Viral RNAs: Lessons from the Enemy

Bryan R. Cullen[1],*
[1]Department of Molecular Genetics & Microbiology, Center for Virology, Duke University Medical Center, Durham, NC 27710, USA
*Correspondence: bryan.cullen@duke.edu
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Viruses are adept at evolving or co-opting genomic elements that allow them to maximize their replication potential in the infected host. This evolutionary plasticity makes viruses an invaluable system to identify new mechanisms used not only by viruses but also by vertebrate cells to modulate gene expression. Here, I discuss the identification and characterization of viral mRNA structures and noncoding RNAs that have led to important insights into the molecular mechanisms of eukaryotic cells.

Viruses have historically represented a valuable experimental system to study the molecular biology of mammalian cells. In part, this is due to the fact that, prior to the advent of RNA interference, viruses represented the most genetically tractable system available to study how mammalian cells function. Moreover, because many viruses express their genomes, mRNAs, and proteins at very high levels, it proved possible to perform biochemical and molecular experiments using viral gene products that were difficult to attempt using cellular gene products. As a result, virus research led to the initial identification of almost all of the known posttranscriptional modifications of mRNA transcripts. These include mRNA capping, first discovered by analysis of reovirus and vaccinia virus mRNAs (Furuichi et al., 1975; Wei and Moss, 1975); mRNA splicing, first discovered by analysis of adenovirus transcripts (Berget et al., 1977; Chow et al., 1977); and polyadenylation, first described for vaccinia virus mRNAs (Kates, 1970). In addition, mutational analysis has also identified several structures located in viral mRNA molecules that play key roles in their transcription, nuclear export, or translation (Table 1, Figur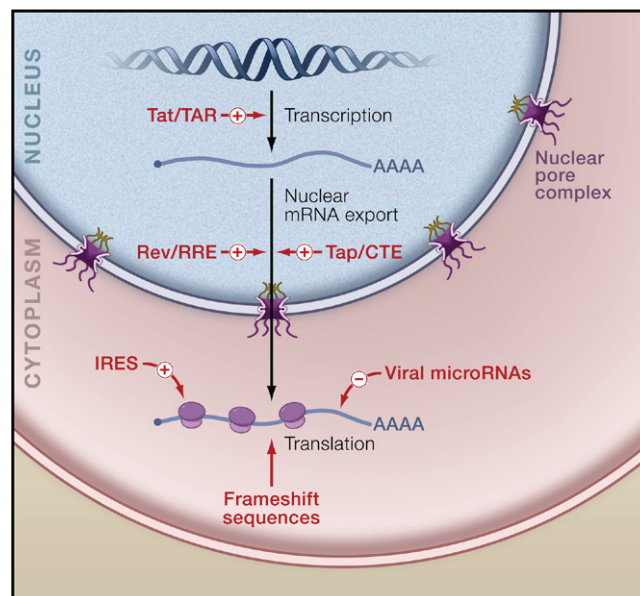e 1). Finally, many viruses express a number of noncod-ing RNAs that enhance viral replication and pathogenesis but whose functions and mechanisms of action often remain poorly understood.

TAR RNA and HIV-1 Transcription

Although viral RNA elements obviously play an important role in directing the transcription of the genomes and antigenomes of RNA viruses, the transacti-

Figure 1. Viral Noncoding RNAs or RNA Structures Influence Eukaryotic Gene Expression
The HIV-1 Tat protein activates transcription of the HIV-1 provirus by recruiting the cellular P-TEFb complex to the viral TAR RNA hairpin. Nuclear export of incompletely spliced HIV-1 transcripts is facilitated by the RRE RNA structure, which recruits a complex consisting of the viral Rev protein and cellular CRM1. A similar function is performed by the CTE RNA structure present in MPMV, which recruits the cellular Tap nuclear export factor. The cytoplasmic translation of picornaviral mRNAs is facilitated by IRES elements, and the translation of retroviral and coronaviral mRNAs is modulated by sequences that induce ribosomal frameshifting. Finally, the translation of both viral and cellular mRNAs can be specifically downregulated by virally encoded microRNAs.

vation response (TAR) element remains unique in that it promotes the efficient transcription of human immunodeficiency virus type 1 (HIV-1) proviral DNA by cellular RNA polymerase II (RNAP II). HIV-1 is a complex retrovirus, that is, it encodes not only the canonical structural proteins and enzymes found in all retroviruses (Gag, Pol, and Env) but also four auxiliary proteins and two regulatory proteins, Tat and Rev. Mutational analysis of HIV-1 mutants lacking a functional *tat* gene showed that these were replication defective due to a dramatic drop in transcription from the long terminal repeat (LTR) promoter element (Fisher et al., 1986; Kao et al., 1987). Subsequent analysis mapped the target site for the Tat protein to a ~50 base pair (bp) element located immediately 3′ to the transcription start site. TAR folds into a simple RNA stem-loop structure that represents the actual target for Tat function (Feng and Holland, 1988).

