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

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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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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

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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 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 in this study. The codon-modified regions are indicated.

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

 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 NC53 in Gag and PR53 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...
named according to the amino-acid location of the replaced sequence: (i) NL h^{CA173-NC53/PR17-23}, (ii) NL h^{CA173-NC53/PR17-RT463} and (iii) NL h^{PR23-RT463}. PCR site directed mutagenesis was performed in the WT NL4.3 plasmid as previously described (28). The codons at amino acid 173 in CA (CA173), amino acid 23 in PR (PR23) and/or amino acid 463 in RT (RT463) 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 CA173 and PR23) generated NL h^{CA173-NC53/PR17-23}. Similarly, replacement of the entire 2230 bp BssHII-NotI region (between amino acids CA173 and RT463) yielded NL h^{CA173-NC53/PR23-RT463} and replacement of the 1610-bp XbaI-NotI region (between amino acids PR23 and RT463) generated NL h^{PR23-RT463}. 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 polyethylamine (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. The RT activity of cell culture supernatants was measured 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 protease 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 MgCl₂. 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 10⁵ 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 in 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 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*CA173-NC53/PR17–23*, 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-NC53 RNAs were purified by gel filtration. DNA primer (NL4255a: 5’ TTCCCTAAAAATTAGCCTGTC 3’) that corresponds to the coding sequences of NC54-60 were chemically synthesized and 5’-end-labeled with [γ-³²P]ATP (Perkin Elmer) and polynucleotide kinase from phage T4 (Fermentas). RT(E4780), lacking RNase H activity, was a gift from G. Bec and was purified according to the 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 ³²P-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 (10nM final concentration) at 37°C for 4 min, in 50 mM Tris–HCl pH 8.3 (37°C), 50 mM KCl, 6 mM MgCl₂ 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 × 10⁵) 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 CO₂-independent media (CO₂10, Gibco BRL) containing 10% Cosmic Calf Serum without antibiotics. Cells were then resuspended in 100 μl CO₂10 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 FACSAria (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 silico 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 (PR23) to amino acid 463 of reverse transcriptase (RT463) were codon modified for analysis (Figure 1). RNA sequences corresponding to coding sequences from amino acid 173 of capsid (CA173) to amino acid 53 of nucleocapsid (NC53) in gag were also codon modified for comparison. A mutant containing codon modification in both gag (CA173-NC53) and pol (PR23–RT463) was also included in the study.

RNA sequences upstream of the coding sequence of CA173 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 NC53 to PR23 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^CA173-NC53/PR17–RT463), showed the largest corresponding shift in total basepair composition, with AU:GC ratios con-

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 intracell-

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
WT, the stability of the dimeric RNA was increased in mutants with codon modification in pol sequences (NL h*CA_{173-NC53}/PR_{17-RT463} and NL h*PR_{23-RT463}) (Figure 5B), whereas the dimer stability of NL h*CA_{173-NC53} 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...
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\(^{CA173-NC53/PR17-23}\) was observed to have only mildly altered RNA dimer stability, the capacity of NL h\(^{CA173-NC53/PR17-23}\) 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 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
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 hCA173-NC53/PR17-23 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 hPR23-RT463 and NL hCA173-NC53/PR23-RT463 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 hCA173-NC53/PR17-23) and the G-rich pol mutants (NL hPR23-RT463 and NL hCA173-NC53/PR23-RT463) 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

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**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.
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

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 RTE478Q 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.
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 expression levels.

In this study, we observed a subtle change in the intracellular protein profile of NL LTRPR23-RT463 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 experimentation is 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 the 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.

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