Colocalization of m^6A and G-Quadruplex-Forming Sequences in Viral RNA (HIV, Zika, Hepatitis B, and SV40) Suggests Topological Control of Adenosine N^6-Methylation

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ABSTRACT: This Outlook calls attention to two seemingly disparate and emerging fields regarding viral genomics that may be correlated in a way previously overlooked. First, we describe identification of conserved potential G-quadruplex-forming sequences (PQSs) in viral genomes relevant to human health. Studies have demonstrated that PQSs are highly conserved and can fold to G-quadruplexes (G4s) to regulate viral processes. Key examples include G4s as a countermeasure to the host’s immune system or G4-guided regulation of replication or transcription. Second, emerging data are discussed concerning the epitranscriptomic modification N^6-methyladenosine (m^6A) in viral RNA installed by host proteins in a consensus sequence favoring 5′-GG(m^6A)-C-3′. The proposed pathways by which m^6A is written, read, and erased in viral RNA genomes and the impact this has on viral replication are described. The structural reason why certain sites are selected for modification while others are not is still mysterious. Finally, we discuss our new observations regarding these previous sequencing data that identify m^6A installation within the loops of two-tetrad PQSs in the RNA genomes of the Zika, HIV, hepatitis B, and SV40 viruses. We hypothesize that conserved viral PQSs can provide a framework (sequence and/or structural) for m^6A installation. We also discuss literature sources suggesting that PQSs as sites of RNA modification could be a general phenomenon. We anticipate our observations will provide ample opportunities for exciting discoveries regarding the interplay between G4 structures and epitranscriptomic modifications of RNA.

POTENTIAL G-QUADRUPLEX FORMING SEQUENCES IN VIRAL GENOMES

In specific guanine-rich (G-rich) sequences of DNA or RNA, G-quadruplex (G4) folds can drive a variety of cellular processes. In human cells, immunofluorescent visualization of G4s identified their formation in the genome and transcriptome. Processes in the genome that destabilize duplex DNA, DNA in the single-stranded regions of telomeres, or R-loops with the correct sequence allow G4s to fold. Functional roles for G4s have been documented in transcription, telomere homeostasis, alteration of the epigenetic landscape, their occurrence at origins of replication, and their function in both DNA and RNA in class-switch recombination, and these sequences may be sites of double-strand breaks to the genome. In RNA, the literature is incongruent with respect to the importance of these folds, although experimental evidence has built a strong case supporting G4 folding in cells, even though they may not persist long-term. In RNA, G4s can alter mRNA expression, regulate pre-mRNA processing (splicing and polyadenylation), and function in microRNAs. Thus, G4s in DNA and RNA are critical for regulating the complex human cellular network, and similar functions have been reported in other eukaryotes, plants, prokaryotes, and viruses. Recent developments regarding PQSs and G4s in viruses are described in this Outlook.

Sequences of DNA or RNA with the pattern 5′-G_x,G_y,G_z,G_w-3′, in which four G runs are closely spaced and separated by loops (L), are potential G-quadruplex-forming sequences (PQSs; Figure 1A). Generally, it is found that n ≥ 3 and x = 1−12 and sometimes up to 20. This pattern allows computational inspection of genomes for PQSs as a first step in G4 studies followed by experimental validation of folding for those deemed interesting. Folded G4s are composed of tetrads comprised of one G from each of the four runs embraced in G-G Hoogsteen pairing (Figure 1B). This G-G pairing directs one lone pair of electrons on each G toward the interior channel to coordinate with the major monovalent cation K^+ (∼140 mM in human cells) and stabilize the structure (Figure 1B). Structural analysis has identified a variety of G4 topologies such as parallel-stranded, antiparallel-stranded, or hybrid structures (Figure 1C). These folds differ in the S′ to 3′ orientation of the G runs and the syn or anti conformations of the G nucleotides.

