Epigenetic and epitranscriptomic regulation of viral replication

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Abstract | Eukaryotic gene expression is regulated not only by genomic enhancers and promoters, but also by covalent modifications added to both chromatin and RNAs. Whereas cellular gene expression may be either enhanced or inhibited by specific epigenetic modifications deposited on histones (in particular, histone H3), these epigenetic modifications can also repress viral gene expression, potentially functioning as a potent antiviral innate immune response in DNA virus-infected cells. However, viruses have evolved countermeasures that prevent the epigenetic silencing of their genes during lytic replication, and they can also take advantage of epigenetic silencing to establish latent infections. By contrast, the various covalent modifications added to RNAs, termed epitranscriptomic modifications, can positively regulate mRNA translation and/or stability, and both DNA and RNA viruses have evolved to utilize epitranscriptomic modifications as a means to maximize viral gene expression. As a consequence, both chromatin and RNA modifications could serve as novel targets for the development of antivirals. In this Review, we discuss how host epigenetic and epitranscriptomic processes regulate viral gene expression at the levels of chromatin and RNA function, respectively, and explore how viruses modify, avoid or utilize these processes in order to regulate viral gene expression.

Although all human cells in a given individual contain the same genome, they differ widely in the genes they express and, hence, in their biological properties. It is therefore well established that gene expression is not determined simply by the sequence information that is encoded into genomic DNA, but rather is subject to multiple levels of control. This Review discusses two complementary regulatory mechanisms that act, respectively, at the DNA and RNA levels. In the case of the host-cell genome, the level of expression of specific genes is regulated not only by flanking promoters and enhancers but also by how accessible the genes embedded within cellular chromatin are to transcription factors. This accessibility in turn is regulated by processes that include DNA methylation, histone remodelling, alternative histone variant usage and the deposition of modifications on histone tails, collectively referred to as epigenetic gene regulation1,2 (Box 1). Similarly, the function of the mRNAs transcribed from a given gene is regulated not only by their nucleotide sequence but also by a range of covalent modifications that are added to individual nucleotides, the most prevalent of which is methylation of the N6 position of adenosine (m6A) (REFS 3,4). These RNA modifications — which have been reported to regulate the stability, the translation and even the immunogenicity of RNA molecules — are referred to as epitranscriptomic modifications.

Epigenetic and epitranscriptomic gene regulation likely initially evolved as means of regulating cell growth and differentiation. However, they are also relevant to several important diseases, including cancer, in which both processes have been reported to be dysregulated5–7. Their importance for the regulation of viral gene expression has only recently begun to emerge. As obligate intracellular parasites, viruses misappropriate parts of the host-cell machinery in order to allow the expression of viral genes. This dependence on cellular gene regulation pathways can, however, also lead to viral gene expression being subject to host repression. Indeed, epigenetic repression has been proposed to function as a form of antiviral restriction used by host cells as an innate immune defence against DNA viruses, in a tug of war that is only revealed when viral countermeasures are experimentally removed8. Alternatively, viruses may repurpose epigenetic repression in order to suppress their own gene expression, as a way to establish latent infections and prevent the production of immunogenic viral proteins9. By contrast, epitranscriptomic modifications generally enhance the function of viral mRNAs, and both DNA and RNA viruses have therefore evolved to maximize the level of these modifications on their transcripts.

In this Review, we discuss epigenetic repression mechanisms, including histone tail modifications and
Histones and proteins including fluorescent dyes can be used to visualize the binding of antibodies labelled with specific markers in microscopy assays. This accessibility is a function of the degree of compaction of the histones bound to that genomic DNA, with open regions being referred to as euchromatin, whereas tightly compacted regions of DNA are referred to as heterochromatin. Chromatin compaction constitutes a form of gene regulation not directly encoded in the DNA nucleotide sequence itself; this is termed epigenetic gene regulation, as it is the molecular mechanism underlying epigenetic inheritance. Epigenetic gene regulation can take the form of DNA modifications, histone tail modifications, chromatin remodelling and alternative histone subunits (see the figure). DNA modifications mainly involve CpG methylation, in which a methyl group is added to deoxyadenosine residues located in CpG-rich genomic regions. CpG methylation is primarily associated with repressive heterochromatin. Histone tail modifications — including methylation, acetylation, phosphorylation and ubiquitylation — are the best understood form of epigenetic regulation. Examples have been found on all four canonical histones (H2A, H2B, H3 and H4). H3 and H4 acetyltransferases are associated with active euchromatin, whereas methylation are more diverse in function. For example, H3 lysine 9 and lysine 27 tri-methylation (H3K9me3 and H3K27me3, respectively) mark heterochromatin, whereas H3K4me3 marks are found on actively transcribed euchromatin. Chromatin remodelling involves ATP-dependent histone chaperones that may slide, evict or load histones, modulating the local density of histones on the chromatin. Finally, chromatin accessibility can be modulated through the use of alternative histones, such as the H2A histone variant in place of H2, or the H3.3 histone variant as a replacement for H3.1. Canonical H3 histone variants H3.1 and H3.2 are deposited by the histone chaperone CAF-1 during DNA replication. However, histones can be displaced by transcription, and they need to be replaced by the histone chaperone complex HIRA, which deposits the replacement histone H3 variant H3.3. Thus, H3.3 previously was thought to mark transcriptionally active chromatin. However, H3.3 later was found also to be loaded by an alternative histone chaperone complex consisting of a heterodimer of Daxx and ATRX, which loads H3.3 onto repressed heterochromatin regions such as pericentromeric regions and telomeres, suggesting that different chaperones may load histone H3.3 with alternative histone tail marks on either actively transcribed or repressed chromatin.

