Review
Future Virology

Involvement of heterogeneous nuclear ribonucleoproteins in viral multiplication

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The study of virus–host interactions is a major goal in molecular virology and provides new effective targets for antiviral therapies. Heterogeneous nuclear ribonucleoproteins (hnRNPs) constitute a group of cellular RNA-binding proteins localized predominantly within the nucleus, which participate in gene transcription and subsequent RNA post-transcriptional modifications. The interaction between hnRNPs and viral components was extensively demonstrated, as well as the ability of virus infections to alter the intracellular localization or the level of expression of different hnRNPs. The involvement of these proteins in the replication of numerous viruses including members from the *Retroviridae*, *Flaviviridae*, *Coronaviridae*, *Arenaviridae*, *Rhabdoviridae*, *Papillomaviridae*, *Orthomyxoviridae*, *Picornaviridae*, *Togaviridae* and *Herpesviridae* families, has been reported. In order to gain an increased understanding of the interactions between virus and cell that result in the productive infection of the latter, in this review we discuss the main findings about the role of hnRNPs in different steps of viral replication, such as RNA synthesis, translation, RNA processing and egress of newly assembled progeny virus.

Virus-induced changes in heterogeneous nuclear ribonucleoprotein expression & localization pattern

The heterogeneous nuclear ribonucleoproteins (hnRNPs) are a group of primarily nuclear proteins that bind to nascent transcripts produced by RNA polymerase II, and which do not stably associate with other RNA–protein complexes [1]. They are involved in the processes of RNA metabolism, such as pre-mRNA splicing, capping and polyadenylation, tRNA maturation, mRNA localization and translation. Binding to nucleic acids is carried out by multiple domains present in the sequences of hnRNPs. The most prevalent domain among the hnRNPs is the canonical RNA recognition motif (RRM), although K homology (KH) domains, quasi-RRM domains and glycine-rich domains may also be found as binding domains to RNPs within the family of hnRNPs [2]. The hnRNP family is composed of at least 20 abundant, major hnRNPs, designated hnRNP A1–U with molecular weights ranging from 34 to 120 kDa. Though most hnRNPs are predominantly nuclear, many of them remain associated with the mRNA as it is transported through nuclear pores, hence undergoing constant nuclear–cytoplasmic shuttling [3].

Changes in hnRNP expression have been described for a few virus–cell systems. For instance, HPV16 and HIV-1 upregulate hnRNP A1 during infection [4,5]. Differences in hnRNP levels in infected and uninfected cells have been also described for Junin virus-persistently infected cells, which showed low levels of hnRNP A/B in comparison to uninfected and acutely infected cells [6].

On the other hand, there is an extensive list of viruses that cause changes in hnRNP intracellular distribution. Inhibition of nuclear trafficking by viruses may contribute to the cytoplasmic accumulation of nuclear factors that would favor virus replication and pathogenesis. Disruption of nucleocytoplasmic transport could also affect the cell antiviral response, impairing nuclear import of signal transducers and activators of transcription involved in the interferon (IFN) response. Infection with viruses from different genera of the *Picornaviridae* family disrupts nucleocytoplasmic trafficking [7–9]. Different hnRNPs have distinct nuclear localization signals, which enable them to be imported from the cytoplasm to the nucleus by passing through the nuclear pore complex, and different types of nuclear localization signals are recognized by specific cellular receptors [9,10]. The analysis of a subset of proteins that constitute the nuclear pore complex revealed that three members of this family, Nup153, p62 and Nup98 were degraded by viral protease 2A during the course of poliovirus (PV) infection, causing hnRNPs A1, I and

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heterogeneous nuclear ribonucleoproteins ■ hnRNP ■ PCBP ■ PTB ■ replication ■ RNA ■ splicing ■ translation ■ virus

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K to accumulate in the cytoplasm [9,11]. Other picornaviruses, such as rhinovirus and coxsackievirus, inhibit nuclear import, inducing Nup153 and p62 degradation [8]; by contrast, cardiovirus L protein would interact with and inhibit the activity of the small GTPase Ran, a critical factor for many nuclear import and export pathways [12]. M protein of vesicular stomatitis virus (VSV), a member of the *Rhabdoviridae* family, is involved in the disruption of a variety of nucleocytoplasmic trafficking pathways by associating with Nup98 and relocalizing hnRNP A1, K and C1/C2 to the cytoplasm [13,14]. Cytoplasmic accumulation caused by an enhancement of hnRNP export that would be mediated by the binding of M to the mRNA export factor Rae1 [15,16]. In the case of T cells transfected with a proviral HIV-1 DNA construct, hnRNP A1 shuttles to the cytoplasm and colocalizes with viral RNA; however, it remains predominantly nuclear when murine macrophages are transfected with the proviral construct, indicating that HIV-1-induced hnRNP A1 compartmentalization is cell type-specific. hnRNP A1 relocalization requires the nuclear export of viral RNA via the activity of Rev, which is known to mediate virus RNA export and, accordingly, the blockade of viral RNA export abrogates hnRNP A1 cytoplasmic accumulation [5].

Several members of the hnRNP family interact with viral RNAs and proteins in functional complexes. Here, we revise the most relevant findings about the participation of hnRNPs in different aspects of virus infections.

Role of hnRNPs in viral RNA synthesis

Picornaviridae

The genome of PV, a positive-strand RNA virus prototype of *Picornaviridae* family, contains 5′ and 3′ terminal noncoding regions (NCRs) with extensive RNA secondary structures, which constitute binding sites for cellular and viral proteins to mediate viral genome expression and replication. PV 5′ NCR contains a cruciform RNA structure (termed stem-loop I, or cloverleaf) necessary for viral RNA replication and an internal ribosome entry site (IRES) named stem-loop IV that allows translation of a virus polyprotein further cleaved by virus-encoded proteinases. RNA replication initiates with the synthesis of genome-length minus-strand RNA, which subsequently serves as the template for the synthesis of plus-strand progeny RNA. We discuss below the main findings regarding the role of hnRNP in picornavirus RNA synthesis (summarized in Figure 1).