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resulting in different loops and grooves in the folds for protein recognition and targeting with small molecules. In DNA, all of these folds have been observed. In contrast, the 2′-OH on ribose in RNA provides an additional hydrogen bond, alters the hydration state of the structure, and leads to a preference for the anti conformation of G as a consequence of the C3′-endo sugar pucker, leading to RNA G4s strongly favoring parallel-stranded folds. Folded G4s in RNA are typically more stable than their DNA counterparts. Stable G4s in viral DNA and RNA with two tetrads \((n = 2\); Figure 1A) are reported. Lastly, folded G4s with bulges, hairpins in the loops, or between two strands are reported, while identification of these sequence types is challenging to predict.

In human viruses PQSs can exist in DNA, RNA, or both polymers depending on the viral replication cycle. Excellent reviews on viral G4s exist that highlight their physiological importance and focus on targeting G4s as a therapeutic approach to fight viral infections; herein, examples of conserved PQSs and folded G4s impacting viral replication are described.

The flaviviruses include dengue, hepatitis C, West Nile, yellow fever, and Zika viruses that have significantly impacted human health. The flaviviruses have positive-sense (+), single-stranded RNA (ssRNA) genomes devoid of a 3′-poly-A tail (Figure 2A). Genome replication occurs in the cytosol through a double-stranded RNA (dsRNA) intermediate via a specialized RNA-dependent RNA polymerase (RdRp) encoded by the virus (Figure 2). The complementary strand to a PQS can adopt an i-motif fold that is a tetraplex structure found in C-rich strands complementary to G4s comprised of (C:C)+ hemiprotonated base pairs that fold under acidic conditions; however, these folds are unstable in RNA and likely are not found in flaviviruses. Potential G-quadruplex-forming sequences can occur in either the positive- or negative-sense strand and impact viral processes when folded. Inspection for PQSs in flavivirus genomes identified seven conserved sequences on the positive strand, while no conserved PQSs were found on the negative strand throughout the genus. This observation was quite surprising because of the high degree of sequence variability in the viral cohort.

As examples, the Zika genome has ~70 PQSs on the positive strand and is devoid of PQSs on the negative strand; in addition, beyond the conserved positive-sense strand PQSs, the hepatitis C viral (HCV) genome also has a PQS on the 3′ end of the negative-sense strand. In vitro analysis demonstrated that a subset of the Zika and HCV PQSs could fold to stable G4s, and addition of a G4-specific ligand stalled polymerase bypass of template G4s from both viruses. Cellular studies identified that HCV replication is attenuated by various G4-specific ligands. These findings support G4 folding in flaviviral genomes, even if ligand induced, and folding impedes replication.

**Why do PQSs persist throughout the flaviviruses, and is there an evolutionary advantage to maintaining these sequences?**
Why do PQSs persist throughout the flaviviruses, and is there an evolutionary advantage to maintaining these sequences? Flaviviruses replicate their RNA genomes in the cytosol and must avoid detection by the RNA decay pathway comprised of the 5′,3′-endonuclease XRN1 that digests foreign RNAs. Foreign RNAs are identified by nonstandard 5′ modifications, absence of appropriate ribonucleoprotein signatures, dsRNAs, or lack of a 3′-poly-A tail, in which the latter three signatures are common to flaviviruses. One approach that flaviviruses utilize to avoid complete nuclease digestion of their genomes is through XRN1-resistant secondary structures, of which G4s represent one such fold. Subgenomic flaviviral genomes are hypothesized to be sacrificial providing an opportunity for replication and packaging of full-length genomes to occur. Recently, a PQS in the RNA genome of the Rift Valley fever virus was shown to block XRN1 degradation. The flaviviral genomes all possess conserved PQSs that can adopt G4s to block XRN1. Thus, we hypothesize that flaviviruses retain PQSs in order to adopt G4s to counteract host-derived surveillance nucleases that combat viral infections. Additional examples of RNA viruses that replicate solely through RNA intermediates and harbor PQSs, some of which can adopt G4s, include the Ebola virus and arenaviruses (e.g., Lassa virus). Further studies regarding G4s as nuclease-resistant structures as a viral countermeasure to host defenses are needed.

