*Published online 23 December 2008* *Nucleic Acids Research, 2009, Vol. 37, No. 3* ***945–956***
*doi:10.1093/nar/gkn1015*

# The A-rich RNA sequences of HIV-1 *pol* are important for the synthesis of viral cDNA

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Received November 11, 2008; Revised December 2, 2008; Accepted December 4, 2008

## ABSTRACT

**The bias of A-rich codons in HIV-1 *pol* is thought to be a record of hypermutations in viral genomes that lack biological functions. Bioinformatic analysis predicted that A-rich sequences are generally associated with minimal local RNA structures. Using codon modifications to reduce the amount of A-rich sequences within HIV-1 genomes, we have reduced the flexibility of RNA sequences in *pol* to analyze the functional significance of these A-rich 'structurally poor' RNA elements in HIV-1 *pol*. Our data showed that codon modification of HIV-1 sequences led to a suppression of virus infectivity by 5–100-fold, and this defect does not correlate with, viral entry, viral protein expression levels, viral protein profiles or virion packaging of genomic RNA. Codon modification of HIV-1 *pol* correlated with an enhanced dimer stability of the viral RNA genome, which was associated with a reduction of viral cDNA synthesis both during HIV-1 infection and in a cell free reverse transcription assay. Our data provided direct evidence that the HIV-1 A-rich *pol* sequence is not merely an evolutionary artifact of enzyme-induced hypermutations, and that HIV-1 has adapted to rely on A-rich RNA sequences to support the synthesis of viral cDNA during reverse transcription, highlighting the utility of using 'structurally poor' RNA domains in regulating biological process.**

## INTRODUCTION

Proteins are often thought to be the prime regulator of biological systems, but it is now appreciated that RNA has a much greater role in the regulation of biological processes than previously thought (1,2). It has been estimated that less than 2% of the human genome codes for proteins, but more than 90% of the human genome is transcribed in different cells (3). This vast difference between mRNA and non-protein coding RNA transcription suggests (a) the existence of an uncharacterized functional RNA-based regulation network and/or (b) the presence of a large quantity of non-functional-junk RNA transcripts within the cell. The existence of these vast quantities of non-coding RNAs in cells implies the significance of these differential RNA species in the regulation of biological processes (1,4). The discovery of RNA interference (RNAi) and microRNA (miRNA) highlights a complex and dynamic coordination network that exists through RNA elements. Recent works have shown that viruses have evolved to manipulate many of these same RNA networks to support their own replications (5,6). It is safe to assume that the microRNA network is not likely to be the last example demonstrating how organisms have developed ingenious ways to regulate biological process via RNA sequences.

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RNA structures are primarily determined by the nucleotide contents of the RNA sequences. With the exception of methionine and tryptophan, all amino acids can be coded by more than one codon. However, synonymous codons are rarely used with equal frequency and patterns of codon usage can vary between individual genes and even complete genomes, and the genomes of RNA viruses are no exception. In a recent analysis of 50 human RNA viruses, a diverse array of codon usage patterns was observed (7). Intriguingly, the nucleotide composition preferred by a given RNA virus does not always conform to the composition observed in the host organism in which it replicates. The striking preference for A-rich codons in the human immunodeficiency virus-type 1 (HIV-1) is well established (8–10) and clearly contrasts with the G-rich codon usage seen in mammalian cellular protein synthesis. The typical nucleotide composition of HIV-1 is ~36% A, 23% G, 19% C and 22% T, with some strains (such as strain O) comprised greater than 40% A (11). The A-rich codon usage bias is apparent in each of HIV-1's three major coding regions; *gag, pol* and *env*, but is most pronounced in the *pol* gene (12–14). Bioinformatic analysis would predict that the bias of A-rich nucleotides in the HIV-1 genome will limit the formation of stable local RNA structures in selected regions of viral genomes, and it is unclear whether these A-rich 'structurally poor' RNA elements are important for the propagation of HIV-1.

There are several non-mutually exclusive hypotheses as to why these A-rich sequences have evolved in the HIV-1 genome. For example, early work suggested that an imbalance in the pyrimidine nucleoside pool of infected cells during reverse transcription could enhance the G-to-A mutation frequency (15,16). It has also been proposed that the low fidelity of reverse transcriptase (RT) may be responsible for HIV-1's codon usage bias. This position emerged from the observation that the most common error made during reverse transcription of HIV-1 in single replication experiments is a G-to-A mutation (17). More recently, it has been thought that the host cytidine deaminase APOBEC3G may have driven the evolution of the A-rich HIV-1 genome (18–22). Nucleic acid editing by APOBEC3G is thought to be an ancient form of host-cell innate immunity (23). It is thought that virion incorporation of APOBEC3G triggers the deamination of cytosine to uracil on the negative sense single-stranded DNA that is produced during reverse transcription and results in the incorporation of A instead of G in the proviral plus-sense DNA strand (24). Viral strategies have evolved to limit G-to-A hypermutation. The role of the HIV-1 accessory protein Vif includes blocking the packaging of APOBEC3G, and thereby removes the opportunity for APOBEC3G to attack the viral genome (25). While Vif is now recognized to be central to HIV-1's evasion of the host-defence response, the evolutionary pathway traversed in the development of this strategy may be visible in HIV-1's A-rich genome.

In a virus renowned for making the most of it is compact genome, it is unlikely that HIV-1's A-rich genome is merely a 'record' left behind by one or all of these hypermutation mechanisms. To assess the role of A-rich structurally poor RNA elements in HIV-1 replication, we have systematically replaced wild-type (WT) A-rich regions of *gag* and *pol* with codon-modified G-rich RNA segments without altering the amino-acid sequences. The A-to-G substitution will have little impact on existing A-U base-pairing as G-U also base pairs. The substitution of A-to-G will also enable original unpaired nucleotides to basepair with either U or C nucleotide. In contrast to the codon optimized expression vectors, partial replacement of the A-rich HIV-1 sequences with G-rich sequences in the context of the full-length HIV-1 genome did not alter the level of overall viral protein production. Codon modification of HIV-1 *pol* led to more pronounced defects in viral replication. These defects in viral replication correlated with an enhancement of genomic RNA dimer stability and a reduction of viral cDNA synthesis. Our results support the notion that the A-rich structurally poor elements of HIV-1 *pol* are important for viral replication by maintaining the structural flexibility of the dimeric RNA that is critical for the synthesis of viral cDNA genomes during infection. This work provides experimental evidence illustrating that the flexibility of RNA molecules is an important regulator of biological processes.

## MATERIALS AND METHODS

### Construction of full-length HIV-1 DNA plasmids

The HIV-1 DNA constructs were derived from the full-length WT HIV-1 plasmid NL4.3 (26). Three codon-modified HIV-1 mutants were created with altered genomic RNA base sequences in *gag* and/or *pol* that retain the WT amino-acid sequences of the expressed viral proteins. WT A-rich regions in the NL4.3 plasmid were replaced with the equivalent regions from a G-rich Gag/Gag-Pol vector (phGag-Pol) (27). The phGag-Pol vector is codon modified (G-rich) throughout, with the exception of the sequences where *gag* and *pol* overlap (between amino acids $NC_{53}$ in Gag and $PR_{23}$ in Pol). These sequences remain A-rich to allow normal production of the Gag-Pol polyprotein via frameshifting (27). The mutants are depicted in Figure 1 and have been

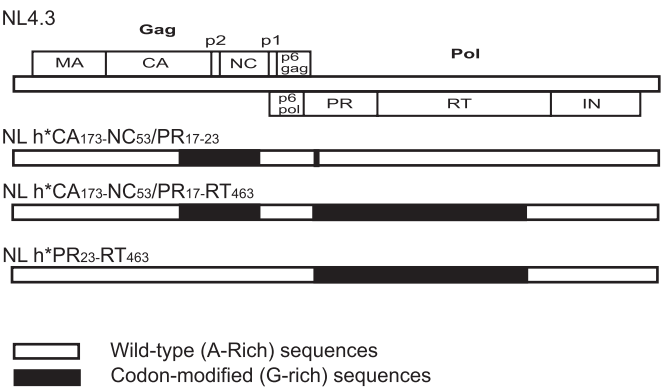


**Figure 1.** Schematic representation of the NL4.3-based constructs used in this study. The codon-modified regions are indicated.

named according to the amino-acid location of the replaced sequence: (i) NL h*$CA_{173}$-$NC_{53}$/$PR_{17–23}$, (ii) NL h*$CA_{173}$-$NC_{53}$/$PR_{17}$-$RT_{463}$ and (iii) NL h*$PR_{23}$-$RT_{463}$. PCR site directed mutagenesis was performed in the WT NL4.3 plasmid as previously described (28). The codons at amino acid 173 in CA ($CA_{173}$), amino acid 23 in PR ($PR_{23}$) and/or amino acid 463 in RT ($RT_{463}$) were modified to introduce the restriction sites BssHII, XbaI and/or NotI, respectively, without altering the amino-acid sequence. The introduced restriction sites were then used to replace the WT A-rich NL4.3 sequences with the corresponding G-rich sequence from phGag-Pol. Replacement of the 620 bp BssHII-XbaI region (between amino acids $CA_{173}$ and $PR_{23}$) generated NL h*$CA_{173}$-$NC_{53}$/$PR_{17–23}$. Similarly, replacement of the entire 2230 bp BssHII-NotI region (between amino acids $CA_{173}$ and $RT_{463}$) yielded NL h*$CA_{173}$-$NC_{53}$/$PR_{23}$-$RT_{463}$ and replacement of the 1610-bp XbaI-NotI region (between amino acids $PR_{23}$ and $RT_{463}$) generated NL h*$PR_{23}$-$RT_{463}$. All mutant sequences were confirmed by sequencing analysis.