It is now known that Tat first interacts with a cellular factor called cyclin T1, which, along with the kinase cdk9, constitutes positive transcription elongation factor b (P-TEFb) (Wei et al., 1998). The resulting complex of Tat and P-TEPb binds directly to HIV-1 TAR via the terminal loop and an adjacent RNA bulge. Once bound

Table 1. Important Viral mRNA Structures

Name	Virus Family	Function
Transactivation response (TAR) element	HIV-1 and other lentiviruses	Binds to a complex containing viral Tat and cellular P-TEFb to activate transcription elongation
Rev response element (RRE)	Lentiviruses; analogous structures found in δ-retroviruses	Recruits a complex of viral Rev and cellular CRM1 to induce nuclear export of incompletely spliced viral mRNAs
Constitutive transport element (CTE)	MPMV and some other simple retroviruses	Recruits the cellular Tap/p15 complex to induce nuclear export of incompletely spliced viral mRNAs
Internal ribosome entry site (IRES)	Picornaviruses and some flaviviruses	Recruits cellular translation factors and ribosomal subunits to viral translation initiation codons in the absence of an mRNA cap

to TAR, P-TEFb mediates the phosphorylation of negative regulators of transcription elongation and of the carboxy-terminal domain of RNAP II molecules that have initiated transcription of HIV-1 proviral DNA. These phosphorylation events render RNAP II elongation competent and allow it to transcribe the entire viral genome (Barboric and Peterlin, 2005; Kao et al., 1987). In contrast, in the absence of Tat or TAR, transcription initiation at the LTR promoter still occurs but almost all of these initiating RNAP II molecules fall off the DNA template within ~200 bp of the transcription start site. Analysis of Tat function led to the realization that not only transcription initiation but also transcription elongation can regulate gene expression levels in animal cells (Barboric and Peterlin, 2005).

Retroviral Nuclear mRNA Export
Almost all retroviruses contain a single RNAP II-dependent promoter element in the viral LTR that drives transcription of an initial genome-length RNA that also acts as an mRNA for translation of the viral Gag and Pol proteins (Cullen, 2003). In the case of HIV-1, this initial transcript can also be processed into fully spliced transcripts encoding the Tat and Rev proteins of HIV-1 as well as the auxiliary protein Nef. Alternatively, this transcript can be processed into partially spliced mRNAs encoding the three other viral auxiliary proteins Vif, Vpu, and Vpr. The HIV-1 replication cycle, therefore, requires that the single initial viral transcript is exported out of the nucleus in several differentially spliced forms. These include an unspliced form that programs Gag and Pol expression and that is packaged into virion progeny; partially spliced forms that program expression of Env, Vif, Vpr, and Vpu; and fully spliced forms that program expression of Tat, Rev, and Nef (Cullen, 2003).

The difficulty with this scenario is that eukaryotic cells do not normally permit the nuclear export of intron-containing mRNAs. Almost all cellular mRNAs are transcribed as intron-containing pre-mRNAs, and these introns are recognized in the nascent transcript by splicing factors, including commitment factors. Commitment factors both commit the pre-mRNA to the splicing pathway and retain the pre-mRNA in the nucleus until all introns are removed (Legrain and Rosbash, 1989).

HIV-1 mRNAs rely entirely on cellular factors for appropriate splicing, and intron-containing HIV-1 transcripts are therefore also retained in the infected cell nucleus by splicing commitment factors. The strategy that HIV-1 has evolved to circumvent this nuclear retention is dependent on the viral Rev protein, which is translated from a fully spliced viral mRNA that is constitutively exported from the nucleus. As a result, HIV-1 mutants lacking a functional *rev* gene are able to express the proteins encoded by fully spliced viral mRNAs, that is, Tat, Nef, and the defective Rev protein itself, but cannot express any of the proteins encoded by incompletely spliced viral mRNAs, including Gag, Pol, and Env. The transcripts encoding these viral structural proteins, however, can be detected in the nucleus of cells infected by Rev-deficient viruses, where they are either degraded or eventually fully spliced and then exported (Cullen, 2003).