**Acetylation**

The addition of a small chemical group called an acetyl group (CH3CO) found on RNA and proteins including histones.

**Histones**

A family of highly basic proteins used to organize genomic DNA (see Box 1).

**Heterochromatic marks**

Epigenetic markers that mark a segment of chromatin for transcriptional repression, which usually coincide with ‘closed chromatin’ that is less accessible to transcription factors.

**Immunofluorescence microscopy**

Microscopy assays in which subjects of interest are visualized through specific binding of antibodies labelled with fluorescent dyes.

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**Box 1 | Relationship of chromatin architecture and histone modifications to gene expression**

The genomic DNA of cells is bound by basic proteins called histones, with every ~147 bp of DNA wrapped around a core octamer containing two molecules apiece of the histones H2A, H2B, H3 and H4, to form chromatin (see the figure). Although chromatin can protect genome integrity, histones can also hinder the accessibility of genomic DNA for transcription. This accessibility is a function of the degree of compaction of the histones bound to that genomic DNA, with open regions being referred to as euchromatin, whereas tightly compacted regions of DNA are referred to as heterochromatin. Chromatin compaction constitutes a form of gene regulation not directly encoded in the DNA nucleotide sequence itself; this is termed epigenetic gene regulation, as it is the molecular mechanism underlying epigenetic inheritance. Epigenetic gene regulation can take the form of DNA modifications, histone tail modifications, chromatin remodelling and alternative histone subunits (see the figure). DNA modifications mainly involve CpG methylation, in which a methyl group is added to deoxyadenosine residues located in CpG-rich genomic regions. CpG methylation is primarily associated with repressive heterochromatin. Histone tail modifications — including methylation, acetylation, phosphorylation and ubiquitylation — are the best understood form of epigenetic regulation. Examples have been found on all four canonical histones (H2A, H2B, H3 and H4). H3 and H4 acetyltransferases are associated with active euchromatin, whereas methylation are more diverse in function. For example, H3 lysine 9 and lysine 27 tri-methylation (H3K9me3 and H3K27me3, respectively) mark heterochromatin, whereas H3K4me3 marks are found on actively transcribed euchromatin. Chromatin remodelling involves ATP-dependent histone chaperones that may slide, evict or load histones, modulating the local density of histones on the chromatin. Finally, chromatin accessibility can be modulated through the use of alternative histones, such as the H2A histone variant in place of H2, or the H3.3 histone variant as a replacement for H3.1. Canonical H3 histone variants H3.1 and H3.2 are deposited by the histone chaperone CAF-1 during DNA replication. However, histones can be displaced by transcription, and they need to be replaced by the histone chaperone complex HIRA, which deposits the replacement histone H3 variant H3.3. Thus, H3.3 previously was thought to mark transcriptionally active chromatin. However, H3.3 later was found also to be loaded by an alternative histone chaperone complex consisting of a heterodimer of Daxx and ATRX, which loads H3.3 onto repressed heterochromatin regions such as pericentromeric regions and telomeres, suggesting that different chaperones may load histone H3.3 with alternative histone tail marks on either actively transcribed or repressed chromatin.

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**Alternative histone variants**

Alternative histone variants that are loaded onto viral DNA upon entry into host-cell nuclei and how viruses avoid or even utilize this repression to enhance viral replication or persistence. We then explore the epitranscriptomic RNA modifications, including methylation and acetylation, found on viral transcripts, and how they enhance viral gene expression or help evade innate immune detection. We highlight the importance of non-encoded regulatory information found on viral chromatin and RNA and discuss how the regulatory pathways that are influenced by this information could serve as novel targets for antiviral therapies.

**Epigenetic repression and its avoidance**

Incoming viral DNA molecules are sensed by target cells and are rapidly loaded with histones bearing heterochromatic marks, thus inhibiting viral gene expression. How cells distinguish viral DNA from their own remains undefined, though epigenetic repression of foreign DNA seems mainly to be orchestrated by proteins associated with the pro-myelocytic leukaemia nuclear bodies (PML-NBs) and by the innate immune DNA sensor interferon-inducible protein 16 (IFI16). Here we discuss how herpesviruses, hepatitis B virus (HBV) and retroviruses can be epigenetically repressed and how these viruses can disrupt or avoid repression in order to initiate productive infections. We also discuss how viral latency can be achieved through the establishment of a dormant viral state through mechanisms involving the establishment of a latent viral infection.

**PML-NB-mediated epigenetic repression.** Epigenetic suppression of viral DNA is frequently associated with nuclear protein aggregates called PML-NBs or nuclear domain 10 (ND10) (Box 1). PML-NBs consist of cage-like structures constructed of PML proteins, which contain other effector proteins that together appear as punctate foci when visualized by immunofluorescence microscopy. Several DNA viruses, including Simian virus 40, adenovirus type 5, herpes simplex virus 1 (HSV-1) and human cytomegalovirus (HCMV), localize to and establish viral replication sites adjacent to PML-NBs, suggesting that this structure is at least spatially, if not functionally, related to viral replication. Through the recruitment of cofactors, PML-NBs can be associated with many cellular functions, including antiviral defence and transcriptional repression. The expression of the PML-NB components Sp100 and PML increases in response to interferon signalling, and PML-NB components including PML, Sp100, Daxx and ATRX suppress viral replication. Daxx and Sp100 also have transcriptional repressive functions, likely through recruiting co-repressors such as HDACs. Moreover, Daxx and ATRX can load the histone variant H3.3 onto heterochromatic regions. All of the functions above implicate
**Interferon**

A family of proteins that are expressed by eukaryotic cells upon invasion by viruses, used to signal neighbouring cells to mount an antiviral response through the expression of a variety of interferon-stimulated genes.