### Virus production

293T cells were used for transient transfection. The cells were maintained in Dulbecco's Modified Eagle's Medium (Gibco BRL) containing 10% heat-inactivated foetal bovine serum (P. A. Biological Co.) and 1% penicillin–streptomycin. Virus stocks were produced using either the calcium phosphate co-precipitation method or polyethylenimine (PEI) for the transient transfection. For calcium phosphate transfection, 10 μg of DNA from each of the HIV-1 constructs was routinely used. The enhanced green fluorescent protein (EGFP, Clonetech) reporter plasmid (2 μg) was included to monitor transfection efficiency. For PEI transfection, 3 μg of DNA from each HIV-1 construct was used. Supernatants and cells were collected at 36 h post-transfection and separated by centrifugation for 30 min at 3000 rpm (Beckman Model GS-6). The RT activity of cell culture supernatants was measured utilizing a micro-RT assay as previously described (29).

### Viral infectivity in peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from HIV-seronegative buffy coats (supplied by the Red Cross Blood Bank, Melbourne, Victoria, Australia) as previously described (30). PBMCs were stimulated with 10 μg/ml of phytohemagglutinin (Murex Diagnostics) and maintained in RPMI (Roswell Park Memorial Institute) 1640 medium (Gibco) containing 10% fetal bovine serum, gentamicin, glutamine and 5% interleukin-2 (Boehringer) for 3 days. Viral supernatants, which were normalized for RT activity, were then mixed with $10^5$ PBMCs in a 96-well tissue culture plate. Eight 10-fold dilutions of each virus were tested in triplicate. A half media change was carried out every 3–4 days. Viral infectivity was assessed by monitoring RT activity, as described above, with supernatants collected on day 3, 7, 10 and 14 post-infection. Infectivity was quantified using $TCID_{50}$.

### Protein isolation, quantification of virion proteins and western blot analysis

Intracellular viral protein was isolated from transfected 293T cells by washing cells twice with 1× phosphate buffered saline (PBS) then lysing with 2× Tris-buffered saline (TBS) lysis buffer containing 1% Nonidet P-40, 20 mM phenylmethyl sulfonyl fluoride (PMSF), 1 μM pepstatin and 1 μM leupeptin. Cell lysates were freeze-thawed in liquid nitrogen and then clarified by centrifugation for 20 min at 14 000 rpm. Virion protein was obtained by ultracentrifugation (Beckman model L-90, SW 41 rotor) of the transfection supernatants at 35 000 rpm for 1 h at 4°C through a 20% sucrose cushion. The viral pellets were resuspended in 50 μl 2× TBS lysis buffer. Western blot analysis was carried out as previously described (31), using pooled sera from HIV-1 infected patients to detect total HIV-1 proteins.

### Analysis of virion RNA packaging

Pelleted virions were prepared by ultracentrifugation as described above and resuspended in 500 μl Trizol (GIBCO). Following incubation for 30 min on ice to allow lysis, the samples were phenol/chloroform extracted and genomic RNA was isolated as previously described (32,33). A standard curve of WT RNA was prepared consisting of 150%, 100%, 50% and 10% of WT HIV-1. Samples were normalized by total virion proteins. Samples were heat denatured at 68°C for 15 min and then separated by electrophoresis in a 1% denaturing agarose gel. Samples were transferred onto a Hybond N membrane (Amersham Pharmacia Biotech). RNAs were hybridized with a radioactive riboprobe (pGEM7zHIV-1), which is complimentary to the 5 end of the HIV-1 genomic RNA (28,30–33).

### Analysis of virion RNA dimer stability

Viral supernatants were purified and concentrated by ultracentrifugation as described above. Virion pellets were resuspended in 500 μl of RNA dimer lysis buffer [10 mM Tris (pH 7.5), 1 mM EDTA, 1% SDS, 50 mM NaCl, yeast tRNA at 50 μg/ml and proteinase K at 100 μg/ml], phenol/chloroform extracted and isolated for melting curve analysis as previously described (34,35). Similar amounts of genomic RNA were used to analyze the stability of the virion RNA dimer as previously described (28,30,36). Briefly, RNA samples that had been heat denatured for 10 min at either 40°C, 43°C, 46°C, 49°C, 52°C or 55°C were separated by electrophoresis in a 1% native agarose gel in 0.5× Tris–borate–EDTA buffer. Samples were transferred onto a Hybond N membrane (Amersham Pharmacia Biotech). Dimeric and monomeric RNAs were hybridized with a radioactive riboprobe (pGEM7zHIV-1) as above.

### Assessment of reverse transcription competency in infected cells

Viral stocks derived from transfected 293T cells were filtered (0.22 μm), concentrated by ultracentrifugation then normalized by p24 Gag ELISA (Vitroniska). The viral

stocks were treated with RNase-free DNase (Roche) for 30 min at 37°C in the presence of 0.01 M $MgCl_2$. An aliquot of each DNase-treated viral stock was heat-inactivated (56°C for 1 h) prior to infection to monitor plasmid DNA contamination from the transfection. WT and mutant virions were used to infect PHA-stimulated PBMCs pooled from two donors at 500 ng p24 per $10^5$ cells. At 24 h post-infection, the PBMCs were washed in PBS and then lysed in PCR lysis buffer [10 mM Tris pH8, 1 mM ethylenediamine tetra-acetic acid, 0.002% (vol/vol), Triton X-100, 0.002% (wt/vol) sodium dodecyl sulfate and proteinase K at 0.8 mg/ml]. The lysates were incubated at 56°C for 1 h, followed by heat inactivation of proteinase K for 10 min at 95°C.

The PBMC lysates were assessed via real-time PCR using the iCycler iQ (BIO-RAD). The HIV-1 based primers utilized to detect early and late reverse transcription products have been previously described (37,38). The primer pair AA55/M667 that amplifies the R-U5 region of the LTR was used to detect the early reverse transcription products of minus-strand strong stop DNA (ssDNA). The primer pair M661/M667 that amplifies within the LTR/*gag* region was used to detect complete HIV-1 cDNA. The PCR reactions were performed using SYBER green I (BIO-RAD) with the PCR conditions previously described by Victoria *et al*. (38). Input cell numbers were normalized by quantifying CCR5 (39).

### *In vitro* reverse transcription assay

A 220-nucleotide-long HIV sequence corresponds to the coding sequence of last 13 amino acids of p2 and first 60 amino acids of NC were cloned out from NL4.3 and NL $h^*CA_{173}$-$NC_{53}$/$PR_{17–23}$, respectively. These sequences were engineered into *in vitro* transcription vector. RNA templates were prepared from plasmids digested by SmaI and *in vitro* transcribed, with T7 RNA polymerase as previously described (40). WT and NL $h^*p2$-$NC_{53}$ RNAs were purified by gel filtration. DNA primer (NL4255a: 5′ TTCCCTAAAAAATTAGCCTGTC 3′ that corresponds to the coding sequences of $NC_{54–60}$) were chemically synthesized and 5′-end-labeled with [$\gamma$-$^{32}$P]ATP (Perkin Elmer) and polynucleotide kinase from phage T4 (Fermentas). $RT^{E478Q}$, lacking RNase H activity, was a gift from G. Bec and was purified according to a protocol kindly provided to us by Dr Torsten Unge (Uppsala, Sweden).

For reverse transcription experiments, template RNAs WT and codon-modified HIV sequences were hybridized with $^{32}$P-labelled primer NL4255a and template RNAs at a 3:1 molar ratio. Hybridization efficiency was checked on a native 12% polyacrylamide gel. Primer/template duplexes (10 nM final concentration) were pre-incubated with RT (10 nM final concentration) at 37°C for 4 min, in 50 mM Tris–HCl pH 8.3 (37°C), 50 mM KCl, 6 mM $MgCl_2$ and 1 mM DTT. Reverse transcription was initiated by the addition of 50 μM of each of the four deoxyribonucleoside triphosphates. Reactions were stopped at 30 s, 1, 5, 15, 30 and 60 min by the addition of formamide and reaction products were analyzed on denaturing 8% polyacrylamide-urea gels and quantified with a Bioimager.