The human immunodeficiency virus (HIV) is a retrovirus responsible for causing acquired immune deficiency syndrome (AIDS). The HIV capsid has two copies of the sense-strand ssRNA genome essential for reverse transcription (RT) to yield a proviral dsDNA (Figure 2). The proviral dsDNA upon integration in the host genome is the template for mRNA synthesis to produce viral proteins and more viral RNA to incorporate into newly formed viral capsids. The HIV genome harbors PQSs that can fold in the DNA and RNA throughout the sequences; i-motif folds in the proviral dsDNA have not been reported. Key PQSs in HIV are the U3 region in the 3′-UTR of the viral RNA that codes for part of the 5′ and 3′ long-terminal repeat (LTR) of the proviral DNA. In the proviral DNA, the PQSs in the U3 region in the 5′-LTR are part of the promoter for regulation of RNA synthesis in the nucleus. The conservation of these PQSs has been extensively documented. Currently, three NMR-based structures have been reported for two different HIV G4s found in the U3 region of the 5′-LTR of the proviral DNA genome. The RNA counterparts in the U3 region were shown to fold on the basis of circular dichroism (CD) analysis and a reverse transcriptase stop assay. In the cellular context demonstration of G4 folding in DNA and RNA was achieved by addition of various G4-specific ligands that slowed viral replication, and mutational studies that demonstrated sequences incapable of G4 formation impacted viral fitness, i.e., the ability of the virus to thrive and replicate.

Another PQS site that can fold in HIV is one between two RNA genomes to yield a bimolecular G4 in the central polypurine track (cPPT) near the dimer initiation site and the gag polypolyne coding region. The close association of the two viral genomes is an essential part of the viral replication strategy, and a G4 fold may aid in this process based on the observation that mutation to abolish G4 folding impacts replication. Additionally, folded G4s were identified in the HIV proviral genome in the nef protein coding region. Thus, HIV DNA and RNA provide many examples of G4s with functional roles during replication.

A fascinating example of G4s functioning as a countermeasure to surveillance by the host’s immune system was documented in the Epstein–Barr virus. Infection with this virus is associated with Burkitt’s lymphoma, nasopharyngeal carcinoma, and Hodgkin’s lymphoma. The Epstein–Barr virus has a dsDNA genome with a series of 13 PQSs in the coding region of the Epstein–Barr virus-encoded nuclear antigen 1 (EBNA1) mRNA that function as cis-acting regulatory elements. Verification of two-tetrad G4 folding was shown by CD and NMR spectroscopies. Cellular studies showed that folded G4s in the EBNA1 mRNA downregulated protein expression, which at first glance appears counterproductive; however, the decrease in EBNA1 protein expression allows the virus to evade detection by the host’s immune system. This example illustrates that viruses may have favorably evolved nucleic acid secondary structures, such as G4s, to increase their survival and dissemination in host communities. Similarly, PQSs in other viruses may function in a similar fashion as that demonstrated for the Epstein–Barr virus.

Identification of important PQSs in other human viruses has been noted. The SV40 virus has a closed-circular dsDNA genome with a PQS in the promoter proposed to adopt a G4 and function in early and late replication of the genome. The human papillomaviruses and herpesviruses have dsDNA genomes, in which PQSs were found in key regulatory regions. Further support for SV40 and papillomaviruses containing folded G4s is derived from both genomes coding for a helicase that unwind G4s. These viruses may harness G4 folds to serve a vital function during replication and then ensure they are unwound when not needed. Future studies in this area will provide more answers and may expand the roles of G4s.

The diverse cellular properties for G4s identified in humans and viruses suggest that they likely are involved in other cellular processes.

Folded G4s in viruses are bound by protein “readers.” Nucleolin was demonstrated to bind G4s from HCV and Epstein–Barr viruses, and a key function ascribed to nucleolin is induction of G4 folds. In HIV, nucleolin binding and folding of PQSs were determined to favor the proviral DNA over the viral RNA PQSs. In contrast to favoring G4 folding by nucleolin, the nuclear proteins HNRNPA2 and HNRNPB1 were found to bind HIV proviral DNA and to unwind folded G4s that may function during regulation and timing of HIV replication. Additionally, the HIV-1 nucleocapsid protein can function as a chaperone for G4 folding. These competing activities of proteins on viral G4s paint a complex cellular picture, and future studies will provide more clues that are desperately needed. Another possible role for viral PQSs suggested by a reviewer is that they may function as scaffolds for binding host proteins to label the viral strands as “self” in an attempt to avoid immune surveillance. The diverse cellular properties for G4s identified in humans...
and viruses suggest that they likely are involved in other cellular processes.