Rev function requires a highly structured RNA target, located in the HIV-1 *env* gene, called the Rev response element or RRE (Malim et al., 1989). The RRE contains a single, high-affinity Rev-binding site and also functions as a scaffold for the multimerization of Rev on viral mRNAs. Rev in turn interacts with a cellular factor called

CRM1 that belongs to the karyopherin family of nucleocytoplasmic transport proteins (Fischer et al., 1995). This interaction is mediated by a leucine-rich motif located toward the carboxyl terminus of Rev that was the first nuclear export signal (NES) to be identified and is the prototype of the leucine-rich class of NESs.

Karyopherin function is regulated by the action of a G protein called Ran, which, like all G proteins, is active when bound by GTP and inactive when bound by GDP (Kohler and Hurt, 2007). Cells contain high levels of a Ran-specific G nucleotide exchange factor (GEF) in the nucleus and of a Ran-specific GTPase activating protein (GAP) in the cytoplasm. As a result, Ran:GTP is largely nuclear and Ran:GDP is mainly cytoplasmic. Ran:GTP binds to CRM1 in the nucleus and activates the binding of CRM1 to leucine-rich NESs. The ribonucleoprotein complex, consisting of Ran:GTP, CRM1, and Rev, that forms on the HIV-1 RRE directs incompletely spliced HIV-1 transcripts to the nuclear pore complex and then into the cytoplasm, where hydrolysis of the GTP moiety by cytoplasmic GAP disassembles this complex.

Although Rev was the first nuclear mRNA export factor to be identified, it soon became clear that CRM1 is not required for the nuclear export of most cellular mRNAs. In fact, CRM1 is involved largely in the nuclear export of small nuclear RNAs (snRNAs) and pre-ribosomal subunits, as well as in protein nuclear export (Kohler and Hurt, 2007). So which factors are required for the export of cellular mRNAs?

An important part of the answer emerged from analysis of a second retrovirus called Mason-Pfizer monkey virus (MPMV). MPMV has a simpler genomic organization than HIV-1 and only encodes the three structural proteins

Gag, Pol, and Env. Nevertheless, MPMV expresses both a genome-length Gag/Pol mRNA and a spliced Env mRNA. As MPMV does not encode a Rev homolog, how do the incompletely spliced genomic MPMV mRNAs reach the cytoplasm? This question led to the discovery of an RNA stem-loop structure within the MPMV genome, the constitutive transport element (CTE), that mediates the nuclear export of incompletely spliced mRNAs in the absence of any viral proteins (Bray et al., 1994). Further analysis revealed that the CTE recruits a heterodimer of two cellular proteins, Tap and p15, that also plays a critical role in the nuclear export of the majority of cellular mRNAs (Grüter et al., 1988; Kohler and Hurt, 2007). Normally, the Tap/p15 heterodimer is only recruited to mature, fully spliced mRNAs. However, the CTE is able to prematurely recruit Tap/p15 to partially spliced mRNAs and thereby circumvents the nuclear retention of incompletely spliced MPMV mRNAs. Although CTEs have now been defined in several other exogenous and endogenous retroviruses, not all CTEs act by directly recruiting Tap/p15. In particular, the avian leukemia virus CTE does not appear to bind to Tap or p15 directly, although Tap may be required for its function (LeBlanc et al., 2007). Further analysis may reveal new insights into how the export of retroviral nuclear mRNAs is regulated.

Viral mRNA Translation, IRES Elements, and Frameshifting

After an mRNA is exported to the cytoplasm, it must recruit cellular ribosomes in order for the translation of the encoded open reading frame to occur (Figure 1). The process of translation initiation is both complex and heavily regulated. For the majority of cellular mRNAs, it is initiated by the recruitment of the eukaryotic initiation factor eIF4F to the mRNA 5′ cap (Martinez-Salas et al., 2008) (see Review by N. Sonenberg and A.G. Hinnebusch in this issue of *Cell*). eIF4F consists of the cap-binding protein eIF4E, the RNA helicase eIF4A, and a third protein, eIF4G. The role of eIF4G is to facilitate recruitment of the 43S preinitiation complex, consisting of the 40S ribosomal subunit, found in a complex with the initiator methionine tRNA (Met-tRNAi) and other eIFs, to the mRNA cap. Once recruited to the cap, the 40S subunit then scans

along the mRNA searching for the translation initiation codon. At this point, the 60S ribosomal subunit is also recruited and translation initiation occurs (Martinez-Salas et al., 2008).