### β-Lactamase entry assay

MT2 cells ($5\times 10^5$) were incubated with virions produced through PEI transfection of 3 μg of appropriate proviral DNA and 1.5 μg pMM310 (a β-lactamase-Vpr plasmid, kindly donated by M. Miller, Merck Research Laboratories) (50 ng p24-Gag) at 37°C for 1 h. Cells that were not exposed to virus were used as a negative control for virus entry. Cells were washed in $CO_2$-independent media ($CO_2$10, Gibco BRL) containing 10% Cosmic Calf Serum without antibiotics. Cells were then resuspended in 100 μl $CO_2$10 containing 2.5 mM probenecid and 20 μl of CCF2/AM dye prepared as per manufacturer's instructions (Invitrogen), then incubated at room temperature for 7 h protected from light. Cells were washed once with PBS and then fixed in a 4% solution of paraformaldehyde. The change in emission fluorescence of CCF2 after cleavage by the β-lactamase-Vpr plasmid was monitored by flow cytometry with a three-laser FACS ARIA (Becton Dickinson). CCF2 fluorescence was excited using a Violet 407 nm laser emission. Blue emission was detected at 450 nm BP (450/40 = range 410–490 nm); green emission was detected with a 502 nm LP and a 520 nm BP (520/30 = range 490–550 nm).

## RESULTS

### Codon modification and *in scilico* analysis of the folding potential of HIV-1 RNA sequences

The unusually high levels of A-rich sequences in *pol* suggest that the local RNA sequences within *pol* would have a flexible structure and a low folding potential to form stable RNA elements. By taking advantage of the existing codon-modified HIV-1 genomes in the literature (27), we have codon modified the HIV-1 *pol* in the context of the full length HIV-1 genome (without changing the amino acids) to examine the role of A-rich RNA sequences in viral replication. Using the full-length NL4.3 strain of HIV-1 as the parental backbone, the RNA sequences corresponding to coding sequences from amino acid 23 of protease ($PR_{23}$) to amino acid 463 of reverse transcriptase ($RT_{463}$) were codon modified for analysis (Figure 1). RNA sequences corresponding to coding sequences from amino acid 173 of capsid ($CA_{173}$) to amino acid 53 of nucleocapsid ($NC_{53}$) in *gag* were also codon modified for comparison. A mutant containing codon modification in both *gag* ($CA_{173}$-$NC_{53}$) and *pol* ($PR_{23}$-$RT_{463}$) was also included in the study.

RNA sequences upstream of the coding sequence of $CA_{173}$ were left untouched due to the existence of a number of well defined cis-acting instability elements in *gag* (41). Similarly, the coding sequence of the major homology region of capsid protein has not been modified as this region has been implicated to be important for the trafficking of HIV-1 RNA (42–44). The coding sequences from $NC_{53}$ to $PR_{23}$ were unmodified as they consist of RNA frameshift elements and overlapping reading frames of Gag and Pol (45). The coding sequence of

integrase was not altered due to the presence of DNA flap sequences (46,47). It is conceivable that some of the yet to be identified RNA structures within these codon-modified segments might be abolished through these codon modifications (48).

As biochemical analysis of RNA structures of HIV-1 *pol* is currently lacking, a bioinformatic approach was employed to estimate the RNA folding potentials of our codon-modified HIV-1 genomes. Energy difference scan (EDscan) (49), a program for analysis of well-ordered folding segments in nucleotide sequences, was used. By fixing a window size of 70, 80, 90 or 100-nucleotides, the lowest free energy of the optimal structure folded by the segment was calculated. Regardless of the window size used for analysis, the codon-modified genomes consistently had lower free energy values than the unmodified control and a higher probability to form stable RNA structures. Furthermore, in accordance with the enrichment of A-rich nucleotides in *pol*, the local minimum free energy values for unmodified *pol* sequences were generally higher than the free energy values for unmodified *gag*, implicating that the RNA structures in HIV-1 *pol* is more flexible than in *gag*. These points were verified with whole genome folds (Figure 2) that considered a range of optimal and sub-optimal configurations for each intact RNA. When measured for the optimal folds, the largest genome-wide change in A content (36% vs. 32%), between WT (NL4.3) and mutant (NL $h^*CA_{173}$-$NC_{53}$/$PR_{17}$-$RT_{463}$), showed the largest corresponding shift in total basepair composition, with AU:GC ratios converting from 46:43% to 40:50%, respectively, and the genome minimum energy (dG) shifting equivalently lower (−2096 kcal vs. –2388 kcal). Despite these significant composition changes, the optimal and suboptimal data also predicted the global topological changes for each RNA are primarily restricted to within a few hundred nucleotides of the altered bases. That is, outside of the *gag–pol* genes themselves, and a few local perturbations in the 3′-most 150 bases, the remainder of the unmodified and codon-modified genomes took similar configurations over a wide range (optimal plus >20 kcal) of sampled energies.

### A-rich RNA sequences of the HIV-1 *pol* are critical for viral replication

The biological impact of the flexibility of HIV-1 *pol* RNA sequences on HIV-1 biology was assessed using WT and codon-modified HIV-1. WT and mutant virions derived from transfection of proviral DNA into 293T cells were normalized for RT activity and assayed for infectivity in three different PBMCs donors. To directly assess the relative infectivity of mutant and WT HIV-1, tissue culture infectious dosage 50% (TCID50) calculations were performed on day 10 of infection (Figure 3A). The two mutants with G-rich *pol* regions, NL $h^*CA_{173}$-$NC_{53}$/$PR_{23}$-$RT_{463}$ and NL $h^*PR_{23}$-$RT_{463}$ were 30–100-fold less infectious than WT, while the mutant NL $h^*CA_{173}$-$NC_{53}$/$PR_{17–23}$, which is codon modified in *gag* but remains A-rich in PR-RT was ∼5-fold less infectious than WT (Figure 3A). The replication kinetics of these viruses also differed (Figure 3B). NL $h^*CA_{173}$-$NC_{53}$/$PR_{23}$-$RT_{463}$ was non-infectious. NL $h^*PR_{23}$-$RT_{463}$ demonstrated dramatically reduced infectivity when compared to WT, but displayed a slight increase in virus production over the time course (Figure 3B). The replication kinetics of NL $h^*CA_{173}$-$NC_{53}$/$PR_{17–23}$ were delayed when compared to WT; however, viral production was similar to that of WT by day 14. Overall, these results point to an important role for HIV-1's A-rich sequences in viral infectivity and suggest that the A-rich RNA sequences in the PR-RT region of the *pol* gene are critical for replication.

### Partial codon modification of HIV-1 can alter virion protein processing but does not enhance viral protein expression

Previous studies have shown that expressions of the codon-modified HIV-1 gene in expression constructs significantly enhance the levels of viral protein expression by a factor of 10 through bypassing the Rev-dependent mechanism (27,50,51). One would expect that if our codon modifications had altered critical unknown *cis*-acting instability elements (INS) in *gag* and *pol*, an enhanced level of viral protein expression would be detected. Western blot analysis was performed on viral proteins produced in transfected 293T cells using HIV-1 pooled patient sera. Loading volumes were normalized for transfection efficiency based on the expression of the co-transfected EGFP. Overall the intracellular protein profiles of the codon-modified mutants were comparable but not identical to WT (Figure 4A). In the intracellular protein profile of NL $h^*PR_{23}$-$RT_{463}$, we observed an additional 90 kDa protein (Figure 4A). Based on size, this protein is most likely to be an uncleaved RT-IN intermediate. Purified viral particles were also analyzed by western blotting. Differences were seen with the protein profiles of the three codon-modified mutants in comparison with the WT (Figure 4B). It is important to note that viral protein expression levels in the three codon-modified constructs were comparable to WT. These results contrast with previous studies (27,50,51), highlighting that our codon-modified HIV-1 RNA genomes (perhaps due to the presences of Rev responsive elements) are not likely to alter the viral RNA trafficking network or influence the levels of viral protein expression.

### Flexibility of HIV-1 RNA sequences in *pol* is a negative regulator of genomic RNA dimer stability

The virion packaging of RNA genomes and the stability of RNA dimers in the codon-modified HIV-1 were also examined. For analysis of genomic RNA packaging, samples were normalized on the basis of total viral protein. A standard curve of WT genomic RNA packaging was constructed from four dilutions (150%, 100%, 50% and 10%). The levels of virion genomic RNA packaging in these codon-modified HIV mutants varies from 50% to 80% of RNA genomes found in WT HIV (Figure 5A). Analysis of genomic RNA dimer stability showed that dimeric RNA was present in all viruses (Figure 5B). WT genomic virion RNA was fully dissociated from dimeric to monomeric RNA at 46°C. When compared to