**THE VIRAL EPITRANSCRIPTOME INCLUDES N⁶-METHYLADENOSINE**

Chemical modifications to the transcriptome are called the epitranscriptome, and those in mRNA are of keen focus at present. Modifications to mRNA include 5-methylcytosine (5mC), pseudouridine (Ψ), inosine (I), N1-methyladenosine (m1A), N6-methyladenosine (m6A), and N6-2′-O-dimethyladenosine (m6Am). By far the best studied, and likely the most abundant modification in mRNA, is m6A (Figure 3), although effects associated with decoupling m6A from m6Am have presented challenges because of their similar structures. Emerging data have identified that m6A can function in nearly all aspects of mRNA biogenesis including splicing, nuclear export, translation efficiency, and decay of the unwanted strands; these are focal points in recent reviews.

One of the great mysteries regarding m6A is why certain DRACH motifs are methylated while others are not. A prominent example found a hairpin structure in RNA that was selectively methylated demonstrating a structural component to site selection; however, hairpin structures do not explain all m6A sites, and other secondary structures and/or protein factors are likely involved.

In mRNA, methyl groups are "written" on A at the N⁶ position via a large hetero-multimeric methyltransferase complex found in the nucleus. Critical for substrate recognition in the complex is METTL14 (Figure 3), and additional factors found to be important include WTAP, KIAA1429, ZC3H13, and HAKAI (Figure 3). Formation of m6A occurs within the consensus sequence 5′-DRACH (D = A, G, or U; R = A or G; H = A, C, or U; A = m6A), in which GGACH is a favored insertion context. Recent studies have identified that m6Am is written into the 5′ cap of mRNA by an RNA polymerase II-associated methyltransferase. In mammalian genomes, m6A has been noted throughout mRNA from highly regulated genes, resulting in methylation of 0.1% of adenosines with an average of 2–3 m6As per target transcript, and the extent of methylation at a site can reach ~90% in certain cases.

In mRNA, m6A is a dynamic modification because an "eraser" (i.e., demethylase) can revert the sequence back to A. Two prominent m6A demethylases identified are the nonheme Fe(II)- and α-ketoglutarate-dependent dioxygenases ALKBH5 and FTO (Figure 3). These erasers strongly select m6A in single-stranded RNA (ssRNA) over double-stranded RNA (dsRNA). When the activity for ALKBH5 or FTO are
knocked out of cells, an increase in global A methylation is observed supporting their function; however, many unanswered questions remain regarding selection of demethylation sites, cell dependency of the erasers, and the substrate scope for the demethylases as well as whether other erasers exist.

The best characterized “readers” of mA in mRNA include YTHDF1, YTHDF2, and YTHDF3 that reside in the cytoplasm, as well as YTHDC1 found in the nucleus and YTHDC2 that is nucleocytoplasmic (Figure 3). The presence of mA in specific contexts of RNA can unfold secondary structures (i.e., structural switches or cis-regulatory elements) allowing HNRNPC and HNRNP G to bind, designating these proteins as mA readers. The list of mA readers in mRNA is continually growing. The three cytoplasmic YTH factors have similar binding constants for mA, and therefore, binding is determined by other factors such as proteins and RNA structure. Specifically, YTHDF1 binds mA and recruits translation factors to increase expression; YTHDF2 binding promotes 3’-poly-A tail deadenylation resulting in mRNA degradation; and YTHDF3 can promote translation or degradation by interacting with YTHDF1 or YTHDF2, although, this is not well understood. Lastly, YTHDC1 facilitates mRNA biogenesis and export from the nucleus, and YTHDC2 has two conflicting roles by promoting translation or degradation. Degradation observed by YTHDC2 is through interaction with XRN1, the activity of this protein can be blocked by G4s. Because of the considerable interest in this field, additional readers and their impact on mRNA biology will likely soon be found. During a viral infection, mA-associated proteins are found in both the cytoplasm and nucleus allowing viral RNA methylation to occur (Figure 3).