hnRNP C

hnRNP C is a predominantly nuclear protein and exists as two alternatively spliced isoforms, C1 and C2. They have an RRM and an acidic auxiliary domain that is implicated in protein–protein interactions. An oligomerization domain has also been identified in hnRNP C1 and, within the cell, hnRNP C forms a heterotetramer consisting of one C2 and three C1 molecules [17]. hnRNP C is relocated to the cytoplasm during PV infection, interacting with the 3′ end of the negative-strand RNA through the RRM domain. Using GST pull-down assays, it was proved that the auxiliary domain mediates the hnRNP C interaction with viral proteins, the precursor of viral polymerase 3D and primary precursors to the nonstructural proteins [18]. In order to assess the biological significance of these protein–protein interactions, *in vitro* RNA replication and translation assays in HeLa cytoplasmic extracts were performed. In the presence of a recombinant mutated hnRNP C1 with a truncated auxiliary domain, a 50% reduction in RNA synthesis was obtained compared with reactions supplemented with wild-type protein. Furthermore, depletion of hnRNP C from *in vitro* replication reactions, using short RNAs representing the PV negative-strand 3′ NCR, led to decreased RNA synthesis [18]. More recently, using RNA-binding assays in conjunction with the immunoprecipitation of RNP complexes by hnRNP C antibodies, this research group demonstrated that hnRNP C also binds to the 5′ end of PV minus-strand RNA. In addition, the authors showed that hnRNP C1 multimerization domain is required for PV *in vitro* replication. Moreover, knockdown of hnRNP C1 expression using siRNAs caused a selective reduction in the amounts of plus-strand RNA synthesized determined by quantitative real-time PCR [19]. Accordingly, another study showed a delayed kinetics of PV replication and a decreased synthesis of plus-strand RNA in SK-OV-3 cells, which express low levels of hnRNP C. These findings suggest that hnRNP C may function as part of the replication complex or as an RNA chaperone favoring viral genomic RNA synthesis at late times after infection, when this hnRNP is strongly relocalized to the cytoplasm [20].

PCBP/hnRNP E

PCBP2, also called hnRNP E2 or αCP-2, detected in the cytoplasm and the nucleus of human cells, contains three KH domains (KH refers to the homology domain initially identified in hnRNP K). PCBP2 has a binding

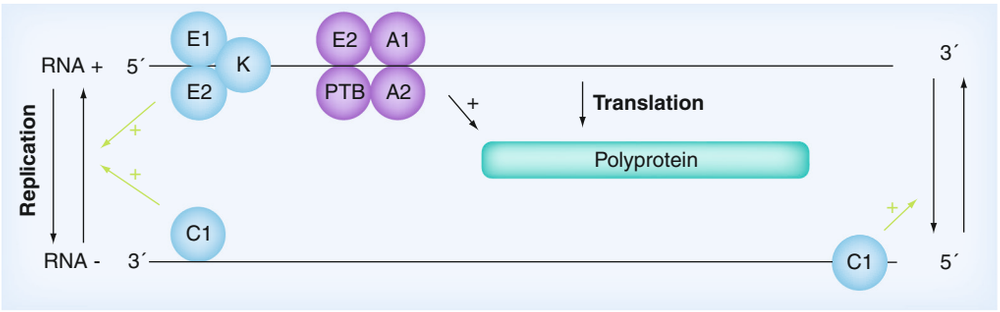

Figure 1. Participation of heterogeneous nuclear ribonucleoproteins in picornavirus RNA synthesis and translation. Picornavirus replication/transcription and translation processes and the interactions between hnRNPs and viral RNA are presented. hnRNPs involved in RNA synthesis are represented as clear circles while hnRNPs implicated in viral translation are represented as dark circles. Arrows with a plus (+) indicate an enhancer effect.

preference for poly(rC), and the first and third KH domains would mediate binding to poly(rC) [21]. PCBP2 forms homodimers and also interacts with other hnRNPs [22,23]. A highly homologous isoform, PCBP1 (also named hnRNP E1 or αCP-1) is 83 and 90% identical at the nucleic acid level and amino acid level, respectively.

PCBP1 and PCBP2 bind to the stem-loop I of PV 5′ NCR and facilitate the interaction of the uncleaved precursor of the protease–polymerase (3CD) with RNA [24]. *In vitro* transcription–replication assays, using a bicistronic PV replicon to discriminate translation from RNA replication, showed that PCBP2 mediates PV RNA translation and replication, while PCBP1, which could not efficiently bind stem-loop IV, mediates only replication [25]. The genomic PV RNA is not only mRNA but also the template for synthesis of negative-strand RNA, and it is unknown how the RNA template is selected either for translation or for RNA synthesis. A model proposed by Walter and colleagues suggests that more PCBP2 (conforming multimers) is required for translation than for replication [25]. Thus, positive-strand RNA would be selected to be translated when sufficient quantities of PCBP2 multimers are available. Afterwards, viral protein accumulation may affect PCBP2 multimerization, allowing RNA synthesis to occur; however, the role of multimers in translation remain to be elucidated.

Experimental evidence supports the idea that protein 3CD would mediate the circularization of PV genomic RNA, either by interacting with PABP1 or by forming homodimers [26,27]. Circularization of the genome would be necessary to approximate stem-loop I RNA and template RNA for the initiation of negative-strand synthesis. Interestingly, PCBP would also play a role in template circularization through its ability to interact with PABP1 [27].

Toyoda *et al.* analyzed the relevance of a C-rich spacer region located between the stem-loop I and the stem-loop IV in PV RNA replication [28]. Genetic analysis by extensive mutagenesis and functional studies revealed that PCBP has little binding affinity for the stem-loop I but requires additional binding to the C-rich region for its function in the synthesis of PV RNA. In line with these studies, the importance of the linker region in Coxsackie B virus negative-strand synthesis was also demonstrated [29].

hnRNP K

hnRNP K, which has been found not only in the nucleus but also in the cytoplasm and mitochondria, contains three KH domains (KH1, KH2 and KH3) and multiple modules that, on one hand, bind kinases, while on the other hand, recruit chromatin, transcription, splicing and translation factors [30]. Through an RNA pull-down assay and proteomics approaches it was shown that hnRNP K binds to the stem-loop I in the 5′ NCR of enterovirus 71 (EV71) [31,32]. The use of siRNA to achieve the silencing of hnRNP K expression rendered significantly reduced viral yields. In addition, a slot-blot assay using a specific RNA probe against either positive- or negative-sense EV71 viral RNA indicated that the synthesis of both strands was delayed in hnRNP K knockdown cells [31].

Flaviviridae

Dengue virus (DENV) and Japanese encephalitis virus (JEV) have a capped positive-strand RNA genome with no poly(A) tail. Both 5′ and 3′ NCRs contain sequences and secondary structures important for the regulation of translation and replication that are highly conserved among flaviviruses. Several hnRNPs (Q, A1, A2/B1, I and H) were found to bind specifically and with high affinity to the DENV 3′ NCR,

suggesting that these molecules may play a biologically significant role in the DENV life cycle [33]. It was also demonstrated that silencing of PTB, also named hnRNP I, which is accumulated within the cytoplasm during DENV infection and colocalizes with NS1 and NS2 viral proteins in the endoplasmic reticulum, inhibits virus replication, while overexpression of PTB results in the activation of DENV replication [34]. On the other hand, *in vitro* binding assays and coimmunoprecipitation analysis revealed a protein–protein interaction between the DENV core protein and hnRNP K [35], and a similar approach was used to analyze the interaction between hnRNP C and NS1, a key glycoprotein involved in the production of infectious virus and the pathogenesis of dengue diseases [36]. Recently, Katoh *et al.* showed that hnRNP A2 is accumulated in the cytoplasm upon infection with JEV, facilitating viral replication by interacting with core and NS5 proteins and with the 5′ NCR of minus-strand JEV RNA [37].