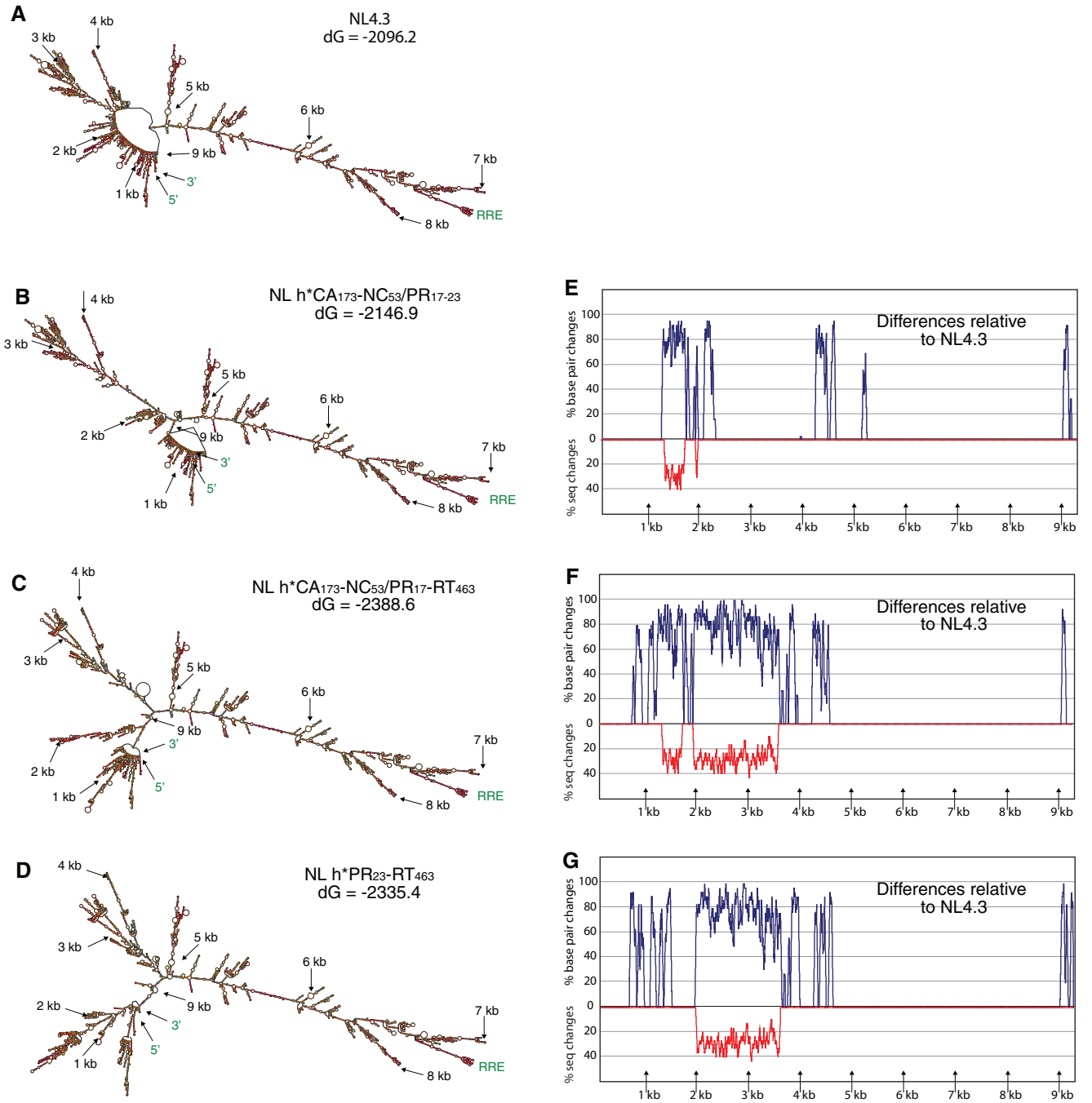


**Figure 2.** Computer-prediction showing the topology of the single stranded RNA of the WT and codon-modified constructs. The folds were prepared by using the mfold program and presented (**A**–**D**) as the global MinE structure with bases colored according to their P-num values (72). Low P-num values (<3%) are represented by red bases, while increasing P-num values are represented by the colors orange, yellow, green (medium) and blue through black (high). The sequence and global MinE structure for NL4.3 were compared to each of the three mutant datasets (**E**–**G**). For each paired series, a sliding window 30 residues wide, tabulated the percent of bases with changed sequence (red), and the percent of bases which took different pairing partners (blue). The data are plotted for every comparative window across the genome length. Genome landmarks include *gag* (b 336–1835), *pol* (b 1631–4639), *env* (b 5767–8329) and the RRE (b 7294–7541).

WT, the stability of the dimeric RNA was increased in mutants with codon modification in *pol* sequences (NL h*$CA_{173}$-$NC_{53}$/$PR_{23}$-$RT_{463}$ and NL h*$PR_{23}$-$RT_{463}$) (Figure 5B), whereas the dimer stability of NL h*$CA_{173}$-$NC_{53}$ was only mildly affected in comparison with the WT control (Figure 5B).

## A-rich sequences in HIV-1 are essential for reverse transcription

One of the key roles of dimeric RNA genomes in retroviruses is to enable inter-mixing of genomic sequences during reverse transcription, which ultimately maintains

A

| Proviral DNA | Virion Infectivity Infectious particles/ml (x1000) |
|---|---|
| NL4.3 | 1780 |
| NL h*$CA_{173}$-$NC_{53}$/$PR_{17-23}$ | 316 |
| NL h*$CA_{173}$-$NC_{53}$/$PR_{17}$-$RT_{463}$ | 18 |
| NL h*$PR_{23}$-$RT_{463}$ | 56 |

B

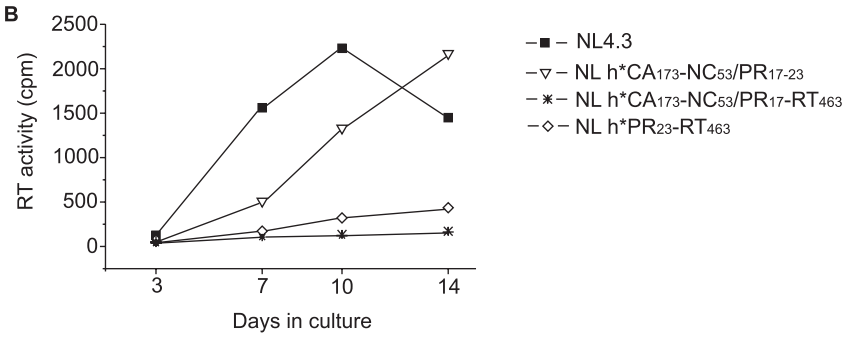


**Figure 3.** Partial codon modification of the HIV-1 genome impairs viral replication in PBMCs. (**A**) Virion infectivity ($TCID_{50}$) of WT and mutant HIV-1 in PBMCs. The values shown are per $10^2$ infectious particles/ml. (**B**) Replication kinetics of the WT and mutant HIV-1 in PBMCs. Freshly isolated PBMCs were stimulated with PHA for 3 days and then infected with either WT (NL4.3) or mutant virions. Infectivity was monitored as RT activity in the culture supernatants, which were collected 3, 7, 10 and 14 days after infection. The results represent the mean of duplicate samples.

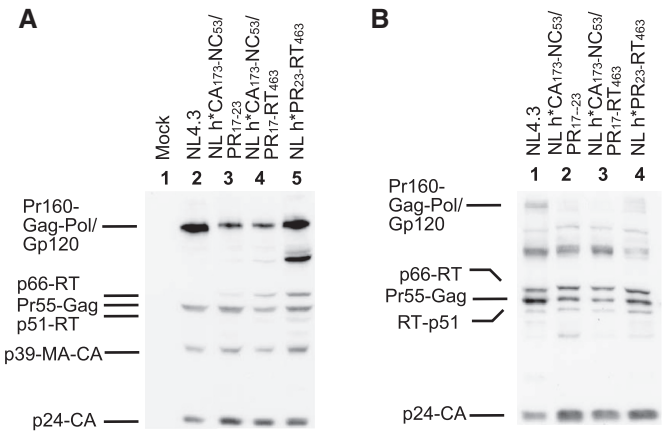


**Figure 4.** Partial codon modification of the HIV-1 genome can alter virion protein processing. Intracellular (**A**) and virion lysates (**B**) were produced from transfections in 293T cells, resolved by SDS–PAGE (10%) and probed with sera from HIV-1 infected individuals.

genome diversity. To determine whether codon modification could interfere with the reverse transcription process, the generation of early and late reverse transcription products was monitored using real-time PCR (38). The M667-AA55 primer pair was used to amplify the minus-strand ssDNA, the M667-M661 primer pair was used to amplify the complete viral cDNA. The primer positions are depicted in Figure 6A. Potential contamination from transfected plasmid DNA was measured in samples infected with heat-inactivated virus, and amplification from these control samples was consistently less than 1% of the WT 'live-virus' infection sample, suggesting that contaminating plasmid DNA was negligible. Results representative of three separate infections are presented in Figure 6B. The level of minus-strand ssDNA detected was dramatically reduced in each of the mutants, and accordingly, the detectable complete viral cDNA is also reduced. It is worth noting that although NL h*$CA_{173}$-$NC_{53}$/$PR_{17–23}$ was observed to have only mildly altered RNA dimer stability, the capacity of NL h*$CA_{173}$-$NC_{53}$/$PR_{17–23}$ to synthesize viral cDNA was significantly suppressed (Figure 6). These results suggest a critical role for A-rich sequences in both HIV-1 *gag* and *pol* to support the synthesis of viral cDNA during reverse transcription.