Enabling next-generation sequencing has allowed advancements in understanding where and how mA functions in mRNA. The challenges in locating mA in a sequence result from this heterocycle coding like an A during PCR workup for sequencing. mA sites when adducted to the protein results in a functional mutation signature (C → T transition) to identify the location of mA (mA-CLIP). Another version of the cross-linking approach feeds cells with 4-thiouridine (4SU) to be metabolically incorporated into the RNA, and during immunoprecipitation photo-cross-linking occurs between the antibody and 4SU yielding a covalent bond (PAR-CLIP). The 4SU site when added to the protein results in a mutation signature (U → C) during sequencing near the mA for identification. Advancements to the photo-cross-linking approach have been reported (i.e., mA-LAIC-Seq). Drawbacks to antibody-based methods include biases to the data, missing clustered modifications, and the generation of data that are not easily quantifiable. Even though new methods and technologies are sorely needed to better understand mA, significant knowledge has been gained with existing techniques.

The presence of mA in viral RNA was first noted in the 1970s in the human viruses SV40, influenza A, and human adenoviruses. Recently, new sequencing approaches allowed three independent reports of multiple mA sites in the HIV-1 RNA genome, additionally, each study conducted a series of knockdown experiments to determine the impact of mA readers, writers, and erasers on viral fitness. Among the data differing observations and interpretations exist; these have been recently reviewed. For the present discussion, key points include overlap of mA sites in the 3′-UTR of the HIV-1 RNA genome in which there are conserved PQSs shown to adopt G4 folds. The sequencing approaches applied to identify mA were low- and high-resolution mA sequencing. Also noted was the binding of mA readers (i.e., YTHDF1-3) to the 3′-UTR region that is critical for the impact of mA on viral fitness; however, whether binding is favorable or detrimental for HIV-1 replication is a core difference in the studies.

Regions of mA in flaviviral genomes were sequenced by two independent laboratories that found similar sites and functions for mA in the viral RNA. Sequencing for mA by the lower resolution methods in the dengue, HCV, West Nile, yellow fever, and Zika viruses identified conserved regions of mA installation across the flaviviruses studied. In both studies, knockdown of established mA-interacting proteins found that when mA installation was suppressed, viral fitness increased, and when mA removal was suppressed, viral fitness decreased. These observations suggest that the host introduces mA in specific regions of flaviviral RNA as a mechanism for combating the infection; however, why these regions are selected out of the many possible DRACH motifs is not known.

A defining role for mA in viral RNA from other human viruses was reported, a great example of which was documented in the hepatitis B virus (HBV) that has a DNA genome and replicates through an RNA intermediate. Installation of mA in RNA from the HBV increased translation but decreased reverse transcription of the RNA to DNA providing a dual role for mA on replication. A key location for mA installation in the HBV within the epsilon loop on the 3′-end of the RNA was identified. The Kaposi’s sarcoma-associated herpesvirus has mA introduced by host writers that aids in splicing of a pre-mRNA for an essential protein; additionally, mA insertion was favored in S′-GGAC sequence contexts. For readers interested in viral epitranscriptomics, excellent reviews on this topic exist.