Picornaviruses presented two mysteries in terms of how these pathogenic viruses are able to translate the single large polyprotein encoded by their positive-sense RNA genome. First, the single genome-length picornavirus mRNA is uncapped. Second, infection by picornaviruses such as poliovirus results in the efficient translation of viral mRNAs, yet cellular mRNA translation is largely blocked. So, why is this uncapped viral mRNA translated more efficiently than capped cellular mRNAs?

The key discovery that led to the resolution of this conundrum was the identification of the poliovirus internal ribosome entry site (IRES), a ~450 nucleotide (nt) highly structured RNA element found in the 5′ untranslated region (5′UTR) of poliovirus mRNAs (Jang et al., 1988; Pelletier and Sonenberg, 1988). The IRES directly recruits several eIFs and the 40S ribosomal subunit to an internal viral translation initiation codon without the requirement for either cap binding or 5′UTR scanning. As a result, poliovirus translation is independent of the host cell cap recognition factor eIF4E. Moreover, although poliovirus translation initiation does require eIF4G, it functions perfectly well with the carboxy-terminal fragment of eIF4G that is generated by the proteolytic cleavage of eIF4G by a virus-encoded protease. Because cap-dependent translation requires full-length eIF4G, this cleavage blocks host cell translation, whereas viral mRNA translation is not only unimpeded but in fact is enhanced by the access of viral mRNAs to the entire pool of available eIFs and ribosomal subunits (Martinez-Salas et al., 2008).

Subsequent work has demonstrated that all picornaviruses as well as some flaviviruses, including hepatitis C virus (HCV), contain IRES elements. Surprisingly, these exist in several functionally distinct classes. For example, the HCV IRES, unlike the poliovirus IRES, can directly recruit 40S ribosomal subunits to the viral internal translation initiation codon in the absence of eIFs, although eIFs do participate in the process of

translation initiation (Martinez-Salas et al., 2008). An even more unusual IRES is found in cricket paralysis virus (CrPV), a picornavirus-like insect virus (Jan et al., 2003; Pestova and Hellen, 2003). The CrPV IRES not only is able to recruit both the 40S and 60S ribosomal subunits to assemble elongation-competent 80S ribosomes on viral mRNAs but also acts as a mimic of Met-tRNAi to permit initiation of the translation of viral capsid proteins in the absence of Met-tRNAi.

Although IRES elements were first discovered in RNA viruses, a subset of cellular mRNAs are now known to also contain IRESs. Interestingly, IRESs seem to be especially prevalent in mRNAs whose expression is activated by stress, when cap-dependent translation may be inefficient. Many IRES-containing host mRNAs encode proteins that protect cells from stress, whereas the proteins encoded by other IRES-containing cellular mRNAs seem to be important during apoptosis (Bushell et al., 2006; Komar and Hatzoglou, 2005).

Another interesting translational phenomenon observed in several virus families, including many species of retroviruses and all coronaviruses, is programmed ribosomal frameshifting (Brierley and Dos Ramos, 2006). In retroviruses such as HIV-1, frameshifting prevents some ribosomes from terminating translation at the end of the open reading frame (ORF) encoding the Gag structural protein and instead induces ribosomes to shift into the overlapping *pol* ORF, resulting in the production of the large Gag-Pol polyprotein. Similarly, in coronaviruses, ribosomal frameshifting is used to produce the 1a/1b replicase polyprotein rather than the shorter 1a variant. Frameshifting is induced by a bipartite element consisting of a 5′ frameshifting site and an adjacent 3′ RNA structure (Brierley and Dos Ramos, 2006; Jacks et al., 1988). The frameshift site has the consensus sequence X_XXY_YYZ (where the translational phase is indicated), which then slips into the −1 frame, that is, XXX_YYY_Z. The actual shift sites found in HIV-1 and the SARS coronavirus are U_UUU_UUA and U_UUA_AAC, respectively. The 3′ RNA structure found in HIV-1 is thought to be a simple RNA hairpin but other −1 frameshifting signals instead contain a