A reduction of viral cDNA synthesis was also observed when codon-modified RNA and corresponding WT *in vitro* transcribed RNAs were used as templates for *in vitro* reverse transcription assay (Figure 6C). Using WT A-rich RNA as template, the completion of viral cDNA synthesized can be detected as early as 5 min after the initiation of the *in vitro* reverse transcription, with 20% of the total labeled primers were detected with the completed viral cDNA at the end of reaction (Figure 6C). In contrast, when codon-modified RNA were used as template for *in vitro* reverse transcription, the synthesis of complete viral cDNA can only be detected 30 min post the initiation of the reaction, with only 2% (10-fold less) of the radioactive primers being part of the viral cDNA 60 min after the reverse transcription has started (Figure 6C).

### Viral entry is unaffected in codon-modified HIV-1

To verify that the observed defect in reverse transcription in the codon-modified mutants was not due to an

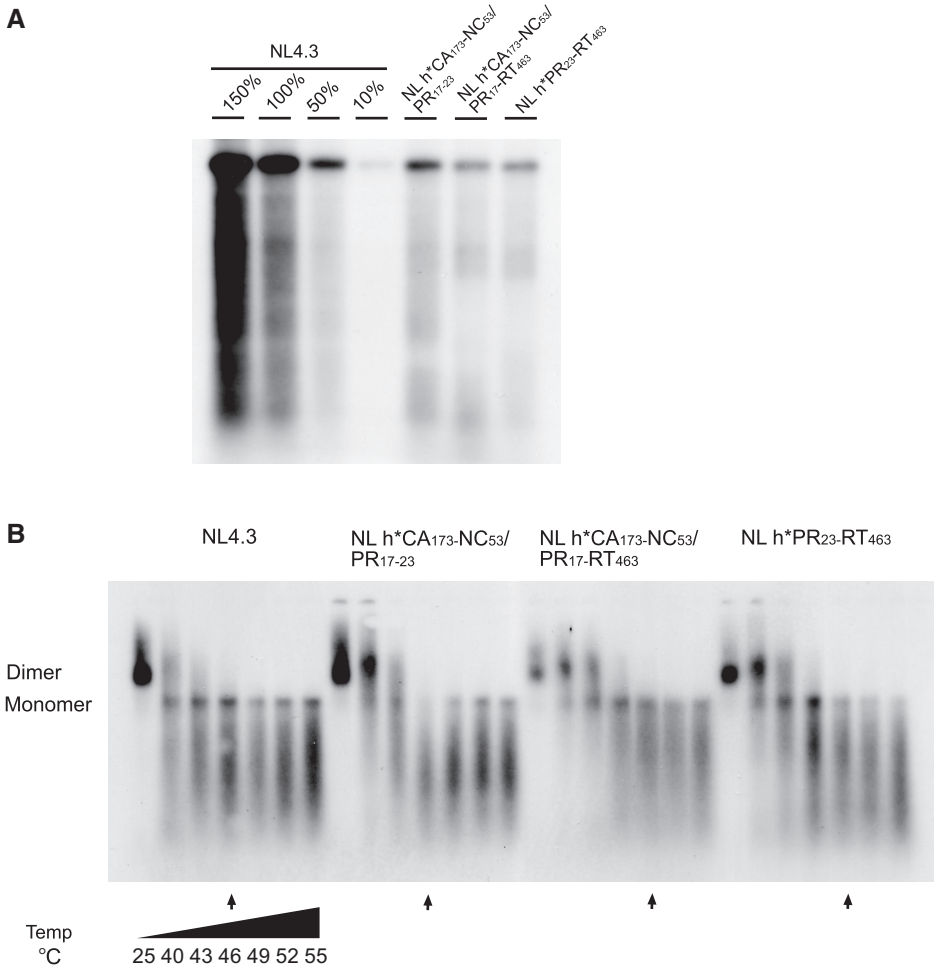


**Figure 5.** The impact of partial codon modification of the HIV-1 genome on virion genomic RNA packaging and dimerization. (**A**) Viral RNA packaging was examined using northern analysis. WT and mutant genomic RNA was heat denatured for 15 min at 68°C. Samples were then electrophoresed in a 1% native agarose gel and probed with an HIV-1 riboprobe. (**B**) Virion RNA dimerization was assessed with melting curve and electrophoretic analysis. WT and mutant virion RNA was resuspended in RNA dimerization buffer and heat denatured for 10 min at 40°C, 43°C, 46°C, 49°C, 52°C and 55°C. Dimers and monomers were electrophoresed in a 1% native agarose gel and probed with an HIV-1 riboprobe. The temperature at which the RNA dimers fully dissociate into monomers is marked with an arrow.

upstream defect in viral entry, a β-lactamase based viral entry assay was utilized (52). Viruses were produced by cotransfecting 293T cells with proviral DNA and the β-lactamase-Vpr (BlaM-Vpr) expression vector. The resultant WT and mutant virions were used to infect target cells MT2 with CCF2 dye. Upon successful virus entry, CCF2 was cleaved by the virion-associated BlaM enzyme, which resulted in a change of its fluorescence emission spectrum from green (520 nm) to blue (447 nm) (52). Our results show that similar amounts of blue cells were detected in our WT and mutant HIV-1 infected samples, indicating codon-modified HIV-1 were not defective in virus entry (Figure 7).

## DISCUSSION

This work provides evidence of a role for the A-rich RNA genome of HIV-1 in viral replication. Each of the mutants with codon modification of *gag* or *pol* showed dramatic impairment in viral replication. NL h*$CA_{173}$-$NC_{53}$/$PR_{17–23}$ was less infectious than WT HIV-1 with 5-fold less infectious particles. The two HIV-1 mutants with codon modification in *pol* were dramatically more impaired, as NL h*$PR_{23}$-$RT_{463}$ and NL h*$CA_{173}$-$NC_{53}$/$PR_{23}$-$RT_{463}$ were non-infectious in PBMCs and had 30–100 less infectious particles than WT (Figure 3). The A-rich bias is most pronounced in the *pol* gene (14), and codon modification to this region produced more dramatic changes to viral replication than changes made primarily to the *gag* gene. The difference in infectivity between the G-rich *gag* mutant (NL h*$CA_{173}$-$NC_{53}$/$PR_{17–23}$) and the G-rich *pol* mutants (NL h*$PR_{23}$-$RT_{463}$ and NL h*$CA_{173}$-$NC_{53}$/$PR_{23}$-$RT_{463}$) also indicates that different segments of HIV-1's A-rich genome may have distinct functions. One possibility is that the PR-RT regions of *pol* contain one or more yet to be identified RNA motifs that are important for viral replication (48).

The fact that an A-rich genome is a conserved feature in all lentiviruses (7,10) points to a critical role for this codon

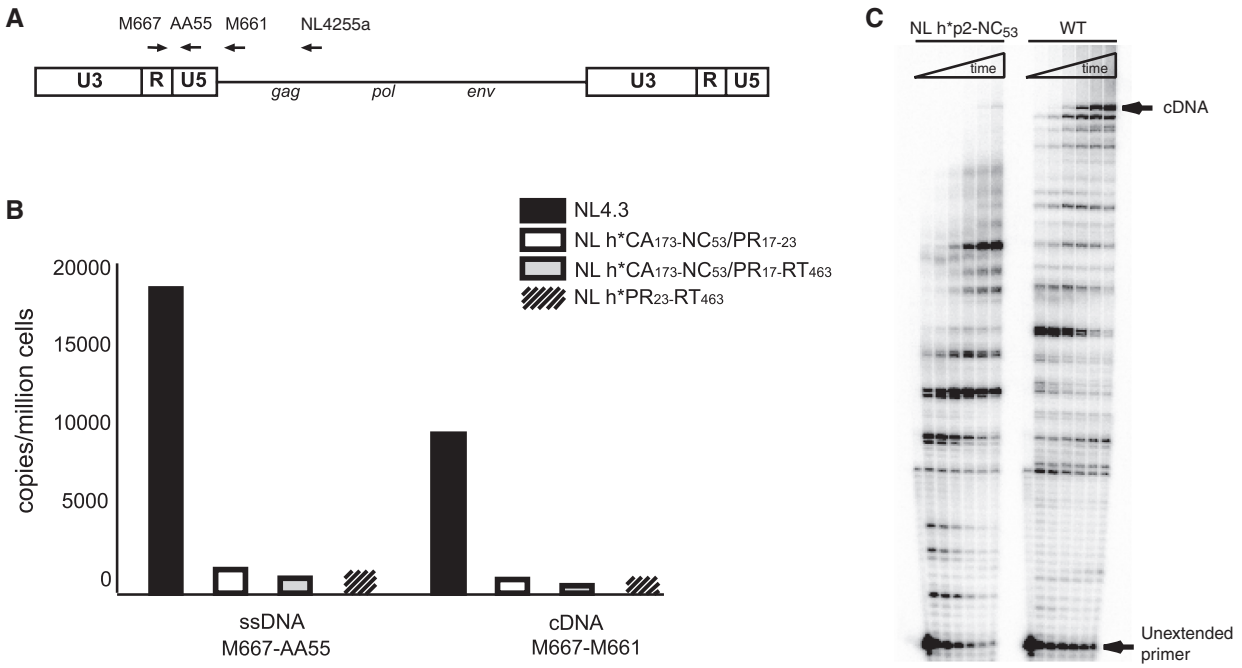


**Figure 6.** Partial codon modification of the HIV-1 genome results in viral particles with decreased reverse transcription activity. (**A**) The location and orientation of HIV-1 specific primers used to amplify viral DNA in real-time PCR is depicted. The primer pair AA55/M667 was used to amplify the minus-strand ssDNA. The primer pair M661/M667 was used to amplify complete HIV-1 cDNA (cDNA). (**B**) Freshly isolated PBMCs were stimulated with PHA for three days prior to infection with the WT (NL4.3) and mutant virions. Samples were lysed 24 h post-infection for analysis of reverse transcription products by real-time PCR. Results are shown as copies of viral DNA per million cells. (**C**) *In vitro* extension of DNA primers on codon-modified and WT RNA templates with primer NL4255a. Ten nanomolars of primer/template were extended with 10 nM of HIV-1 $RT^{E478Q}$ for 0 s, 30 s, 1 min, 5 min, 15 min, 30 min and 60 min. Reaction products were analyzed on denaturing 8% polyacrylamide-urea gels and quantified with a Bioimager. These figures are representative of three distinct experiments.