Most experimental evidence about the importance of hnRNPs in flavivirus RNA synthesis was obtained by studying HCV replication (Figure 2). The HCV genome is an uncapped linear ssRNA molecule with positive polarity that contains 5′ and 3′ NCRs flanking an open reading frame encoding a polyprotein, which is processed into structural and nonstructural proteins by host and viral proteases. Four distinct RNA domains in the HCV 5′ NCR have been described; stem-loops 1 and 2 are necessary for RNA replication, whereas stem-loops 2, 3 and 4 are identified as the IRES region. Within the replication initiation complex there is a polymerase (NS5B) that synthesizes minus-strand RNA that serves as a template for the production of the plus-strand viral RNA. Several hnRNPs including hnRNP Q, L, PTB and PCBP were shown to bind the 5′ and/or 3′ end of HCV RNA [38].

PCBP/hnRNP E

PCBP2 binds to the replication region of the 5′ NCR and is associated with HCV nonstructural proteins in detergent-resistant membrane fractions, which are the sites of the HCV replication complex. *In vivo* knockdown of PCBP2 inhibits HCV replication, and blocking of PCBP2 by antibodies reduced HCV RNA replication *in vitro*, indicating that PCBP2 is implicated in HCV RNA replication. Moreover, binding studies and electron microscopy analysis indicate that PCBP2 interacts with 5′ and 3′ NCRs and induces genome circularization, providing advantages for viral replication, such as the coordination of translation and RNA synthesis, proper localization of the viral polymerase and stabilization of viral genome integrity [38]. However, another recent study showed that the RNA level of HCV was unaffected by either the overexpression or absence of PCBP2 in R1b cells [39], thus it is not yet possible to unequivocally attribute a function to PCBP in HCV RNA synthesis.

PTB/hnRNP I

PTB, also known as hnRNP I, interacts with multiple pyrimidine tracts in the HCV genome that are found in the 5′ NCR and within the 3′ NCR [40,41]. Using a subgenomic replicon system, Domitrovich *et al.* found that PTB partially represses RNA replication [42]. Recombinant NS5B was UV cross-linked with a radiolabeled 3′ NCR probe in the presence of PTB and the amount of NS5B bound to RNA was determined. NS5B binding gradually decreased with increasing amounts of PTB, indicating that binding of PTB to these regulatory elements may affect the replicase function. As described

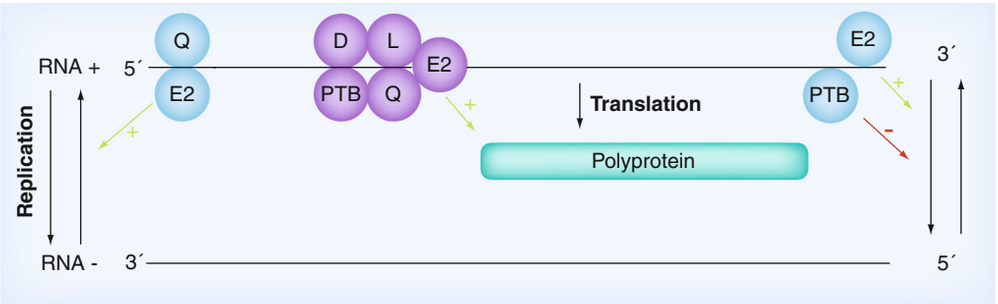

Figure 2. Participation of heterogeneous nuclear ribonucleoproteins in HCV RNA synthesis and translation. HCV replication/transcription and translation processes and the interactions between hnRNPs and viral RNA are presented. hnRNPs involved in RNA synthesis are represented as clear circles, while hnRNPs implied in viral translation are represented as dark circles. Arrows with symbol plus (+) or minus (–) indicate an an enhancer or inhibitory effect, respectively.

below, binding of PTB to 5′ NCR HCV RNA is required for IRES-dependent translation, and it was also demonstrated that PTB interacts with NS5B and NS3 viral proteins. Thus, the authors proposed that the binding of PTB to 3′ NCR may block the initiation of replication from the 3′ end, while binding to 5′ NCR may exert a positive influence on the 5′ IRES-dependent translation. Once a threshold level of viral proteins is synthesized, interaction of these proteins with PTB may affect its ability to enhance translation for subsequent RNA synthesis [42]. By contrast, using siRNAs, Aizaki *et al.* found that silencing of PTB reduced the replication of an HCV RNA replicon [43]. Moreover, in a cell-free replication system, these authors showed that HCV RNA synthesis was inhibited by anti-PTB antibody; hence, the function of PTB in HCV replication should be further studied to better understand these controversial observations.

hnRNP Q

hnRNP Q, also known as SYNCRIP (synaptotagmin-binding, cytoplasmic RNA-interacting protein) or NSAP1, is distributed throughout the cytosol instead of being localized in the nucleus. hnRNP Q binds to RNA *in vitro*, preferentially to poly(A) or poly(U), in a phosphorylation-dependent manner. In HCV-infected cells, hnRNP Q is associated with a detergent-resistant membrane fraction where HCV RNA replication occurs and is colocalized with newly synthesized HCV RNA [44]. A cell-free RNA synthesis assay was performed using hnRNP Q immunodepleted membrane fractions. Treatment with anti-hnRNP Q antibody inhibited the replication activity in a dose-dependent manner [44].

Coronaviridae

Coronavirus genomic RNA is a capped, polyadenylated and positive-strand RNA that contains a sequence termed 'leader RNA' at the 5′ end. The leader sequence is also present at the 5′ ends of all subgenomic (sg) mRNAs and an NCR follows this sequence. There is another NCR at the 3′ end of the genome, followed by a poly(A) chain of variable length. Once viral RNA is released into the cytoplasm, genomic RNA is used to translate proteins of the replicase–transcriptase complex that synthesize a negative-strand RNA, which, in turn, serves as a template for the transcription of multiple sgmRNAs in virus-infected cells. Coronavirus mRNAs share identical 3′ ends but extend for various distances toward the 5′ terminus. The initiation point of each mRNA corresponds to a stretch of consensus sequences, called intergenic sequences (IGs) on the genomic RNA. The sgmRNA synthesis occurs through a discontinuous-transcription mechanism in which the leader RNA is joined to the 5′ end of each mRNA, involving a fusion between the leader and the IG regions, and there is also evidence that 3′ end may interact with the upstream transcription regulatory sequences [45]. The role of hnRNPs in coronavirus RNA synthesis is discussed below (summarized in Figure 3).