Table 2. Selected Viral Noncoding RNAs

Name	Virus	Size	Function
LAT intron	HSV-1	~2 kb	Unclear, but may modulate translation in latently infected neurons
PAN	KSHV	~1.1 kb	Unclear, but contains an element that stabilizes un-spliced transcripts
β2.7	hCMV	~2.7 kb	Binds to mitochondrial enzyme complex I to stabilize ATP production and inhibit apoptosis
VA1	adenovirus	~160 nt	Binds to cellular PKR to block PKR activation by viral dsRNA and the resultant inhibition in translation

pseudoknot 3′ to the frameshift signal (Brierley and Dos Ramos, 2006). It has been proposed that the function of the 3′ RNA structure is to induce transient ribosomal pausing at the frameshift site to facilitate ribosomal slippage in the −1 direction. Although frameshifting in HIV-1 occurs with an efficiency of ~5%, frameshifting efficiency in other viruses can be as high as ~25% and may be facilitated by a direct interaction between the paused ribosome and the downstream pseudoknot structure. Programmed frameshifting is not unique to viruses but is found also in a small number of cellular genes in both eukaryotes and bacteria (Shigemoto et al., 2001; Tsuchihashi and Kornberg, 1990).

Viruses, RNA Interference, and MicroRNAs

RNA interference (RNAi) was first discovered by genetic analysis in nematodes (Fire et al., 1998); however, it is likely that RNAi first evolved as an innate immune response to viral infection. Indeed, RNAi continues to represent a key component of the antiviral response in plants and invertebrates (Cullen, 2006). The triggers for RNAi in these species are the long double-stranded RNAs (dsRNAs) that form critical intermediates in the replication of all RNA viruses except retroviruses. These dsRNAs are bound by the RNase III-related enzyme Dicer, which progressively cleaves these dsRNAs into ~22 bp RNA duplexes containing terminal 2 nt 3′ overhangs (see Review by R.W. Carthew and E.J. Sontheimer in this issue of *Cell*). One strand of this duplex, called a small-interfering RNA (siRNA), is then incorporated into the RNA-induced silencing complex (RISC), where it acts as a guide for RNA to target RISC to complementary regions of viral genomic, anti-genomic, or mRNA species. RISC then cleaves these viral RNAs, leading to their degradation. The first siRNAs to be identified were in fact antiviral siRNAs produced in tobacco cells infected by a pathogenic RNA virus, potato virus X (Hamilton and Baulcombe, 1999).

RNAi is a critical component of the antiviral immune response in plants and invertebrates, but emerging evidence indicates that RNAi responses to viral infection are not induced in mammalian somatic cells (Cullen, 2006). Instead, mammalian cells have evolved other innate responses that are induced by viral dsRNAs, including the interferon response. Because of the importance of RNAi as an antiviral defense in plants and insects, many RNA viruses that infect these species have evolved gene products that inhibit RNAi and, hence, enhance virus replication. Conversely, the absence of antiviral RNAi responses in mammalian cells means that the RNAi machinery in these cells generally remains active during viral infection (Figure 1).

Although the role of RNAi as an antiviral response appears to have been lost in mammalian somatic cells, the residual RNAi machinery still plays a very important role by mediating the function of cellular microRNAs (miRNAs). Unlike siRNAs, which are derived from long dsRNAs (frequently of exogenous origin), miRNAs are encoded within the cell's genome as part of one arm of an ~80 nt RNA hairpin located in a larger RNAP II transcript called a primary miRNA (Bartel, 2004). After excision, by the sequential action of the host cell RNase III-related enzymes Drosha and Dicer, miRNAs are loaded into RISC and downregulate the expression of cellular mRNAs. Unlike viral mRNA targets of viral siRNAs, cellular mRNAs are rarely fully complementary to cellular miRNAs.

As full complementarity is a prerequisite for efficient cleavage by RISC, cellular mRNAs are generally not subject to degradation by cellular miRNAs. Instead, cellular miRNAs can induce the translational inhibition of cellular mRNAs by binding to partially complementary target sites (Bartel, 2004).

As most mammalian viruses do not seem to interfere with the loading or function of RISC, miRNAs remain active in infected cells, thus offering the possibility for viruses to use the cellular RNAi machinery to regulate cellular or viral gene expression by programming RISC with viral miRNAs. Analysis of a range of virally infected cells has revealed that several DNA viruses, including herpesviruses, encode multiple distinct miRNAs. Of note, most viral miRNAs appear to be processed by the same Drosha and Dicer dependent pathway used by the majority of cellular miRNAs, although there are a few examples of viral miRNAs that are transcribed by RNA polymerase III, not RNAP II, and then excised directly by Dicer (Gottwein and Cullen, 2008). Similarly, RISCs programmed by viral miRNAs appear to function in the same way as RISCs programmed by cellular miRNAs.