**Tegument**

The space in viral particles between the outer membrane and the inner protein capsid shell; the term is most commonly used in the herpesvirus family, where proteins packaged in this space are termed tegument proteins.

**Ubiquitin**

A small peptide that can be ligated onto other proteins, best known to mark proteins for degradation through the proteasome.

**Proteasome**

A large complex of proteins involved in the breakdown of peptides. Known as the 26S proteasome, it is located in the cytoplasm and the cell nucleus.

**Herpesviruses**

A large family of viruses that include herpes simplex virus (HSV), varicella-zoster virus (VZV), cytomegalovirus (CMV), and Epstein-Barr virus (EBV). They are known for their ability to establish latency and reactivate under stress.

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PML-NBs as subnuclear hubs of the epigenetic repression of viral DNA.

**IFI16-mediated epigenetic repression.** Invading viral DNA can also be detected by the innate immune DNA sensor IFI16 (fig. 1b). IFI16 contains an oligomerizing pyrin domain (PYD) and two double-stranded DNA (dsDNA)-binding HIN200 domains, and it oligomerizes on histone-free segments of dsDNA longer than 70bp. IFI16 was initially characterized as a DNA sensor that induces interferon-β through the signalling factors STING, TBK1 and IRF3 (REF.29), and it was shown to repress gene expression from transfected plasmids as well as from viruses including herpesviruses, papillomaviruses and HBV. IFI16 is a key player in the epigenetic repression of viral DNA, it activates interferon-independent manner, with IFI16 directly recruiting H3K9 methyltransferases to HSV DNA. Interestingly, IFI16 suppression of HSV-1 and Kaposi sarcoma-associated herpesvirus (KSHV) transcription involves the deposition of suppressive H3K9me3 marks in an interferon-independent manner, with IFI16 directly recruiting H3K9 methyltransferases to KSHV DNA. Although IFI16 has a key role in the epigenetic repression of viral DNA, it should be noted that IFI16 function is likely closely connected to the inhibition by PML-NBs, as IFI16 is known to interact with PML-NB components.14

**Herpesviruses.** Epigenetic repression of viruses is best understood for herpesviruses (fig. 1). Herpesvirus DNA is packaged into virions ‘naked’ (that is, free of histones)36–37. Upon release into the nucleus, viral DNA is detected and then rapidly loaded with histones harbouring suppressive marks, as a form of innate immune response. Upon infection of fibroblasts by the alphaherpes virus HSV-1, within 2h viral DNA is loaded with histones, mainly consisting of Daxx–ATRX and HIRA-loaded H3.3 with repressive H3K9me3 marks39–41. HSV-1 disarms this repression in two stages. First, the viral protein VP16, which is packaged into the tegument layer of incoming virions, recruits host proteins, including host-cell factor 1 (HCF-1) and octamer-binding factor (Oct-1), in order to form a complex that recruits the histone demethylases lysine-specific demethylase 1 (LSD1) and Jumonji domain 2 (JMJD2) family members as a means to remove repressive H3K9 marks from viral immediate early promoters42,43 (fig. 2a). This VP16 complex also recruits the methyltransferases Set1 and MLL1, which deposit H3K4me3 activating marks on the histone H3 bound to viral DNA44. This allows expression of the immediate early viral protein ICP0, which orchestrates the second stage of de-repression. ICP0, an E3 ubiquitin ligase, induces the ubiquitylation and degradation of the PML-NB components PML and Sp100 (fig. 2b). Once these structural components of PML-NBs are degraded, Daxx and ATRX are dispersed away from viral replication sites, de-repressing regions of the viral genome not already activated by VP16. Indeed, ICP0 promotes the gradual removal of repressive histone marks H3K9me3 and...
H3K27me3 from the entire viral DNA genome, ensuring a productive infection. In parallel, ICP0 has also been implicated in the degradation of IFI16 (Fig. 2c). IFI16 suppresses gene expression from ICP0-deficient mutants of HSV-1, with IFI16 directly binding viral DNA, promoting the deposition of repressive H3K9me3 marks and the removal of active H3K4me3 marks. However, the role of ICP0 in counteracting IFI16 is somewhat controversial, with one study reporting that inhibition of HSV-1 gene expression by IFI16 occurs only in the absence of ICP0, whereas another study reported that IFI16 is also active against wild-type HSV-1 (refs 11,29). This discrepancy may be partially explained by the finding that ICP0-mediated degradation of IFI16 is efficient in primary cells yet less so in transformed cell lines such as HeLa. Regardless, IFI16 clearly represents a key component of the host-cell viral-DNA recognition machinery.

The betaherpesvirus HCMV expresses a different tegument protein, pp71, that degrades Daxx and mislocalizes ATRX, ensuring the presence of open chromatin on viral DNA bearing H3Ac and minimal H3K9me3 marks (Fig. 2b). Similarly, the gammaherpesvirus Epstein–Barr virus (EBV) packages the tegument protein BNRF1, which localizes to PML-NBs and disassembles the Daxx–ATRX histone chaperone complex, whereas HCMV pp71 degrades Daxx. Hepatitis B virus (HBV) protein HBx induces the degradation of Smc5 or Smc6. HCMV IE1 can also induce the degradation of IFI16, an alternative antiviral epigenetic repression factor, with ICP0 implicated as being involved in this degradation. Retroviruses avoid epigenetic repression by integrating their DNA into host euchromatin, where it can no longer be identified as foreign DNA. HBV, murine leukaemia virus.