PTB/hnRNP I

PTB binds to the mouse hepatitis virus (MHV) plus-strand leader RNA, and deletion analysis allowed the identification of UCUAA pentanucleotide repeats as the PTB-binding site. This sequence interacts with the IG regions where the leader joins sgmRNAs, and the importance of PTB binding in these interactions was assessed using a defective interfering RNA reporter system [46]. Moreover, UV-crosslinking experiments showed that several cellular proteins, including PTB, bind specifically to a 350-nucleotide region on the minus-strand RNA that is complementary to the 3′ of the viral genome. Defective interfering RNAs carrying mutations that interfere with PTB binding exhibited a substantially reduced ability to synthesize sgmRNAs. Interestingly, PTB binding induces changes in the secondary structure of the genomic RNA, which are necessary for transcription activity [47]. Unexpectedly, overexpression of PTB exerts a dominant-negative effect, inhibiting MHV RNA synthesis but not translation. A possible explanation for these results lies in the interaction between PTB and the replicase complex, thus excess of PTB may titrate out one or more components of this complex, resulting in replication/transcription inhibition. It is interesting to note that cytoplasmic relocalization of PTB during MHV infection has not been detected, thus it is probable that PTB cytoplasmic level is sufficient for its function in MHV transcription/replication [48].

PTB interaction with 5′ NCR and IG sequences in the transmissible gastroenteritis coronavirus (TGEV) genome was shown by RNA affinity chromatography and mass spectrometry analysis. In contrast with results reported for MHV, knockdown of PTB expression in Huh7 cells infected with TGEV or transfected with a TGEV-derived replicon caused a significant increase in mRNA levels, indicating that PTB has a negative effect on viral mRNA

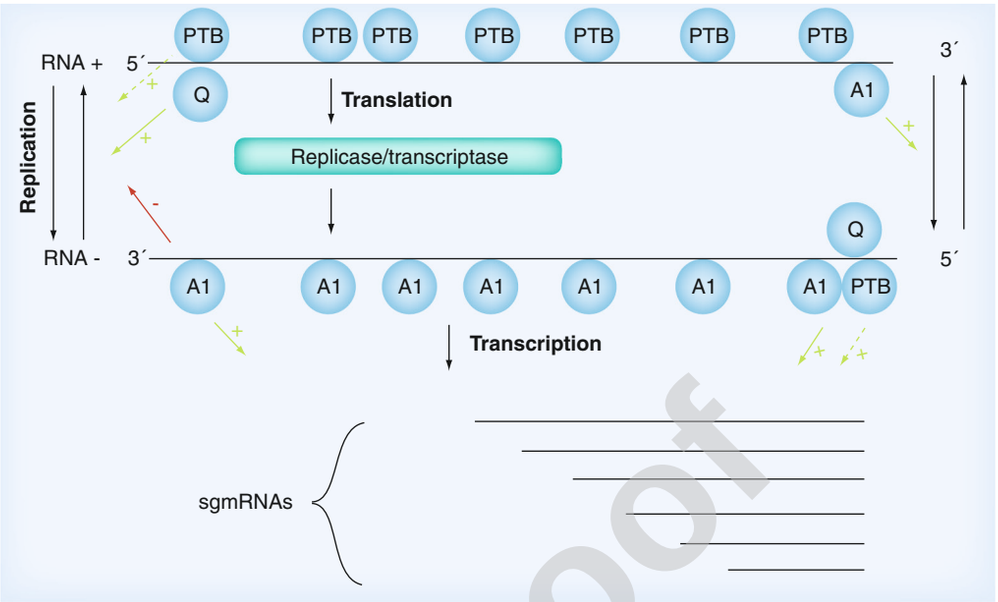

Figure 3. Participation of heterogeneous nuclear ribonucleoproteins in mouse hepatitis virus RNA synthesis. A schematic representation of MHV replication/transcription processes and the interactions between hnRNPs and viral RNA are presented. Arrows with a plus (+) or minus (–) indicate an enhancer or inhibitory effect, respectively. Dotted arrows indicate that the enhancer effect only occurs at low levels of hnRNP expression within the cell cytoplasm.
hnRNP: Heterogeneous nuclear ribonucleoprotein; MHV: Mouse hepatitis virus; sgmRNA: Subgenomic mRNA.

accumulation. Interestingly, during TGEV infection, PTB levels in the cytoplasm progressively increased, thus the early active synthesis of viral sgmRNAs could be associated with low levels of cytoplasmic PTB. Furthermore, confocal microscopy analysis showed that cytoplasmic PTB localized to novel discrete granules, which do not contain components of viral replication–transcription complex. The presence of stress granule markers in these granules suggests that PTB may play a regulatory role in the transition of viral transcription to subsequent stages in the viral cycle [45].

hnRNP A

hnRNPs from the A/B type, such as A1/A1b, A2/B1, B2 and A3, have a highly conserved amino-terminal domain, which contains two tandemly repeated RRMs and a divergent glycine-rich domain at the carboxy terminus implicated in protein–protein interactions [49]. hnRNP A1 binds to the leader and IG sequences of the negative strand and to the 3′ region of the positive strand of MHV RNA [50]. Lai and colleagues found, using northern blot assays, that overexpression of hnRNP A1 enhances the synthesis of genomic and sgmRNAs [51]. In apparent contradiction with this report, another research group stated that MHV is able to replicate in a murine cell line (CB3) that does not express hnRNP A1 [52]. Further studies performed in CB3 cells gave an explanation for these controversial results, as it was proved that other members of the A/B family (A2/B1 and A3) were able to replace hnRNP A1 function in HCV RNA replication [49].

hnRNP Q

The interaction between hnRNP Q and the 5′ NCR of MHV genomic RNA and the 5′ NCR of antigenomic RNA was determined by RNA affinity purification. Binding occurs not only *in vitro* but also in MHV-infected cells, which overexpressed hnRNP Q. Moreover, a dominant-negative mutant of the hnRNP with a C terminus deletion interferes with virus replication without affecting viral translation, indicating the involvement of hnRNP Q in RNA synthesis [53]. Furthermore, RNA affinity protein purification assays showed that hnRNP Q binds 3′ end of the TGEV genome and knockdown of hnRNP Q caused a significant reduction of viral RNA synthesis [54].

Other viruses

The genome of Sindbis virus, a member of the *Togaviridae* family, consists of a single, plus-strand RNA, which is first translated to render nonstructural proteins responsible for RNA synthesis. A minus-strand RNA is synthesized,

which serves as a template for the transcription of two plus-strand RNAs, a genomic (G) RNA and a subgenomic (SG) RNA, which encodes the structural proteins. Interaction of hnRNP K with nonstructural proteins and SG RNA [55], and hnRNP A1 binding to Sindbis virus 5′ NCR [56] were demonstrated. Further analysis revealed that hnRNP A1 associates with G, and SG RNA promoters and enhances the synthesis of G and SG RNAs [57].

