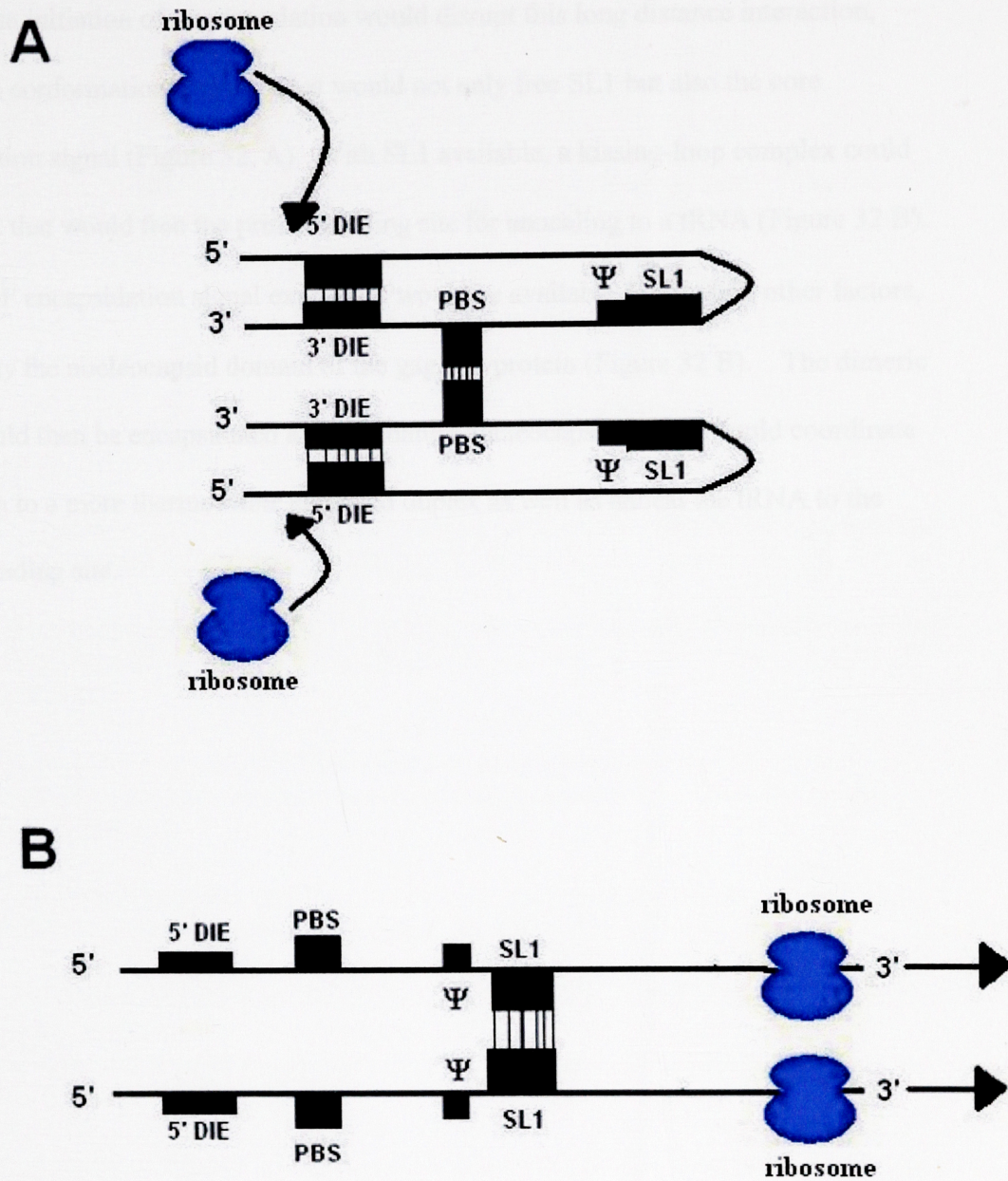


Figure 33. Schematic Representation of conformational changes in the leader region of HIV-2 RNA. (A) The initiation of gag translation begins at the AUG start codon located in the 3' dimer interfering element. (B) The disruption of the long distance interaction between the 5' and 3' dimer interfering elements allows a conformational change in the RNA. This conformational switch results in SL1 being available for dimerization. Also, the primer binding site is now poised to accept a tRNA and the Ψ encapsidation signal is freed to interact with other factors.

The initiation of *gag* translation would disrupt this long distance interaction, allowing a conformational switch that would not only free SL1 but also the core encapsidation signal (Figure 32, A). With SL1 available, a kissing-loop complex could be formed that would free the primer binding site for annealing to a tRNA (Figure 32 B). With the Ψ encapsidation signal exposed it would be available for binding other factors, most likely the nucleocapsid domain of the *gag* polyprotein (Figure 32 B). The dimeric RNA would then be encapsidated and the mature nucleocapsid protein could coordinate the switch to a more thermostable extended duplex as well as anneal the tRNA to the primer binding site.

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