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Hepatitis B virus. Epigenetic repression by host factors also affects other nuclear DNA viruses. HBV, a hepatavirus, causes chronic liver infections that can result in cirrhosis or hepatocellular carcinoma. The transcriptionally active form of viral DNA is covalently closed circular DNA (cccDNA). Epigenetic repression of cccDNA becomes apparent when the HBV X protein (HBx) is mutationally inactivated. In this context, the structural maintenance of chromosome 5 and 6 (Smc5/6) proteins, which colocalize with PML-NBs, act as restriction factors for HBV transcription. In a striking parallel to herpesviruses, the HBx protein recruits the cellular E3 ubiquitin ligase DDB1 in order to induce the ubiquitylation and degradation of Smc5/6 (refs 58–60) (Fig. 2b). Importantly, artificial depletion of the PML-NB components PML and Sp100 using RNA interference not only results in the nuclear redistribution of Smc6 but also allows the transcription of HBV cccDNA in the absence of HBx. Thus, HBV, like herpesviruses, disrupts PML-NBs as a means to circumvent the epigenetic silencing of viral DNA.

Retroviruses. As we argued above, host cells can recognize incoming viral DNA as targets for epigenetic silencing. Whereas herpesviruses and HBV use viral proteins to disperse or degrade PML-NB components and so prevent silencing, retroviruses appear to avoid proviral DNA silencing through the alternative strategy of chromosomal integration (Fig. 2d). In the absence of integrase function, the potency of epigenetic repression becomes apparent, as unintegrated retroviral DNA is poorly transcribed1–2. Retroviral particles contain RNA genomes, which are reverse transcribed in the cytoplasm. Like herpesviral DNA, retroviral DNA enters the nucleus ‘naked’ and is rapidly loaded with histones3,4. Specifically, unintegrated HIV-1 DNA is loaded with the histone variant H3.3 bearing the repressive H3K9me3 mark and only low levels of the active mark H3Ac. In the case of murine leukaemia virus (MLV), epigenetic repression requires the DNA-binding protein NP220, which recruits epigenetic repressors including the H3K9me3 methyltransferase SETDB1, along with the human silencing hub (HUSH) complex5, which has previously been identified as a repressor of endogenous retrotransposons and has been proposed to have a role in the epigenetic silencing of latent HIV-1 proviruses6 (Fig. 1c). However, the previous study also demonstrated that the HUSH complex has no role in silencing unintegrated HIV-1 proviruses; these are targeted for epigenetic repression remains to be determined. It is also unclear how the repressive marks deposited on unintegrated proviral DNA are removed after integration; however, HIV-1 proviruses are known to preferentially integrate into actively transcribed chromatin, which may allow active chromatin marks of the surrounding region to spread to the proviral DNA.7 Regardless, the ability of cells to effectively silence unintegrated retroviral DNA means that integrase inhibitors are currently among the most clinically effective antiretroviral drugs.

If unintegrated retroviral DNA is indeed epigenetically silenced, this implies that a factor that induces the transcription of unintegrated retroviral DNA should be able to rescue the replication of integrase-deficient (ΔIN) retroviruses. Indeed, this has recently been demonstrated. Specifically, the Tax protein encoded by human T cell leukaemia virus 1, which is a potent activator of the cellular NF-kB transcription factors RelA and RelB, was shown to rescue the robust replication of ΔIN HIV-1 in T cells8. This rescue correlated with the effective recruitment of RelA and RelB to the two NF-kB binding sites located in the HIV-1 enhancer element as well as with the acquisition of active, and the loss of repressive, epigenetic marks on unintegrated HIV-1 DNA. Thus, in the presence of Tax, ΔIN HIV-1 replicates as transcriptionally active DNA circles that are analogous to HBV cccDNAs.

Latent viral infections. Upon infection of certain cell types, viruses may be epigenetically repressed, resulting in a latent infection. The successful repression of viral DNA, while it prevents the killing of individual cells by lytic viral replication, does not necessarily reflect a clear victory for the host, as the establishment of latency can allow viruses to maintain long-term infections that avoid the host adaptive immune response yet revert to a lytic replication cycle at a later time. This is the case with latent infection of neurons by HSV-1, of B lymphocytes by EBV and even of resting T cells by HIV-1. Each of these examples of latent infection is associated with the addition of repressive epigenetic marks to viral DNA, including H3K9me3 and H3K27me3, and with low levels of active markers9. Interestingly, latent infection by HSV-1 in neuronal cells may be partly explained by VP16 and its host cofactor HCF-1 being sequestered in the cytoplasm and thus unable to de-repress nuclear viral DNA10. The fact that HCF-1 is uniquely localized to the cytosol in neurons but not in other cell types suggests that HSV-1 may have evolved to utilize the cytosolic localization of HCF-1 to identify cell types in which latency is the preferred strategy.

Latency establishment is an intricately regulated process that allows viruses to temporarily adopt an alternative viral state. Taking EBV as an example, upon infection of B lymphocytes, the virus first activates all eight latency genes to aid in the activation of naïve B cells (type III latency), and then certain genes are switched off (type I/II latency), to minimize the number of viral proteins that are detectable by the immune system, with different latency types utilizing distinct viral promoters that are associated with active chromatin marks only when needed10–12. Meanwhile, lytic viral genes remain transcriptionally silent and are associated with heterochromatic markers during latency13,14. Thus, herpesviruses appear to have subverted what may have originally evolved as an innate antiviral defence mechanism (the epigenetic silencing of viral DNA) as a means to maintain viral latency by selectively silencing viral genes. In this way, herpesviruses can establish a long-lived viral reservoir that avoids detection by the host adaptive immune system.