Studies performed with VSV constitute the first example of the involvement of PCBP in the gene expression of a negative-strand RNA virus. The VSV genome consists of a negative-sense RNA strand that encodes five structural proteins. Once inside the cytosol, genes encoding L and P viral proteins undergo primary transcription and upon translation of the transcripts, newly synthesized proteins are used for further steps in the infection process, including genome replication and secondary transcription. Recent studies showed that siRNA-mediated silencing of PCBPs promoted VSV replication and, conversely, that overexpression of PCBPs inhibited VSV replication. The interaction of PCBPs with VSV P protein in infected cells was also demonstrated. A detailed analysis of the effect of overexpression of PCBPs on different steps of viral replication indicated that PCBP2 negatively regulates primary transcription and genome replication whereas PCBP1 inhibits only primary transcription [58].

The involvement of hnRNPs in the replication of another negative-strand RNA virus from the *Orthomyxoviridae* family, influenza virus, was poorly studied up to now. A proteomic analysis revealed that hnRNP M and H1 are part of the complexes formed intracellularly by the influenza virus polymerase [59]. It was also demonstrated that depletion of hnRNP F, which interacts with the nonstructural protein NS1, enhanced virus propagation, appearing to act as a negative regulator of influenza virus replication [60].

The role of hnRNPs on HIV-1 RNA synthesis has been also analyzed. After entry into the cell, reverse transcription of the RNA genome to a dsDNA takes place. Afterwards, a DNA copy of the viral genome inserts into the host cell chromosome and the integrated DNA serves as the template for the synthesis of viral RNA. Transcription of the integrated proviral DNA genome, mediated by the viral protein Tat and several transcription factors, generates a single transcript. This viral transcript undergoes a series of splicing events that render 22 different mRNAs of approximately 4 kb and 22 different mRNAs of approximately 2 kb. The unspliced (9 kb) mRNA is packaged within the newly assembled virions as a viral genome.

Overexpression of the hnRNPs A1, A2, A3, H or F caused a marked reduction in the level of viral mRNAs synthesis in human HEK 293 cells cotransfected with a proviral vector and plasmids expressing the hnRNPs. Accordingly, knockdown of these hnRNPs promoted an increase in mRNA synthesis without any effect on their stability [61].

Role of hnRNPs in RNA translation

Picornaviridae

The picornavirus IRES elements have been classified into four distinct groups based on primary sequence, secondary structure and other criteria. IRES-dependent translation requires *trans*-acting factors, collectively known as IRES-specific transacting factors, which allow the binding of translation factors and ribosomal subunits. Several RNA-binding proteins, including hnRNPs (Figure 1), have been identified as IRES-specific transacting factors that stimulate picornavirus IRES translation [62].

PCBP2/hnRNP E2

Picornaviruses containing a type I IRES, such as PV, CBV and human rhinovirus (HRV) require PCBP2 in translation initiation [62]. PCBP1 and PCBP2 contain nearly identical KH domains; however, significant amino acid differences were observed in the linker region between the KH2 and KH3 domains. The addition of recombinant PCBP2 but not PCBP1 can rescue PV IRES-mediated translation in HeLa cytoplasmic extracts depleted of PCBPs [63]. Mobility shift assays revealed that PCBP1/PCBP2 chimeric proteins containing the PCBP2 linker domain were able to bind IRES RNA and promote translation, indicating the relevance of the PCBP2 linker domain in PV translation [64].

PTB/hnRNP I

PTB exhibits a significant stimulatory effect on the activity of HRV [65] and human hepatitis A virus [66] IRES elements, moderately enhances foot-and-mouth-disease virus IRES activity [67] and has little effect on encephalomyocarditis virus IRES-dependent translation [68]. A single PTB molecule binds to IRES, a finding that was confirmed by mass spectrometry of PTB/IRES complexes. PTB would stimulate PV IRES activity by inducing the translation factor eIF4G to bind in the optimal position and orientation to promote internal ribosome entry [69].

Table 1. Role of heterogeneous nuclear ribonucleoproteins in viral infections.

hnRNP	Virus	Function	Ref.
hnRNP A/B	MHV	Required for mRNAs synthesis	[49,51]
	HIV	Negative regulator of RNA synthesis; splicing regulation	[61,80,82,83]
	EV71	Required for RNA translation	[32]
hnRNP A1	HPV	Splicing regulation; involvement in viral persistence	[4,99]
	Sindbis virus	Required for RNA synthesis	[57]
	HRV	Required for RNA translation	[70]
	HIV	Required for RNA translation; regulation of RNA trafficking and assembly	[5,79,84,93]
hnRNP C	PV	Required for plus-strand RNA synthesis	[18–20]
hnRNP D	HCV	Required for viral translation	[77]
	HIV	Regulation of mRNA trafficking	[80]
hnRNP E1 (PCBP1)	PV	Required for RNA replication	[24,25]
	HIV	Negative regulator of protein expression	[81]
hnRNP E2 (PCBP2)	PV	Required for RNA replication and translation	[24,25,63,64]
	Coxsackievirus B	Required for RNA translation	[62]
	HRV	Required for RNA translation	[62]
	HCV	RNA replication (controversial evidence); RNA translation (controversial evidence).	[38,39,71–73]
	Influenza virus	Negative regulator of virus replication	[60]
	VSV	Negative regulation of primary transcription and RNA genome replication	[58]
hnRNP E (PCBP)	MNV	Involvement in pathogenesis	[96]
	Porcine reproductive and respiratory syndrome virus	Modulation of innate immune response	[105]
hnRNP H	HPV	Regulation of late gene expression	[90]
hnRNP I (PTB)	HCV	RNA synthesis (controversial evidence); required for RNA translation	[42,43,66]
	MHV	Required for RNA synthesis, though its overexpression exerts a negative effect	[47]
	TGEV	Suppression of mRNA synthesis	[45]
	HRV	Required for RNA translation	[66]
	Hepatitis A virus	Required for RNA translation	[66]
	Foot-and-mouth disease virus	Required for RNA translation	[67]
	HPV	Regulation of late gene expression	[91]
	MNV	Involvement in pathogenesis	[96]
	PV	Involvement in pathogenesis	[97]
hnRNP F	HIV	Negative regulator of RNA synthesis	[61]
hnRNP H	HIV	Negative regulator of RNA synthesis	[61]
hnRNP K	EV71	RNA replication	[31]
	HSV-1	Required for viral release	[94]
	DENV	Required for viral release	[95]
hnRNP L	HCV	Required for RNA translation	[76]
hnRNP M	HPAI	Involvement in pathogenesis	[98]
hnRNP Q	HCV	Required for RNA synthesis and for RNA translation	[44,74,75]
	MHV	Required for RNA synthesis	[53]
	TGEV	Required for RNA synthesis	[54]