Although beyond the scope of this article, it is interesting to note that several cellular and viral mRNA targets of viral miRNAs have now been defined (Gottwein and Cullen, 2008). In general, it appears that viral miRNAs either downregulate cellular or viral genes that increase the sensitivity of virally infected cells to host innate or adaptive immune responses or, in the case of herpesvirus miRNAs, stabilize viral latency by downregulating the expression of the viral immediate early proteins, which favor entry into the lytic replication cycle.

Viral Noncoding RNAs

In addition to miRNAs, a number of DNA viruses also encode long noncoding RNAs that play a role in regulating viral replication and pathogenesis (Table 2; reviewed in Sullivan and Cullen, 2009; see Review by C.P. Ponting, P.L. Oliver, and W. Reik in this issue of *Cell*). But how do viruses use noncoding RNAs to promote their replication? One interesting noncoding RNA is the latency associated

transcript (LAT) encoded by herpes simplex virus type 1 (HSV-1). LAT is an ~8.3 kb capped polyadenylated RNA that is the only viral RNA expressed at high levels in neurons latently infected with HSV-1. LAT is spliced to give an unstable ~6.3 kb exonic RNA and a stable ~2 kb intron. The instability of the 6.3 kb LAT RNA appears to be due to the fact that it is processed into several viral miRNAs that may play a key role in regulating HSV-1 latency (Umbach et al., 2008). The role of the stable 2 kb LAT intron is less clear, but evidence has been presented arguing that the LAT intron is exported out of the nucleus by CRM1 and associates with cellular ribosomes, thus suggesting a role in modulating mRNA translation in neurons latently infected with HSV-1 (Atanasiu and Fraser, 2007).

Another interesting viral noncoding RNA is the polyadenylated nuclear (PAN) RNA encoded by Kaposi's sarcoma-associated herpesvirus (KSHV). PAN is an unspliced RNAP II transcript that is the most highly expressed viral RNA during lytic KSHV infection, comprising up to 80% of all viral RNAs. Remarkably, the function of this RNA in the viral life cycle is still unclear. However, recent data demonstrate that PAN contains a novel ~80 nt-long RNA element that stabilizes PAN RNA in the infected cell nucleus. Insertion of this viral RNA element in *cis* also increases the nuclear abundance of cellular mRNAs, such as β-*globin* mRNAs, that are normally unstable when expressed in an intronless form (Conrad et al., 2006). It is unclear whether this element simply stabilizes PAN RNAs or whether it also acts in *trans* to stabilize other KSHV-coding mRNAs, which are also largely intronless.

Finally, several viral noncoding RNAs seem to be inhibitors of cellular innate antiviral immune responses. For example, the β2.7 noncoding RNA encoded by human cytomegalovirus (hCMV) binds to mitochondrial enzyme complex I of the host cell. This interaction stabilizes the production of ATP in infected cells and also inhibits virally induced apoptosis (Reeves et al., 2007). Another noncoding RNA, the VA1 RNA expressed by adenovirus, also binds to a cellular factor to inhibit an antiviral response. In this case, the target is protein kinase R (PKR), a cellular protein that binds to the long dsRNAs produced by adenoviruses and many other pathogenic viruses. Binding of dsRNA by PKR induces PKR dimerization and autophosphorylation as well as phosphorylation of cellular eIF2α, which results in a global inhibition of translation in the infected host cell. VA1, a highly structured ~160 nt-long noncoding RNA, binds to PKR with high affinity and blocks PKR dimerization and activation. This prevents the inhibition of translation induced by adenovirus-derived dsRNAs and allows virus replication to proceed unimpeded (Mathews and Shenk, 1991). Interestingly, both hCMV β2.7 and adenovirus VA1, like KSHV PAN and HSV-1 LAT, are also expressed at high levels in infected cells. β2.7 comprises up to 20% of all viral transcripts in hCMV-infected cells, and adenovirus VA1 is expressed at an extraordinarily high number of copies (~10^8) per infected cell. Presumably, these high expression levels facilitate the saturation of cellular binding sites for these RNAs.

Conclusion
Efforts to understand the replication cycles of viruses are often motivated by the pathogenic potential of these intracellular parasites. However, such analyses have also led to several key insights into how not only infected cells but also uninfected cells regulate the expression of their genome. Moreover, as noted in the brief discussion of viral noncoding RNAs, our knowledge of how virally encoded transcripts work in the host cell remains far from complete. Clearly, future research into virus replication will provide unexpected and exciting insights into the complex molecular machinery that makes human cells tick.

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