In conclusion, viral episomal DNA can be epigenetically silenced by the host using mechanisms analogous to host heterochromatic gene repression, while DNA viruses including herpesviruses and HBV package and/or encode viral proteins that can overcome
this suppression. Interestingly, the identified targets of viral de-repressor proteins have highlighted PML-NBs as a key structure in the epigenetic suppression of viral DNA. Although it remains enigmatic how PML-NBs differentiate between host and episomal viral DNA, one hypothesis suggests that IFI16 may act as the DNA sensor that recruits PML-NB components to viral DNA, because IFI16 associates with PML-NB components and because both IFI16 and PML-NBs are recruited to incoming HSV-1 DNA. By contrast, some reports suggest that IFI16 and PML-NBs represent distinct mechanisms. Retroviruses appear to avoid epigenetic repression through integration of their DNA into euchromatic regions of the host-cell genome, as the epigenetic repression of unintegrated retroviral DNA closely mirrors that seen with mutant DNA viruses that are unable to mount the necessary countermeasures. Alternatively, when viruses invade host cells that are at least transiently non-permissive, viruses may utilize epigenetic suppression to enter a controlled, dormant state, thereby forming viral reservoirs that have so far proven impossible to eradicate.

**Epitranscriptomic regulation**

RNA transcripts are subject to a range of different covalent modifications at the single-nucleotide level, and over 100 such modifications have been identified, particularly on tRNAs and other non-coding RNAs (ncRNAs); several of these modifications have been shown to regulate ncRNA function. The repertoire of epitranscriptomic modifications (primarily but not exclusively methylations) found on eukaryotic mRNAs is more limited than that found on ncRNAs, and functional data exist for only a fraction of these mRNA modifications. For the purposes of this Review, we only discuss the four epitranscriptomic modifications that so far have been reported to affect viral gene expression — N⁶-methyladenosine (m⁶A), 5-methylcytidine (m⁵C), N⁴-acetylcytidine (ac⁴C) and 2’-O-methylation of the ribose moiety of all four ribonucleosides (collectively Nm) (Fig. 3). Quantification of the levels of several different epitranscriptomic modifications on highly purified samples of the genomic RNA of both HIV-1 and the model animal retrovirus MLV has revealed that these viral RNAs contain far higher levels of m⁶A, m⁵C and Nm residues than do cellular mRNAs expressed in infected cells. Specifically, m⁶C was detected at a level 14–30 times higher on these viral RNAs than on cellular poly(A)-RNA; Nm levels were also 10–20 times higher, and m⁶A modifications were 2–10 times more prevalent. The level of ac⁴C was not examined in these two studies, but it was reported to be at a level comparable to that of m⁶A on HIV-1 transcripts in a third study that, despite having used virion RNA samples of unknown purity, obtained m⁶A and m⁵C levels similar to those in the previous reports. Regardless, the very high prevalence of m⁶A, m⁵C, Nm and likely ac⁴C modifications on these viral RNAs indicates that viruses have evolved to maximize their addition to viral transcripts, which strongly suggests that these epitranscriptomic modifications are facilitating one or more steps in the viral replication cycle (Fig. 3).

**N⁶-methyladenosine.** The most common epitranscriptomic modification found on eukaryotic cellular mRNAs is m⁶A, representing 0.2–0.4% of all adenosine residues. As a result, m⁶A has been a major focus of epitranscriptomic research, resulting in the identification of methyltransferase-like 3 (METTL3) as the enzyme that adds m⁶A to mRNAs (Table 1). METTL3, which is referred to as the m⁶A ‘writer’, functions as part of a complex with several cofactors, including METTL14 and WTAP, and uses S-adenosylmethionine (SAM) as the methyl donor (Fig. 3). This complex is predominantly nuclear, and the addition of m⁶A to mRNAs has been reported to occur co-transcriptionally. Once deposited, m⁶A residues can be detected by five m⁶A ‘readers,’ including the predominantly nuclear protein YTHDC1 and the cytoplasmic factors YTHDC2, YTHDF1, YTHDF2 and YTHDF3. All five of these proteins contain a YTH domain, which directly binds m⁶A residues, and all five m⁶A readers appear to bind to all m⁶A sites equivalently. Two m⁶A demethylases (also known as ‘erasers’), called ALKBH5 and FTO, have also been reported, and it has been proposed that mRNA modification by m⁶A may be dynamic and reversible, though this claim remains controversial.

Although the presence of m⁶A on viral transcripts was reported as long ago as the 1970s, analysis of the effect of m⁶A residues on viral gene expression and replication only recently became technically feasible, with the development of techniques that allow epitranscriptomic modifications, including m⁶A, to be mapped to specific sites on RNAs (Box 2). Initially, we and others reported that m⁶A promotes HIV-1 gene expression and replication; knockdown of either METTL3 or the most highly expressed m⁶A reader, YTHDF2, inhibited HIV-1 gene expression, whereas knockdown of the ALKBH5 eraser or YTHDF2 overexpression promoted HIV-1 gene expression. Subsequent studies from our laboratory and others — using a combination of gene knockdown, knockout and overexpression strategies — demonstrated that m⁶A residues also promote the replication of a range of other viruses, including influenza A virus (IAV), the picornavirus enterovirus 71, respiratory syncytial virus (RSV), human metapneumovirus (HMPV) and the polyomavirus SV40. In the case of IAV, HMPV and RSV, viral variants carrying silent mutations that eliminated a subset