DENV: Dengue virus; EV71: Enterovirus 71; hnRNP: Heterogeneous nuclear ribonucleoprotein; HPAI: Highly pathogenic avian influenza; HRV: Human rhinovirus; MHV: Mouse hepatitis virus; MNV: Murine noravirus; PV: Poliovirus; TGEV: Transmissible gastroenteritis coronavirus; VSV: Vesicular stomatitis virus.

hnRNP A

Interaction between hnRNP A1 and HRV IRES was established by using UV cross-linking assays, and it was also determined that cytoplasmic redistribution of hnRNP A1 after HRV infection leads to enhanced HRV IRES activity [70]. Knockdown of hnRNP A1 or A2 has very little effect on the EV71 IRES activity [56]; however, when both hnRNP A1 and hnRNP A2 were knocked down there was a dramatic reduction in EV71 IRES activity, indicating that hnRNP A1 can be replaced by hnRNP A2 (reviewed in [32]).

Flaviviridae

Several hnRNPs appear to be implicated in the translation of HCV proteins (Figure 2). PTB stimulates translation of HCV RNAs *in vivo* [42,66]. This effect was not observed with PTB mutants lacking RRMs located in the C-terminal third of the molecule, indicating the relevance of these domains in PTB activity [66]. On the other hand, it has previously been shown that PCBP2-depleted rabbit reticulocyte lysate still supports translation from HCV RNA [71], and sequestration of PCBP via ribohomopoly(C) competitor RNA does not affect HCV IRES-dependent translation [72]. However, more recent reports agree that PCBP2 would be required in HCV cap-independent translation [38,73].

hnRNP Q interacts with an adenosine-rich region encoding the N-terminal part of the HCV polyprotein. Overexpression of hnRNP Q augments HCV IRES-dependent translation, whereas knockdown of this hnRNP reduces HCV IRES activity [74]. Recently, Park and colleagues demonstrated that hnRNP Q facilitates HCV IRES-dependent translation by assisting the proper positioning of the 40S ribosomal subunit on the HCV mRNA [75]. This would occur through the interaction(s) between hnRNP Q and 40S ribosomal subunit r-proteins. HCV IRES RNA also binds hnRNP L with high specificity, and the use of *in vitro* selection techniques, called systematic evolution of ligands by exponential enrichment (SELEX), allowed the isolation of RNA aptamers against hnRNP L which specifically interfered with HCV translation in assays performed *in vitro* and in liver cells [76]. Promotion of HCV translation by hnRNP D, which interacts with the stem-loop 2 of 5′ NCR, has been reported. Overexpression of hnRNP D in mammalian cells enhances HCV IRES-dependent translation, whereas knockdown of hnRNP D inhibits this process. In addition, sequestration of hnRNP D with an interacting DNA oligomer inhibits the translation of HCV mRNA in an *in vitro* system, reinforcing its role in the translation process [77].

Retroviridae

The mechanisms used by retroviruses to employ host factors for translational control have been previously reviewed [78]. Here, we summarize the main findings about the role of hnRNPs on HIV-1 protein expression.

Knockdown of hnRNP A1 and A2 in HeLa cells transfected with a bicistronic dual luciferase construct harboring the HIV-1 5′ IRES reduced Gag translation [5]. Accordingly, overexpression of hnRNP A1 increases p24 production in persistently HIV-infected cells (astrocytoma cell line TH4-7-5) used as model of virus reservoir cells [79], reinforcing the requirement of hnRNP A1 in HIV translation.

Depletion of hnRNP D reduced the expression of HIV-1 Env and Gag structural proteins in a cell line stably transduced with a nonreplicative HIV-1 provirus. Further analysis showed that hnRNP D is required for efficient cytoplasmic accumulation of unspliced (9 kb) and spliced (4 kb) mRNAs. Four different isoforms of hnRNP D exist, and the analysis of individual isoforms revealed that two (p37 and p40) inhibited, while the other two (p42 and p45) increased *Gag* expression from the integrated provirus. Hence, the relative abundance of hnRNP D isoforms would modulate cell permissiveness for the replication of HIV-1. In agreement with these results, selective depletion of p45 and p42 isoforms resulted in the loss of HIV-1 structural protein expression [80].

Finally, overexpression of PCBP1, but not of PCBP2, caused a substantial decrease in Gag, Env and Rev production, and two carboxy-terminal KH domains of PCBP1 were required for this inhibitory effect [81]. It is interesting to note that despite their high degree of similarity, PCBP1 and PCBP2 may have distinct, nonredundant roles in modulating HIV-1 gene expression [81].

Role of hnRNPs in the regulation of RNA processing

Retroviridae

Alteration of the balanced splicing of HIV-1 mRNAs dramatically affects viral infectivity. Regulation of splicing is dependent on the presence of intronic and exonic sequences, as well as cellular splicing factors that interact with these elements. Several reports agreed that hnRNP A1 acts as a splicing repressor by binding to multiple splicing silencer sequences [61,80,82,83].

Interestingly, hnRNP A2 and hnRNP A3 differentially modulate viral splicing [80]. Intracellular levels of hnRNP F and H also modulate splicing events thus hnRNPs from the A/B, H and F groups appear to be key host factors for the balanced production of the different viral mRNAs [61].

In addition, the export of unspliced HIV-1 RNA from the nucleus is mediated by hnRNP A1 and synergistically stimulated by Rev [84]. On the contrary, in the case of human T-cell leukemia virus type 1, hnRNP A1 competes with the Rex regulatory viral protein, responsible for the nuclear export of unspliced and incompletely spliced viral mRNAs, inhibiting Rex-mediated unspliced RNA export [85].

Papillomaviridae

HPV, a small virus with a circular double-strand DNA genome, replicates in the nucleus of cutaneous and mucosal epithelial cells. The HPV16 genome has an early region containing six early genes and a late region with two late genes (*L1* and *L2*). The early region and the late region are followed by the proximal early (pAE) and the distal late polyadenylation signals, respectively. During a productive infection, transcription of HPV16 genome yields polycistronic RNAs, which, after alternative splicing, generate mature mRNAs [86]. The HPV life cycle is dependent upon epithelial differentiation. Early proteins are produced throughout the virus life cycle, but expression of capsid proteins, L1 and L2, is limited to terminally differentiated epithelial cells. Viral RNA elements and cellular RNA binding factors (reviewed in [87]) are important players in HPV gene regulation.