**IS THERE SYNERGY BETWEEN PQSs AND mA?**

Upon inspection of the viral PQS data and mA epitranscriptomic sequencing data, we observed instances in which sites of mA occurred within the loops of PQSs. This observation led us to ask the question whether a synergy exists between some sites selected for mA insertion and folded G4s. In the first example, sequencing for mA in flaviviral genomes was conducted by low-resolution methods. The discussion here will focus on the Zika genome, for which a map...
of the PQSs exists from our prior work (Figure 4A). There are 12 regions within the Zika genome that showed enrichment of m6A above the background input control; these are illustrated with the blue bars below the map in Figure 4A. When we overlaid the PQS and m6A enrichment maps, 8 of the 12 enriched peaks had PQSs with the DRACH motif in a loop region (Figure 4A). In five of the eight overlapping peaks there was no ambiguity, all possible DRACH motifs were in PQS contexts. In the remaining three overlap peaks, DRACH motifs existed either in PQSs or adjacent to PQSs. Inspection of the Zika genome found >300 possible m6A sites according to sequence, while only 12 of those potential sites were methylated. Interestingly, among the ~70 PQSs in the Zika genome, 8 out of the 12 m6A sites were associated with PQSs. This argument does not constitute a strong statistical proof of our observation; nonetheless, it is fascinating to find many examples of PQSs in Zika that have a strong potential to be methylated in predicted loops.

In the second example, sequencing m6A in the HIV-1 RNA genome from two laboratories found modification in the 3′-UTR. In one of the studies, PAR-CLIP was applied to sequence m6A to pinpoint three m6A sites adjacent to G runs that define conserved PQSs in the U3 region of the HIV RNA genome (Figure 4B). Previous studies determined that the region in which m6A was installed has the potential to adopt G4 folds (U3-G4 II, U3-G4 III, and U3-G4 IV; Figure 4B) that impact viral replication. Inspection of the sequence LOGO generated from alignment of 1527 HIV-1 3′-UTR sequences demonstrated strong conservation of the PQSs along with two of the three A nucleotides that are methylated, and the third A is the dominate nucleotide found at this position (Figure 4B). To reiterate, the nucleotides showing strong conservation in HIV-1 strains, as well as HIV-2 and SIV in the U3 region of the 3′-UTR, are the Gs needed for G4 formation as well as the A nucleotides that are methylated.

Additional examples illustrating m6A and PQS overlap were found in the RNA from HBV and SV40. At position 1907 in the HBV pregenomic RNA, an A is methylated adjacent to a two-tetrad PQS (5′-UU GGG U GG CUUU GGGG CAU GG A C-3′ underline = Gs in PQS). Changes to nucleotides adjacent to G4 folds can significantly impact structure. In the HBV case, the m6A site was confirmed by mutational analysis to be critical for viral replication. Other m6A sites were not further interrogated and prevent our PQS inquiry into their data. In the SV40 mRNA, PAR-CLIP sequencing found many examples of m6A in PQSs, and one example is an A at position 2444 (5′- CA GG A GG ACACAGA GGG U GG AU-3′). In SV40, m6A functions to enhance viral replication and gene expression.

Figure 4. Locations for the overlap of PQSs and sites of m6A in the Zika and HIV viral RNAs. (A) OVERLAP of an m6A enrichment map (red line) compared to the input control (gray line) reported for the Zika viral genome and the PQS map for the same genome to illustrate m6A sites with DRACH motifs in PQS loops. The 12 sites that are enriched above background are shown with the blue bars in comparison to the Zika genome diagram at the bottom. (B) Diagram for the LTR and UTR regions of the HIV DNA and RNA genomes, respectively, to identify the U3 region of the 3′-UTR in the viral RNA, in which PQSs that adopt G4 folds (U3-II, U3-III, and U3-IV G4s) have m6A sites in loops. Sequence conservation in this region is shown by the LOGO obtained from 1527 HIV-1 genomes downloaded from www.hiv.lanl.gov.
expression. These observations provide additional examples of established m6A sites in PQS contexts.

As a consequence of the only recent emergence of sequencing m6A in viruses, more data are not yet available to provide stronger generalizations. With this limitation in mind, examples exist from viruses of critical m6A sites existing in PQS contexts that appear to impact these human viruses significantly. In this Outlook, we bring attention to the possible synergy between these two disparate fields of study and hope that researchers will add data to support or refute the G4-m6A correlation in the future. As more m6A sequencing data are collected on viral RNAs, inspection for m6A within PQSs and how these G-rich sequences can impact local secondary structure may provide missing clues as to why these regions are selected by proteins for methylation while others are not. Moreover, how m6A impacts G4 folds can aid in drawing conclusions regarding the impact of methylation on viral fitness. Currently, whether the PQS context favors methylation (writing m6A) or disfavors demethylation (erasing m6A) is not known.