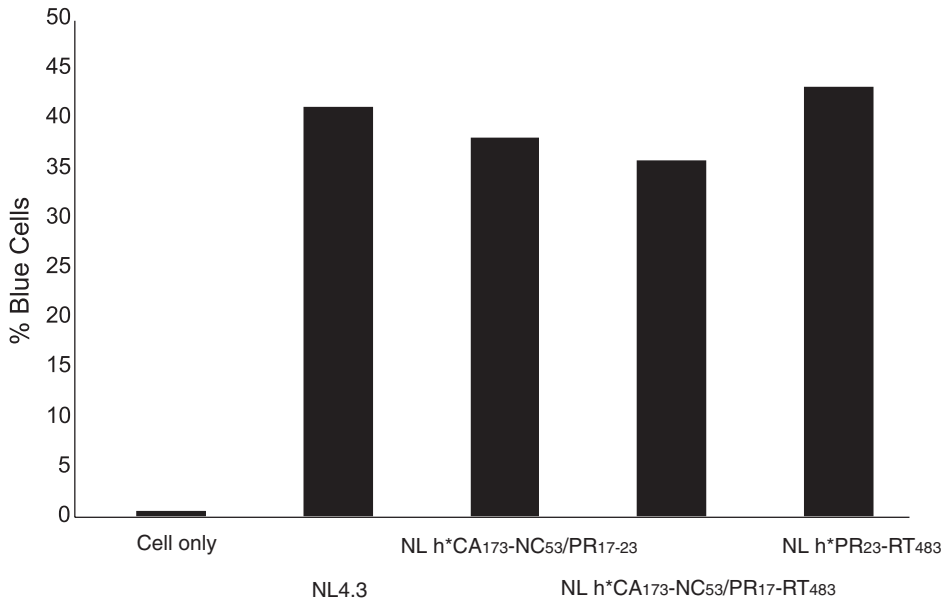


**Figure 7.** Partial codon modification of the HIV-1 genome does not impact upon viral entry. A mock infected cell only control was used to record the background levels of blue cells. Percentage of blue cells indicates the relative amounts of WT and codon-modified HIV-1 to enter MT2 cells. No detectable difference was observed between WT and codon-modified HIV-1 to enter T-lymphocytes. This figure is representative of three distinct experiments.

usage bias in HIV-1 replication that has not yet been explored. HIV-1, like all retroviruses, packages two copies of its RNA genome covalently linked near the 5′ end. The tertiary structure of HIV-1's dimeric RNA genome is essential for viral cDNA synthesis, the translation of viral proteins and the assembly and maturation of viral particles (53). These multifaceted roles require that the RNA genome can interact with itself as well as viral

and host cell proteins, making a dynamic and structurally versatile genome critical for optimal viral function (53,54). While many of the RNA structures that mediate these interactions are known (41,53,55–57), the knowledge of RNA elements existing across the full length of the HIV-1 genome is still in its infancy. Despite this, current data consistently point to important roles for RNA structure in viral function. For example, cis-acting instability elements (INS) are selected regions of high AU content and AUUUA motifs within the HIV-1 viral genome. INS have a well documented role in RNA stability and the nuclear retention of unspliced and singly spliced HIV-1 mRNAs (58,59), which ultimately influence the expression of viral proteins. Similarly, our understanding of the role of RNA structures in virus–host interactions is also rapidly expanding and it appears that both viruses and hosts actively compete for the control of the miRNA pathways to facilitate their own survival (60–63).

The structural genes encoding *gag, pol* and *env* contain multiple INS that make the protein expression dependent on the Rev- and CRM1-nuclear export pathways. The removal of the INS by codon modification allows access to Rev- and CRM1-independent nuclear export pathways that are not utilized by HIV-1 (50,58,64). The most likely explanation for the unchanged viral protein expression observed in our G-rich full length HIV-1 mutants is that in the context of the full-length HIV-1 genome, dependency on HIV-1's standard nuclear export pathways is maintained as the INS and RRE RNA elements have not been altered in *env*. As viral protein expression is not affected by the inclusion of these G-rich *gag* or *pol* sequences in the full-length genome, the observed suppression of viral infectivity in these mutants indicates that the A-rich sequences found in the HIV-1 genome have a role in viral replication that is independent of viral protein production levels.

In this study, we observed a subtle change in the intracellular protein profile of NL $h^{*}PR_{23}$-$RT_{463}$ with the appearance of an additional band at 90 kDa. Alterations to protein processing may be explained by changes in the kinetics of protein folding, which results from the anticipated change to RNA folding. Alternatively, there is evidence to suggest that RNA is important for protein processing in HIV-1 as the presence of RNA is required for proteolytic cleavage of HIV-1 NCp15 (65,66). As genomic RNA and the viral protease may interact for efficient cleavage of viral proteins, changing the RNA codons and consequently altering RNA folding may affect the proper RNA–viral protease interaction required for viral protein processing. It is also conceivable that this 90 kDa protein is derived from alternated spliced RNA that is the by-product of codon modification and the refolding of HIV RNA genomes. Further experimentations are required to validate these hypotheses.

We observed a correlation between the extent of the replication defect and the increase in RNA dimer stability. The increase in the stability of the RNA dimers may be due in part to increased hydrogen bonding strength between monomer strands of the G-rich mutants within the codon-modified RNA sequences. The increased dimer stability may also reflect a modification of RNA elements in *pol* that are required for normal dimer formation. The Dimerization Initiation Site (DIS) stem-loop (SL1) is the primary site involved in RNA dimer formation, while the DIS is indeed the nucleation point for genomic RNA dimerization, additional RNA sequences along the genome participate in RNA dimer formation (53). The involvement of additional sequences has been suggested by the finding that partial or complete deletion of the DIS stem loop does not abolish the formation of dimeric RNA (30). Moreover, evidence from avian retroviruses (67,68) has suggested that retroviral RNA interacts at multiple sites along the genome. The HIV-1 vector system used by Sakuragi *et al*. (69) provided some of the first evidence that RNA elements within *pol* may in fact negatively regulate the stability of RNA dimers (69). Consistent with the observation by Sakuragi *et al*. (69), the introduction of the codon-modified sequences may have obstructed the 'RNA elements' in *pol* required for normal RNA dimer formation, generating more stable dimers akin to those identified (69) when the *pol* region was deleted.

All three codon-modified constructs displayed greatly impaired reverse transcription (Figure 6B). Altering the codon bias of HIV-1 genomic RNA may impair viral infectivity by altering the conformation of the genomic RNA incorporated into the virion, so that the reverse transcription complex is rendered non-functional due to impaired interactions between the viral RNA and NC and other factors such as RNA helicase (70). Alternatively, the negative effects on reverse transcription may be due to changes in RNA structure that hinder the polymerases path, this notion is supported by *in vitro* reverse transcription assay where codon-modified RNA templates are less efficient to support the synthesis of viral cDNA than native HIV-1 RNA genome (Figure 6C). Unsuitable secondary structures introduced by codon modification may cause abortive reverse transcription, and this interpretation is consistent with previous reports showing RNA conformation is an important determinant for the synthesis of viral cDNA (71).

In this study, we have shown that A-rich structurally poor RNA sequences in HIV-1 genomes are not merely 'records' of hypermutations during viral evolution. These elements influence RNA dimer stability and are important for viral cDNA synthesis. Further investigations are needed to reveal the precise mechanistic contribution to HIV-1 biology and/or the regulation of RNA networks within the infected host cells.

## FUNDING

This work was supported by grants from the Australian National Health and Medical Research Council (NHMRC) and was supported in part by the intramural Research Program of the NIH, NCI, Center for Cancer Research. C.K. is recipient of the AMA J.G. Hunter Research Fellowship. R.P.S. is a recipient of the Northcote graduate scholarship. J.M. is a recipient of the Pfizer Foundation Fellowship. Funding for open access

charge: Macfarlane Burnet Institute for Medical Research and Public Health.