### Table 1 | Epitranscriptomic RNA modifications that regulate virus replication

| RNA modification | Writer | Readers | Reported phenotypic effect |
|------------------|--------|---------|----------------------------|
| N⁶-methyladenosine (m⁶A) | METTL3 (N) | YTHDC1 (N) | Increased viral RNA and protein expression; avoidance of RIG-I activation |
| 5-methylcytidine (m⁵C) | NSUN2 (N) | Increased viral mRNA translation |
| N⁴-acetylcytidine (ac⁴C) | NAT10 (N) | Unknown | Increased viral mRNA stability |
| 2’-O-methylated nucleosides (Nm) | FTSJ3 (No) | Unknown | Avoidance of MDA5 activation |

C, cytoplasmic localization; N, nuclear localization; No, nucleolar localization.
clusters of m^A residues, yet it was also reported that knockdown of the m^A writer METTL3 increased Zika virus replication, while knockdown of the m^A eraser ALKBH5 or FTO exerted an inhibitory effect on viral replication\(^{99,100}\). This result, which is opposite to what was seen with the other RNA viruses discussed above, is difficult to understand, as it is not apparent why m^A sites would be evolutionarily conserved across several different flavivirus RNA genomes if they act in cis to inhibit viral gene expression.

Recently, it was reported that the human m^A reader YTHDF3 can inhibit HIV-1 replication, though the reported effect — a less than twofold inhibition in wild-type A3R5 T cells, when compared to YTHDF3 knockout cells over an ~4-day infection period — was very modest\(^{101}\). YTHDF3 was reported to be packaged into HIV-1 virions and to then reduce reverse transcription by ~30\(^{\%}\). The authors also reported that virion-associated YTHDF3 was efficiently degraded by the HIV-1 protease, which they propose serves as a viral countermeasure. By contrast, we previously reported that YTHDF2 overexpression in T cells increased HIV-1 replication, whereas YTHDF2 knockout reduced HIV-1 replication\(^{95}\). One possibility that was not considered is that YTHDF3 may act by competing with YTHDF2 for binding to m^A sites on HIV-1 RNA, thus reducing the positive effect on viral gene expression exerted by YTHDF2.

**5-methylycistidine.** Another relatively common epitranscriptomic mRNA modification is m^C, which represents ~0.05–0.1% of all cytidine residues found in cellular mRNAs, but up to ~1.4% of the cytidine residues in retroviral transcripts\(^{79-83}\). Although cells encode at least eight cytidine methyltransferases that act on RNA, only one of which belong to the NSUN family of proteins, current data indicate that NSUN2 is the nuclear writer responsible for the majority of m^C residues added to mRNAs, including viral mRNAs\(^{102}\). At present, no m^C readers are known. Analysis of the effect of loss of NSUN2 expression, and hence loss of m^C addition to mRNAs, in HIV-1-infected T cells revealed a specific loss of translation efficiency for HIV-1 and for cellular transcripts that are normally highly m^C-modified\(^{83}\). By contrast, cellular mRNAs that normally lack m^C, including several housekeeping genes, were unaffected by the loss of NSUN2 expression. Therefore, m^C seems to increase viral gene expression and replication by acting predominantly at the level of mRNA translation.

**N^4-acetylcystidine.** Recently, ac^C residues were detected on cellular mRNAs at a level of ~1 ac^C residue per mRNA, and the ac^C writer was identified as nuclear N-acetyltransferase 10 (NAT10)\(^{103,104}\) \(\left(\text{Fig. 3}\right)\). However, no ac^C readers are currently known \(\left(\text{TABLE 1}\right)\). In the case of cellular mRNAs, ac^C was reported to enhance both the stability and the translation of cellular mRNAs, in the latter case by acting in open reading frames to improve ribosomal decoding, especially when ac^C was present in the wobble position of codons\(^{104}\). This may relate to the fact that ac^C can form more stable base pairs with guanosine residues\(^{105}\). Recently,
we reported the mapping of ac4C residues in HIV-1 transcripts (Box 2) and reported that the loss of NAT10 expression results in a decline in HIV-1 gene expression in infected T cells106. However, unlike mC, ac4C was found to increase HIV-1 gene expression by enhancing RNA stability, while viral mRNA translation was unaffected. In confirmation of this result, we also observed that the mutagenesis of ac4C clusters in the env gene of

**Box 2 | Techniques used to map epitranscriptomic modifications**

Although high-performance liquid chromatography linked to tandem mass spectrometry (HPLC–MS/MS) can identify and precisely quantify RNA modifications, these methods do not provide location information. Methods to map the location of modifications usually involve RNA deep sequencing, which can be roughly separated into antibody-dependent methods, modification-interacting protein pulldowns and chemical methods. The figure depicts the core mechanisms of modification identification used in various mapping techniques, with immunoprecipitation-based techniques on the left and chemical methods on the right. The simplest method used to map N6-methyladenosine (m6A) sites on mRNA is m6A-seq (also known as methylated RNA immunoprecipitation sequencing (mERIP-seq)), in which RNA is typically fragmented by alkaline hydrolysis and then incubated with an m6A-specific antibody. The resulting RNA–antibody complexes are then recovered and sequenced132,133. However, the resolution of this mapping technique is limited by the average RNA fragment size, which is generally around 125 nucleotides, resulting in fairly broad peaks (that is, it identifies broad regions that contain one or more m6A residues). One variation of this method, called m6A individual-nucleotide-resolution crosslinking and immunoprecipitation (miCLIP), uses ultraviolet (UV) light crosslinking (wavelength of 254 nm) of the antibody to the motif characteristic of m6A modification sites (minimally 5′-RAC-3′; with R representing G or A), resulting in a cytidine-to-thymidine conversion of the cytidine immediately 3′ to the m6A modification in the reverse-transcribed cDNA library, allowing single-nucleotide resolution mapping134.