Increased levels of the splicing factor SF2/ASF, which binds exonic sequence enhancers, facilitates the extensive splicing of virus late transcripts [86], whereas hnRNP A1 binds exonic sequence silencers to inhibit such splicing [88,89]. A significant increase in nuclear hnRNP A1 expression was detected during differentiation of HPV16-infected epithelial cells, suggesting that increased levels of hnRNP A1 may regulate appropriate alternative splicing necessary for late transcript synthesis [4]. On the other hand, pAE plays a major role in the transition between early and late stages of the viral life cycle. hnRNP H binds to GGG motifs located downstream of the pAE, and mutation within these motifs resulted in a gradual decrease in polyadenylation. A high level of hnRNP H expression in the lower layers of the cervical epithelium and a reduction in hnRNP H production in the superficial layers were detected, suggesting that downregulation of hnRNP H would cause a decrease in early polyadenylation, facilitating late gene expression [90].

PTB also regulates HPV16 splicing by interfering with splicing inhibitory sequences. Moreover, PTB could also activate HPV16 late gene expression in chronically infected cells that contained episomal genomic DNA [91].

Role of hnRNPs in virus assembly & egress

hnRNP A2 exhibits an important role in the trafficking of mRNAs during HIV infection. Two *cis*-acting RNA trafficking sequences (hnRNP A2RE-1 and A2RE-2) have been identified in HIV-1 Vpr and Gag mRNAs, and were found to confer cytoplasmic RNA trafficking. Viral replication was markedly compromised in A2RE-2 mutant viruses, and this correlated with reduced genomic RNA encapsidation levels [92]. Genomic RNA localization at the microtubule-organizing center as a result of hnRNP A2 knockdown had little effect on Gag synthesis, but negatively influenced virus production and infectivity [93].

On the other hand, newly assembled HSV-1 particles acquire a secondary envelope at the trans Golgi network and infectious particles are then released by the exocytic pathway. Downregulation of hnRNP K caused a blockade in the release of virus particles to the extracellular milieu [94].

A similar phenomenon was described for DENV, where dissociation of a complex formed by vimentin, hnRNP K and NS1 viral protein by acrylamide treatment reduced viral release [95].

Future perspective

hnRNPs in viral pathogenesis

New insights in the field of viral pathogenicity can be drawn from studies of hnRNPs and viruses. In the case of murine norovirus (MNV; *Caliciviridae*), viral RNA exhibits three stem-loops and a single-stranded polypyrimidine (p[Y]), tract of variable length between MNV isolates within the terminal stem-loop structure that bind PTB and PCBP. Viruses lacking the p(Y) tract are viable, indicating that this interaction was not essential for virus replication; however, the loss of the p(Y) tract was associated with a fitness cost. Furthermore, a p(Y)-deleted mutant displayed a reduced virulence in the STAT1$^{-/-}$ mouse model, pointing out the importance of RNA–hnRNP interactions in norovirus pathogenesis [96]. Similar findings

have been reported for PV infection. Data suggest that the low levels of PTB available in the CNS, coupled with a reduced binding of PTB to the attenuated Sabin3 IRES, leads to the CNS-specific restriction of this PV strain [97].

On this line, the polymerase protein complex from highly pathogenic avian influenza A viruses that have recently emerged from wild and domestic birds interacts with mammalian hnRNP M. It is thought that adaptation of the viral polymerase to interact with mammalian rather than avian host proteins contributes to disease severity, revealing the importance of these virus–host interactions in the emergence of pathogenic strains [98].

hnRNPs in the modulation of the immune response

It is known that escape of the immune response is a trait exploited by many viruses to persist in their hosts. The HPV16 *L1* coding region suppresses the use of a 3′ splice site by interacting with hnRNP A1, determining the ratio between partially spliced L2/L1 mRNAs and spliced L1 mRNAs, which would affect the ability of the virus to establish a persistent infection remaining undetectable for host immune surveillance. Since HPV 16 infection is a risk factor involved in cervical cancer development, and high-risk HPV types are more likely to persist than low-risk types, variations in the composition of the splicing silencer at the *L1* 3′ splice site in different HPV types may affect the outcome of the infection [99].

IFN expression (types I and III) is a key factor in the onset of innate immune response against viruses. Synthesis of IFN is triggered by different stimuli generated by the infection that involve interaction with cellular receptors, named pattern recognition receptors (PRRs) [100]. Stimulation of these receptors by the corresponding pathogen-associated molecular patterns (PAMPs) activates signaling cascades that lead to an antiviral state characterized by the set up of cellular restriction factors, and establishes the basis for the eventual mounting of the adaptive immune response. Although on many occasions viral proteins are the trigger of PRRs, viral nucleic acids are the main activators for this response. PRR–PAMP interactions are the initial step of different signaling pathways which are driven by different adaptor molecules – for example, RIG-I signaling is directed via the MAVS protein for induction of IFN [101].

Once IFN is produced by the infected cell, it may act in an autocrine and paracrine manner, activating IFN-stimulated genes (ISGs) to prevent or stop viral infection. One of the pathways activated by IFN involves the activation of the STAT complexes, which are imported to the cell nucleus and induce transcription of ISGs [102]. Besides the traditional ISGs reported up to now, a novel antiviral mechanism induced by IFN has recently been described. IFN treatment of HCV infected cells inhibited viral replication and also impaired IRES-dependent translation. This action was mediated by an augmentation in hnRNP M expression, which negatively modulates both processes [103].

On this basis, several reports studied how the interaction of viruses and hnRNPs may affect innate immunity. VP24 from Ebola virus is able to bind to NPI-1 subfamily KPNA nuclear import proteins. Binding of VP24 prevents KPNA interaction with phospho-STAT1, inhibiting its import into the cell nucleus in the form of a complex with hnRNP C1/C2. VP24 also binds to hnRNP C1/C2, which in turn interacts with KPNA, redistributing hnRNP C1/C2 from the nucleus to the cytoplasm, impairing IFN production [104]. Binding of PCBPs to the 3′ NCR of STAT-1 and two mRNAs facilitates the IFN response; thus, reduction of PCBP expression by HCV infection would favor viral multiplication [39]. Similar findings were described for porcine reproductive and respiratory syndrome virus infection where interaction of PCBPs with Nsp1 abolishes the innate immune response. At the same time, PCBPs bind to the 5′ NCR and promote RNA replication without affecting the initial polyprotein synthesis [105]. In a similar fashion, considering that hnRNP A1 is also a key factor in PKR expression induced by IFN [106], the relocalization of this hnRNP to the cytoplasm provoked by enteroviruses [32] may have a negative effect on PKR expression. On this line, IRF2 acts as inhibitor of IFN synthesis and its expression is IRES-dependent and positively modulated by PTB [107]. Also, PTB and hnRNP L destabilize mRNA of the *iNOS* gene induced by IFN [108]. Then, manipulation of PTB levels during TGEV infection of cells [45] or hnRNP L in the case of HCV [76], may alter the response to IFN by modulating IRF2 and/or iNOS expression. Modulation of hnRNP L by viruses may also influence the adaptive immune response by altering the biology of thymic pre-T cells. In accordance with recent findings, hnRNP L would regulate T-cell differentiation and migration [109]. The role of hnRNP L in alternative splicing upon T-cell activation is cooperatively