*Conflict of interest statement*. None declared.

## REFERENCES

1. Mattick,J.S. (2005) The functional genomics of noncoding RNA. *Science*, **309**, 1527–1528.
2. Matera,A.G., Terns,R.M. and Terns,M.P. (2007) Non-coding RNAs: lessons from the small nuclear and small nucleolar RNAs. *Nat. Rev. Mol. Cell Biol.*, **8**, 209–220.
3. Amaral,P.P., Dinger,M.E., Mercer,T.R. and Mattick,J.S. (2008) The eukaryotic genome as an RNA machine. *Science*, **319**, 1787–1789.
4. Mattick,J.S. and Makunin,I.V. (2006) Non-coding RNA. *Hum. Mol. Genet.*, **15(Spec No. 1)**, R17–R29.
5. Gottwein,E. and Cullen,B.R. (2008) Viral and cellular microRNAs as determinants of viral pathogenesis and immunity. *Cell Host Microbe*, **3**, 375–387.
6. Haasnoot,J., Westerhout,E.M. and Berkhout,B. (2007) RNA interference against viruses: strike and counterstrike. *Nat. Biotechnol.*, **25**, 1435–1443.
7. Jenkins,G.M. and Holmes,E.C. (2003) The extent of codon usage bias in human RNA viruses and its evolutionary origin. *Virus Res.*, **92**, 1–7.
8. Berkhout,B. and Van Hemert,F.J. (1994) The unusual nucleotide content of the HIV RNA genome results in a biased amino acid composition of HIV proteins. *Nucleic Acids Res.*, **22**, 1705–1711.
9. Berkhout,B., Grigoriev,A., Bakker,M. and Lukashov,V.V. (2002) Codon and amino acid usage in retroviral genomes is consistent with virus-specific nucleotide pressure. *AIDS Res. Hum. Retroviruses*, **18**, 133–141.
10. van Hemert,F.J. and Berkhout,B. (1995) The tendency of lentiviral open reading frames to become A-rich: constraints imposed by viral genome organization and cellular tRNA availability. *J. Mol. Evol.*, **41**, 132–140.
11. Vartanian,J.P., Henry,M. and Wain-Hobson,S. (2002) Sustained G—a hypermutation during reverse transcription of an entire human immunodeficiency virus type 1 strain Vau group O genome. *J. Gen. Virol.*, **83**, 801–805.
12. Grantham,P. and Perrin,P. (1986) AIDS virus and HTLV-I differ in codon choices. *Nature*, **319**, 727–728.
13. Sharp,P.M. (1986) What can AIDS virus codon usage tell us? *Nature*, **324**, 114.
14. Kypr,J. and Mrázek,J. (1987) Unusual codon usage of HIV. *Nature*, **327**, 20.
15. Martinez,M.A., Vartanian,J.P. and Wain-Hobson,S. (1994) Hypermutagenesis of RNA using human immunodeficiency virus type 1 reverse transcriptase and biased dNTP concentrations. *Proc. Natl Acad. Sci. USA*, **91**, 11787–11791.
16. Vartanian,J.P., Plikat,U., Henry,M., Mahieux,R., Guillemot,L., Meyerhans,A. and Wain-Hobson,S. (1997) HIV genetic variation is directed and restricted by DNA precursor availability. *J. Mol. Biol.*, **270**, 139–151.
17. Mansky,L.M. and Temin,H.M. (1995) Lower in vivo mutation rate of human immunodeficiency virus type 1 than that predicted from the fidelity of purified reverse transcriptase. *J. Virol.*, **69**, 5087–5094.
18. Harris,R.S., Bishop,K.N., Sheehy,A.M., Craig,H.M., Petersen-Mahrt,S.K., Watt,I.N., Neuberger,M.S. and Malim,M.H. (2003) DNA deamination mediates innate immunity to retroviral infection. *Cell*, **113**, 803–809.
19. Sheehy,A.M., Gaddis,N.C. and Malim,M.H. (2003) The antiretroviral enzyme APOBEC3G is degraded by the proteasome in response to HIV-1 Vif. *Nat. Med.*, **9**, 1404–1407.
20. Zhang,H., Yang,B., Pomerantz,R.J., Zhang,C., Arunachalam,S.C. and Gao,L. (2003) The cytidine deaminase CEM15 induces hypermutation in newly synthesized HIV-1 DNA. *Nature*, **424**, 94–98.
21. Yu,Q., Konig,R., Pillai,S., Chiles,K., Kearney,M., Palmer,S., Richman,D., Coffin,J.M. and Landau,N.R. (2004) Single-strand specificity of APOBEC3G accounts for minus-strand deamination of the HIV genome. *Nat. Struct. Mol. Biol.*, **11**, 435–442.
22. Lee,Y.N., Malim,M.H. and Bieniasz,P.D. (2008) Hypermutation of an ancient human retrovirus by APOBEC3G. *J. Virol.*, **82**, 8762–8770.
23. Jones,K.L. and Mak,J. (2005) Innate immunity and intracellular trafficking: insights for novel anti-HIV-1 therapeutics. *Curr. Pharmacogenomics*, **3**, 97–117.
24. Yu,Q., Chen,D., Konig,R., Mariani,R., Unutmaz,D. and Landau,N.R. (2004) APOBEC3B and APOBEC3C are potent inhibitors of simian immunodeficiency virus replication. *J. Biol. Chem.*, **279**, 53379–53386.
25. Sheehy,A.M., Gaddis,N.C., Choi,J.D. and Malim,M.H. (2002) Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. *Nature*, **418**, 646–650.
26. Adachi,A., Gendelman,H.E., Koenig,S., Folks,T., Willey,R., Rabson,A. and Martin,M.A. (1986) Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. *J. Virol.*, **59**, 284–291.
27. Huang,Y., Kon,W.-P. and Nabel,G.J. (2001) Human immunodeficiency virus type 1-specific immunity after genetic immunization is enhanced by modification of Gag and Pol expression. *J. Virol.*, **75**, 4947–4951.
28. Shehu-Xhilaga,M., Crowe,S.M. and Mak,J. (2001) Maintenance of the Gag/Gag-Pol ratio is important for human immunodeficiency virus type 1 RNA dimerization and viral infectivity. *J. Virol.*, **75**, 1834–1841.
29. Goff,S., Traktman,P. and Baltimore,D. (1981) Isolation and properties of Moloney murine leukemia virus mutants: use of a rapid assay for release of virion reverse transcriptase. *J. Virol.*, **1**, 239–248.
30. Hill,M.K., Shehu-Xhilaga,M., Campbell,S.M., Poumbourios,P., Crowe,S.M. and Mak,J. (2003) The dimer initiation sequence stem-loop of human immunodeficiency virus type 1 is dispensable for viral replication in peripheral blood mononuclear cells. *J. Virol.*, **77**, 8329–8335.
31. Shehu-Xhilaga,M., Kraeusslich,H.G., Pettit,S., Swanstrom,R., Lee,J.Y., Marshall,J.A., Crowe,S.M. and Mak,J. (2001) Proteolytic processing of the p2/nucleocapsid cleavage site is critical for human immunodeficiency virus type1 RNA dimer maturation. *J. Virol.*, **75**, 9156–9164.
32. Shehu-Xhilaga,M., Hill,M., Marshall,J.A., Kappes,J., Crowe,S.M. and Mak,J. (2002) The conformation of the mature dimeric human immunodeficiency virus type 1 RNA genome requires packaging of pol protein. *J. Virol.*, **76**, 4331–4340.
33. Hill,M.K., Shehu-Xhilaga,M., Crowe,S.M. and Mak,J. (2002) Proline residues within spacer peptide p1 are important for human immunodeficiency virus type 1 infectivity, protein processing, and genomic RNA dimer stability. *J. Virol.*, **76**, 11245–11253.
34. Fu,W. and Rein,A. (1993) Maturation of dimeric viral RNA of Moloney murine leukemia virus. *J. Virol.*, **67**, 5443–5449.
35. Fu,W., Gorelick,R.J. and Rein,A. (1994) Characterization of human immunodeficiency virus type 1 dimeric RNA from wild-type and protease-defective virions. *J. Virol.*, **68**, 5013–5018.
36. Buxton,P., Tachedjian,G. and Mak,J. (2005) Analysis of the contribution of reverse transcriptase and integrase proteins to retroviral RNA dimer conformation. *J. Virol.*, **79**, 6338–6348.
37. Zack,J.A., Arrigo,S.J., Weitsman,S.R., Go,A.S., Haislip,A. and Chen,I.S. (1990) HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure. *Cell*, **61**, 213–222.
38. Victoria,J.G., Lee,D.J., McDougall,B.R. and Robinson,W.E. Jr. (2003) Replication kinetics for divergent type 1 human immunodeficiency viruses using quantitative SYBR green I real-time polymerase chain reaction. *AIDS Res. Hum. Retroviruses*, **19**, 865–874.
39. Zhang,L., Lewin,S.R., Markowitz,M., Lin,H.H., Skulsky,E., Karanicolas,R., He,Y., Jin,X., Tuttleton,S., Vesanen,M. *et al.* (1999) Measuring recent thymic emigrants in blood of normal and HIV-1-infected individuals before and after effective therapy. *J. Exp. Med.*, **190**, 725–732.
40. Marquet,R., Baudin,F., Gabus,C., Darlix,J.-L., Mougel,M., Ehresmann,C. and Ehresmann,B. (1991) Dimerization of human