Another variation is photo-crosslinking-assisted m6A-seq (PA-m6A-seq), in which cells are first pulsed with the highly photoactive uridine analogue 4-thiouridine (4SU). The 4SU-containing RNA is then isolated and UV-light-crosslinked (wavelength of 365 nm) in solution to an m6A antibody135. Because this method utilizes RNase to degrade any RNA not protected by the bound antibody, recovered bound RNA fragments reflect the sequence protected by the antibody, which is ~32 nucleotides. In addition, UV-light crosslinking of proteins to 4SU results in a thymidine-to-cytidine conversion during reverse transcription, which allows the removal of all background reads during final data analysis136. Antibody-based methods can easily be adapted to mapping different modifications, as we have previously demonstrated by performing PA-m6A-seq with mC and ac4C antibodies137,138, whereas m6A-seq has also been adapted to ac3C139. Adapting miCLIP to other antibodies would require additional testing to discover the preferred mutation induced by UV-light crosslinking of the specific antibody.

Antibody-mapping results can be corroborated using the photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP) technique139 to map the binding sites for modification-specific readers in living cells, if known. For example, the m6A reader YTHDF2 can be utilized in this way139. In a method for mapping 5′-methycytidine (m5C), confusingly also named miCLIP, mutation of the m5C writer NSUN2 at a key cysteine residue (C271A) causes NSUN2 to covalently crosslink to the target cytidine residue on the substrate RNA; thus, m5C modifications could be identified by immunoprecipitation of NSUN2-C271A-bound RNAs followed by RNase treatment, RNA fragment recovery and deep sequencing137.

For sites of 2′-O-methylation (Nm), no antibody is currently available, and mapping thus depends on chemical methods. RiboMethSeq exploits the fact that a methylation at the 2′O position can block alkaline hydrolysis at that position138,139. In 2′OMe-Seq, reverse transcription stops on an Nm site under low-dNTP conditions140. Nm-seq and RiboOxiSeq utilize the resistance of 3′-end Nm to periodate cleavage (IO4−)141,142. All three of these chemical Nm-mapping methods provide single-nucleotide resolution. However, because RiboMethSeq and 2′OMe-Seq depend on the absence of sequencing reads ending at the modification site, they require very high sequencing read depths. We note that the oxidation and elimination processes of Nm-seq and RiboOxiSeq lead to the loss of most of the RNA sample. Thus, all current Nm methods require large amounts of input RNA. Bisulfite sequencing has been used to map mC residues on RNAs, exploiting the fact that mC residues are resistant to the cytidine-to-uridine conversion induced by bisulfite treatment143. However, we have observed that cytidine residues located in RNA stems are also resistant to bisulfite treatment, potentially resulting in their incorrect identification as mC residues.
HIV-1, which forms part of the 3′ untranslated region of the viral gag mRNA, nevertheless reduced Gag mRNA and protein levels equivalently. Thus, in addition to RNA methylation, acetylations in the form of ac4C can also be utilized to enhance viral gene expression, through the stabilization of viral RNA transcripts.

**2′O-methylation.** The fourth and final internal epitranscriptomic modification that has, so far, been reported to affect viral replication is 2′O-methylation of the ribose moiety of all four ribonucleosides (Am, Cm, Gm and Um, collectively known as Nm). Each of the four Nm residues represents ~0.1% of the level of the relevant nucleoside found in cellular mRNAs, yet this level was found to be up to 20 times higher when HIV-1 or MLV genomic RNAs were analysed[56]. The Nm writer that acts on retroviral transcripts has been identified as the nucleolar protein FTSJ3 (REF[105]), which was previously shown to function in pre-rRNA processing[108] (FIG. 5). We note that FTSJ3 was reported to be incapable of adding 2′O-methyl groups to cytidine residues[107], which appears inconsistent with the high levels of Cm detected on HIV-1 (1.02%) and MLV (0.74%) genomic RNAs[58,59]. Moreover, preliminary data suggest that the yeast FTSJ3 homologue (Spb1) is able to methylate cytidine residues[110]. Only one report has so far examined the phenotypic effect of Nm residues on HIV-1 replication, and these researchers did not report any effect of Nm residues on HIV-1 gene expression. Instead, they found that HIV-1 virions produced in cells in which FTSJ3 was knocked down by RNA interference were potent activators of the cytoplasmic viral RNA sensor MDA5, a key component of the host antiviral immune response, when the virions were used to infect dendritic cells[111]. Others have also reported that specific epitranscriptomic RNA modifications, including not only Nm but also pseudouridine, can attenuate cellular innate immune responses to transfected mRNA molecules[110–113], whereas m6A was recently reported to prevent the activation of a second cytoplasmic viral RNA sensor, RIG-I[114]. Clearly, it will be important to examine whether other viruses also use epitranscriptomic RNA modifications, including but not limited to m6A and Nm, to escape viral RNA detection by host innate immunity factors.