What possible biological outcomes could arise from m6A installation in PQS contexts? Each sequence context is unique and would need to be studied on a case-by-case basis, although some general speculations can be made; one possibility is that N6 methylation of A could drive the formation of G4 folds at the expense of other secondary structures destabilized by m6A (Figure 5A). A structure switching role for m6A in the context of an RNA hairpin has been demonstrated to modulate protein factor binding.87,120 The impact on secondary structure by m6A can occur via destabilization of base pairing to U and/or

Figure 5. Alternative pathways for installation of m6A in the PQS context can create a structural switch in viral genomes. (A) An unfolded PQS can be methylated to facilitate G4 folding. (B) A folded G4 can be methylated at an A nucleotide in the loop, resulting in loss of the G4 structure or retention/alteration of the fold. To maintain brevity, only the writing of m6A in the PQS context is illustrated; however, erasing m6A may also be impacted by secondary structure.

Figure 6. Structures for chemically modified structures in mRNA.
the hydrophobicity of the new methyl group may alter RNA hydration\cite{10} to favor parallel-stranded RNA G4 folding in these sequences. This G4 switch would display sequences for protein recognition differently, resulting in a downstream phenotypic change. Stabilization of G4 folds would also result in stronger blocks to polymerases during replication of the viral genome. In this scenario, m\textsuperscript{6}A in a G4 would slow viral replication, especially if this increases binding of nucleolin,\cite{72} and this proposal is consistent with the decrease in replication shown for adenosine methylation in flavivirus.\cite{115,114}

On the other hand, m\textsuperscript{6}A could destabilize G4 folds and drive the RNA to another structure (Figure 5B). This is likely because G4s can be sensitive to changes in hydrogen bonding between loop nucleotides.\cite{31} Consequently, methylation of A would disfavor G4 folds and diminish the challenges these structures can pose to replication and protein recruitment. Disrupting the G4 could facilitate polymerase bypass of these sites and favor increased replication. This situation would be consistent with two of the studies regarding m\textsuperscript{6}A impact on HIV-1 in which methylation favored viral replication.\cite{104,105} Further, m\textsuperscript{6}A is readily bypassed by polymerases and would not pose an impasse to replication.\cite{122} These potential pathways in which synergy exists between m\textsuperscript{6}A and G4 folds may provide missing links to understanding the viral epitranscriptomic impacts observed.

Lastly, epitranscriptomic modification of viral genomes in PQS contexts may represent a small window to a larger phenomenon. In the human transcriptome, we note an example of a primary miRNA with m\textsuperscript{6}A in a PQS context that could be a structural switch,\cite{123} and recent nanopore sequencing data of the human transcriptome identified methylguanosines (m\textsuperscript{7}G) in human RNA.\cite{125,126} Other epitranscriptomic modifications that exist (e.g., m\textsuperscript{6}Am, m\textsuperscript{1}A, I, Ψ, and Smc; Figure 6),\cite{77,79} and this list may now include internal N7-methylguanosines (m\textsuperscript{3}G) in human RNA.\cite{124,125} Further, m\textsuperscript{6}A could destabilize G4 folds and drive the RNA to another structure (Figure 5B). This is likely because G4s can be sensitive to changes in hydrogen bonding between loop nucleotides.\cite{31} Consequently, methylation of A would disfavor G4 folds and diminish the challenges these structures can pose to replication and protein recruitment. Disrupting the G4 could facilitate polymerase bypass of these sites and favor increased replication. This situation would be consistent with two of the studies regarding m\textsuperscript{6}A impact on HIV-1 in which methylation favored viral replication.\cite{104,105} Further, m\textsuperscript{6}A is readily bypassed by polymerases and would not pose an impasse to replication.\cite{122} These potential pathways in which synergy exists between m\textsuperscript{6}A and G4 folds may provide missing links to understanding the viral epitranscriptomic impacts observed.

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**Notes**

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