immunodeficiency virus (Type 1) RNA: stimulation by cations and possible mechanism. *Nucleic Acids Res.*, **19**, 2349–2357.
41. Felber,B.K., Zolotukhin,A.S. and Pavlakis,G.N. (2007) Posttranscriptional control of HIV-1 and other retroviruses and its practical applications. *Adv. Pharmacol.*, **55**, 161–197.
42. Mouland,A.J., Xu,H., Cui,H., Krueger,W., Munro,T.P., Prasol,M., Mercier,J., Rekosh,D., Smith,R., Barbarese,E. *et al.* (2001) RNA trafficking signals in human immunodeficiency virus type 1. *Mol. Cell Biol.*, **21**, 2133–2143.
43. Beriault,V., Clement,J.F., Levesque,K., Lebel,C., Yong,X., Chabot,B., Cohen,E.A., Cochrane,A.W., Rigby,W.F. and Mouland,A.J. (2004) A late role for the association of hnRNP A2 with the HIV-1 hnRNP A2 response elements in genomic RNA, Gag, and Vpr localization. *J. Biol. Chem.*, **279**, 44141–44153.
44. Levesque,K., Halvorsen,M., Abrahamyan,L., Chatel-Chaix,L., Poupon,V., Gordon,H., DesGroseillers,L., Gatignol,A. and Mouland,A.J. (2006) Trafficking of HIV-1 RNA is mediated by heterogeneous nuclear ribonucleoprotein A2 expression and impacts on viral assembly. *Traffic*, **7**, 1177–1193.
45. Jacks,T., Power,M.D., Masiarz,F.R., Luciw,P.A., Barr,P.J. and Varmus,H.E. (1988) Characterization of ribosomal frameshifting in HIV-1 gag-pol expression. *Nature*, **331**, 280–283.
46. Zennou,V., Petit,C., Guetard,D., Nerhbass,U., Montagnier,L. and Charneau,P. (2000) HIV-1 genome nuclear import is mediated by a central DNA flap. *Cell*, **101**, 173–185.
47. Arhel,N.J., Souquere-Besse,S., Munier,S., Souque,P., Guadagnini,S., Rutherford,S., Prevost,M.C., Allen,T.D. and Charneau,P. (2007) HIV-1 DNA flap formation promotes uncoating of the pre-integration complex at the nuclear pore. *EMBO J.*, **26**, 3025–3037.
48. Wang,Q., Barr,I., Guo,F. and Lee,C. (2008) Evidence of a novel RNA secondary structure in the coding region of HIV-1 pol gene. *RNA*, **14**, 2478–2488.
49. Le,S.Y., Chen,J.H., Konings,D. and Maizel,J.V. Jr. (2003) Discovering well-ordered folding patterns in nucleotide sequences. *Bioinformatics*, **19**, 354–361.
50. Kotsopoulou,E., Kim,V.N., Kingsman,A.J., Kingsman,S.M. and Mitrophanous,K.A. (2000) A Rev-independent human immunodeficiency virus type 1 (HIV-1)-based vector that exploits a codon-optimized HIV-1 gag-pol gene. *J. Virol.*, **74**, 4839–4852.
51. Wagner,R., Graf,M., Bieler,K., Wolf,H., Grunwald,T., Foley,P. and Uberla,K. (2000) Rev-independent expression of synthetic gag-pol genes of human immunodeficiency virus type 1 and simian immunodeficiency virus: implications for the safety of lentiviral vectors. *Hum. Gene Ther.*, **11**, 2403–2413.
52. Cavrois,M., De Noronha,C. and Greene,W.C. (2002) A sensitive and specific enzyme-based assay detecting HIV-1 virion fusion in primary T lymphocytes. *Nat. Biotechnol.*, **20**, 1151–1154.
53. Paillart,J.C., Shehu-Xhilaga,M., Marquet,R. and Mak,J. (2004) Dimerization of retroviral RNA genomes: an inseparable pair. *Nat. Rev. Microbiol.*, **2**, 461–472.
54. Berkhout,B. (2000) Multiple biological roles associated with the repeat (R) region of the HIV-1 RNA genome. *Adv. Pharmacol.*, **48**, 29–73.
55. Yeung,M.L., Bennasser,Y., Le,S.Y. and Jeang,K.T. (2007) RNA interference and HIV-1. *Adv. Pharmacol.*, **55**, 427–438.
56. Abbink,T.E. and Berkhout,B. (2007) HIV-1 reverse transcription: close encounters between the viral genome and a cellular tRNA. *Adv. Pharmacol.*, **55**, 99–135.
57. Lever,A.M. (2007) HIV-1 RNA packaging. *Adv. Pharmacol.*, **55**, 1–32.
58. Schneider,R., Campbell,M., Nasioulas,G., Felber,B.K. and Pavlakis,G.N. (1997) Inactivation of the human immunodeficiency virus type 1 inhibitory elements allow Rev-independent expression of Gag and Gag/protease and particle formation. *J. Virol.*, **71**, 4892–4903.
59. Schwartz,S., Felber,B.K. and Pavlakis,G.N. (1992) Mechanism of translation of monocistronic and multicistronic human immunodeficiency virus type 1 mRNAs. *Mol. Cell Biol.*, **12**, 207–219.
60. Christensen,H.S., Daher,A., Soye,K.J., Frankel,L.B., Alexander,M.R., Laine,S., Bannwarth,S., Ong,C.L., Chung,S.W., Campbell,S.M. *et al.* (2007) Small interfering RNAs against the TAR RNA binding protein, TRBP, a Dicer cofactor, inhibit human immunodeficiency virus type 1 long terminal repeat expression and viral production. *J. Virol.*, **81**, 5121–5131.
61. Haasnoot,J., de Vries,W., Geutjes,E.J., Prins,M., de Haan,P. and Berkhout,B. (2007) The Ebola virus VP35 protein is a suppressor of RNA silencing. *PLoS Pathog.*, **3**, e86.
62. Triboulet,R., Mari,B., Lin,Y.L., Chable-Bessia,C., Bennasser,Y., Lebrigand,K., Cardinaud,B., Maurin,T., Barbry,P., Baillat,V. *et al.* (2007) Suppression of microRNA-silencing pathway by HIV-1 during virus replication. *Science*, **315**, 1579–1582.
63. Berkhout,B. and Jeang,K.T. (2007) RISCy business: microRNAs, pathogenesis, and viruses. *J. Biol. Chem.*, **282**, 26641–26645.
64. Graf,M., Bojak,A., Deml,L., Bieler,K., Wolf,H. and Wagner,R. (2000) Concerted action of multiple cis-acting sequences is required for Rev dependence of late human immunodeficiency virus type 1 gene expression. *J. Virol.*, **74**, 10822–10826.
65. Sheng,N. and Erickson-Viitanen,S. (1994) Cleavage of p15 protein *in vitro* by human immunodeficiency virus type 1 protease is RNA dependent. *J. Virol.*, **68**, 6207–6214.
66. Sheng,N., Pettit,S.C., Tritch,R.J., Ozturk,D.H., Rayner,M.M., Swanstrom,R. and Erickson-Viitanen,S. (1997) Determinants of the human immunodeficiency virus type 1 p15NC-RNA interaction that affect enhanced cleavage by the viral protease. *J. Virol.*, **71**, 5723–5732.
67. Lear,A.L., Haddrick,M. and Heaphy,S. (1995) A study of the dimerization of Rous sarcoma virus RNA in vitro and in vivo. *Virology*, **212**, 47–57.
68. Ortiz-Conde,B.A. and Hughes,S.H. (1999) Studies of the genomic RNA of leukosis viruses: implications for RNA dimerization. *J. Virol.*, **73**, 7165–7174.
69. Sakuragi,J.-I. and Panganiban,A.T. (1997) Human immunodeficiency virus type 1 RNA outside the primary encapsidation and dimer linkage region affects RNA dimer stability in vitro. *J. Virol.*, **71**, 3250–3254.
70. Roy,B.B., Hu,J., Guo,X., Russell,R.S., Guo,F., Kleiman,L. and Liang,C. (2006) Association of RNA helicase a with human immunodeficiency virus type 1 particles. *J. Biol. Chem.*, **281**, 12625–12635.
71. Paillart,J.C., Berthoux,L., Ottmann,M., Darlix,J.L., Marquet,R., Ehresmann,B. and Ehresmann,C. (1996) A dual role of the putative RNA dimerization initation site of human immunodeficiency virus type 1 in genomic RNA packaging and proviral DNA synthesis. *J. Virol.*, **70**, 8348–8354.
72. Palmenberg,A.C. and Sgro,J.Y. (1998) Topological organization of picornaviral genomes: statistical prediction of RNA structural signals. *Semin. Virol.*, **8**, 231–241.