In conclusion, the current evidence suggests that several different epitranscriptomic RNA modifications are able to promote viral replication, either directly, by increasing viral mRNA stability or translation, or indirectly, by viruses to elude the recognition of their transcripts as foreign by cytoplasmic viral RNA sensors. It is therefore not surprising that viruses appear to have evolved so as to maximize the level of several epitranscriptomic modifications that are added to their mRNAs. By contrast, mammalian cells have evolved the capacity to recognize viral DNA as foreign and seek to silence that DNA epigenetically as one form of innate immune response. Viruses, in turn, have evolved mechanisms that allow them to avoid epigenetic silencing, either by the targeted destruction of relevant cellular factors, such as the components of PML-NBs, or by hiding in host-cell chromosomal DNA, as is seen for retroviruses.

**Future perspectives** In this Review, we have argued that cells use epigenetic gene regulation as a potential mechanism to silence incoming viral DNA molecules, whereas viruses have evolved to recruit the cellular epitranscriptomic modification factors in order to heavily modify viral transcripts, as a means to boost viral gene expression and/or replication. Although the field of epigenetic gene regulation is fairly mature, the question of how cells distinguish between viral DNA and host-cell DNA remains unresolved. In particular, how unintegrated HIV-1 proviral DNA is recognized and silenced remains unknown. By contrast, the field of epitranscriptomic gene regulation is still in its infancy. For example, how writers select specific bases for modification remains largely unknown, and even in the case of m6A — for which the consensus editing sequence 5′-RAC-3′ has been defined, where R is either G or A[115] — only a minority of sites with that sequence are, in fact, modified. Moreover, although five m6A readers have been identified, the readers for all other RNA modifications remain unknown, and even for m6A, how the readers exert their phenotypic effects remains largely undefined. It will be important to understand why m6A clearly promotes viral replication in most published studies, yet has also been suggested in other reports to inhibit viral replication, as discussed above.

Both epigenetic and epitranscriptomic regulatory pathways could in principle be targeted for antiviral drug development. In the case of epigenetic viral gene regulation, two strategies have emerged. In an approach termed ‘shock-and-kill’, drugs that promote the formation of active chromatin, such as histone deacetylase (HDAC) inhibitors, have been proposed as tools to activate latent viruses, including EBV[116–118] and HIV-1 (REF[117]). In the case of HIV-1, this strategy envisions using these drugs to reactivate latent proviruses, in individuals who are also on antiretrovirals, as a means of selectively eliminating latently infected cells. However, this strategy has yet to prove clinically useful. An alternative approach envisions the use of drugs that instead keep viral DNA epigenetically suppressed, including drugs that inhibit the H3K9 demethylases LSD1 and JMJD2, which are recruited by HCF-1 to HSV-1 viral DNA in order to remove repressive marks. Inhibitors of LSD1 and JMJD2 have indeed been shown to suppress HSV-1 gene expression, replication and reactivation from latency, both in vitro and in vivo[119–121]. These drugs have also proved effective against other HCF-1-dependent herpesviruses, including HCMV and varicella zoster virus (VZV), and they might prove useful in the treatment of pathologies that result from the reactivation of latent herpesviruses, such as shingles. Although in principle LSD1 and JMJD2 inhibitors could be used to entirely repress the reactivation of latent DNA viruses, these drugs would then need to be taken long-term, and it seems unlikely that inhibition of host H3K9 demethylases for months or even years would be well-tolerated.

Inhibitors that block the epitranscriptomic modification of viral RNAs clearly would be potentially even more interesting, as this should inhibit viral gene expression and/or promote antiviral immune responses. These drugs would presumably be targeted to the cellular...
writers that add epitranscriptomic marks to mRNAs (TABLE 1), and the emergence of drug-resistant viral mutants would therefore seem to be unlikely. Conversely, as such drugs would also prevent the epitranscriptomic modification of cellular mRNAs, toxicity might be an issue, and we therefore envision that such drugs would be used only briefly, during the 5–7-day acute phase of infection by viruses such as IAV, RSV and possibly severe acute respiratory syndrome coronavirus 2, in order to reduce the peak viral load and limit viral pathogenicity until the adaptive immune system becomes effective.

Because the addition of m^A to mRNAs has been implicated as a driver in some forms of cancer,120–122 several biotech companies are already attempting to identify effective inhibitors of METTL3 function, and it would clearly be of interest to test these, once they have been identified, in animal models that support pathogenic infections by human viruses. In the interim, some data in fact already suggest that drugs that inhibit mRNA methylation could prove to be effective pan-viral inhibitors. Specifically, several drugs, including 3-deaza-adenosine (DAA), are known to act as inhibitors of S-adenosylhomocysteine (SAH) hydrolase and, as a result, to deplete cells of SAM, the methyl donor used not only by METTL3 but also by NSUN2, and likely also by the enzymes that add Nm residues to mRNAs. Importantly, DAA was shown to inhibit m^A addition to mRNAs, while the formation of 7-methylguanosine, which forms part of the mRNA 5' cap, was largely unaffected.121 DAA acts as a potent pan-viral inhibitor in culture and is able to effectively inhibit a wide range of DNA and RNA viruses at doses that are >100-fold lower than the level found to exert a toxic effect on cultured primary cells.121 In vivo, DAA is also a remarkably effective broad-spectrum antiviral. For example, a single dose of DAA given one or two days after infection reduced peak viraemia in Ebola-virus-infected mice by >1,000 fold and resulted in the survival of almost all the treated animals, whereas untreated animals showed a 0% survival rate.122 Similarly, DAA drastically reduced viral titers in cotton rats infected with RSV at doses of DAA that were not detectably toxic.124 Although DAA may not itself be a potentially useful drug, these studies do make the point that the epitranscriptomic modification of viral mRNAs may represent a potential viral Achilles heel, and that the identification of inhibitors of this process could therefore lead to the development of a novel class of potent, broad-spectrum antivirals.

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