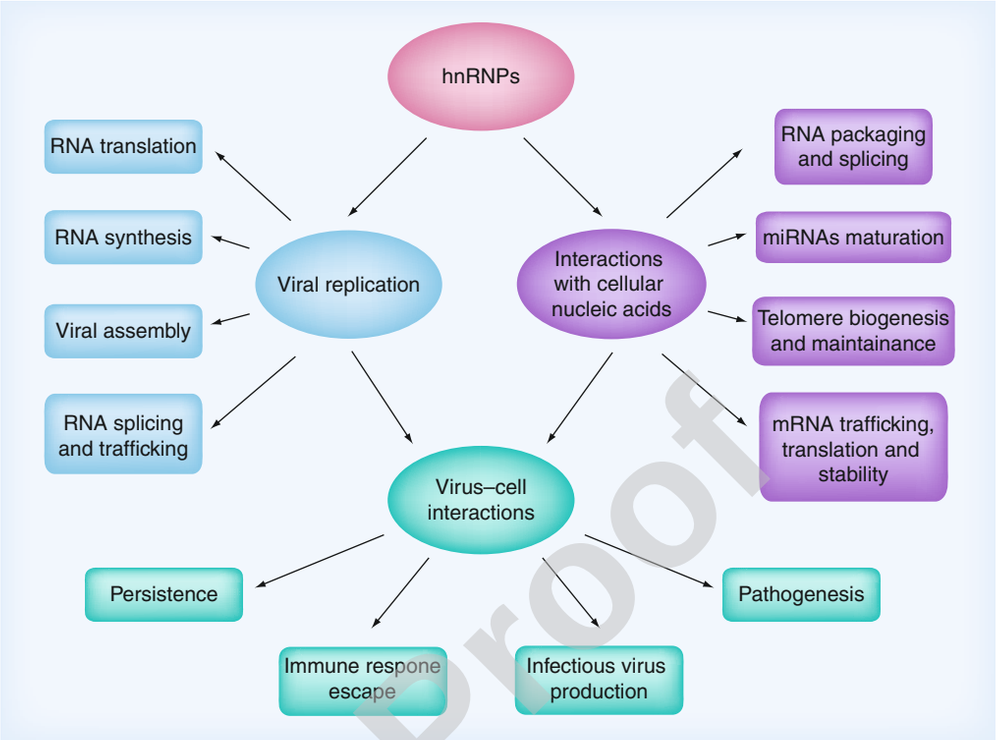

Figure 4. Involvement of heterogeneous nuclear ribonucleoproteins in viral and cellular processes that affect virus–cell interaction. The main functions described for hnRNPs contributing to different aspects of virus–cell interactions are shown.
hnRNP: Heterogeneous nuclear ribonucleoprotein.

complemented by hnRNP K and E2 [110], a fact to be considered in cells infected with viruses that alter expression/localization of these hnRNPs.

miRNAs, small ubiquitously expressed noncoding RNAs, are not only strong regulators of cellular post-transcriptional gene expression, but they also constitute part of the innate immunity against RNA and DNA viruses [111]. Among the novel roles of hnRNPs more recently described, there is evidence that these proteins participate in the generation of miRNAs. hnRNP A1, M4 and H, TDP-43 and FUS (hnRNP P) are components of the miRNA processor complex involved in miRNA maturation [112,113]. The antiviral activity of several miRNAs has been reported for the retroviruses HIV-1 and primate foamy virus [111,114]. Since miRNAs are also involved in cellular differentiation, it may be speculated that cell permissiveness would be regulated by miRNA generation because a virus would be able to replicate in cells where miRNAs are less or not produced. Interestingly, viruses have developed mechanisms to suppress miRNA inhibition and, furthermore, a positive role of miRNAs in the replication of HCV has also been described. On the other hand, virus-encoded miRNAs also regulate viral genome expression [111,114,115]. In the case of the polyomavirus simian virus 40, miRNAs mediate the degradation of the transcript encoding the large T antigen, helping the virus to escape the cytotoxic T-cell response [114]. Thus, changes in the level of expression or intracellular localization of hnRNPs due to viral infection or the cell differentiation process would be key players in the control of viral gene expression.

Conclusion

Interactions among hnRNPs and viral proteins are the basis for the distinctive biological characteristics observed for many virus–host systems, both *in vitro* and *in vivo*. Table 1 summarizes the participation of hnRNPs in viral infections described to date. In this respect, comprehension of the role of hnRNPs in different aspects of the replication of viruses will improve our knowledge about the complex virus–cell interactions that lead to a productive infection and provide the basis for the development of new antiviral approaches. Involvement of hnRNPs in viral and cellular processes that affect virus–cell interaction are represented in Figure 4.

Executive summary

Virus induced changes in heterogeneous nuclear ribonucleoprotein expression

- Heterogeneous nuclear ribonucleoproteins (hnRNPs) are primarily nuclear cellular proteins involved in RNA metabolism – that is, processing, stabilization, localization and translation.
- Viruses change hnRNP intracellular distribution by disruption of the nucleocytoplasmic trafficking mechanisms.

Role of hnRNPs in viral RNA synthesis

- hnRNPs bind to viral genomic and antigenomic RNA, and modulate transcription and replication in plus-strand RNA viruses.
- hnRNP C, K, Q and PCBP favor RNA synthesis of different RNA plus-strand viruses.
- hnRNPs A1, A2/B1, A3, Q and PTB participate in the generation of subgenomic mRNAs of coronavirus by binding 5′ RNA noncoding region and the intergenic sequence at the initiation point of each mRNA.
- Overexpression of certain hnRNPs may exert a negative effect on HIV, HCV and vesicular stomatitis virus RNA synthesis.

Role of hnRNPs in RNA translation

- hnRNP A, PTB and PCPB2 have been identified as IRES-specific transacting factors that stimulate picornavirus internal ribosome entry site translation by allowing the binding of translation factors and ribosomal subunits.
- hnRNP D, L, Q, PTB and PCBP promote translation of HCV RNA by augmenting internal ribosome entry site activity.
- Several hnRNPs enhance HIV translation, whereas PCBP1 causes a substantial reduction in Gag, Env and Rev production.

Role of hnRNPs in the regulation of RNA processing

- Intracellular levels of hnRNPs modulate splicing events in order to achieve a balanced production of the different HIV mRNAs.
- hnRNP A1, H and PTB are involved in the regulation of late transcripts of HPV implicated in the development of persistence in differentiated epithelial cells.

Role of hnRNPs in virus assembly & egress

- hnRNP A2 participates in the HIV RNA trafficking from nucleus to cytoplasm.
- Disruption of hnRNP A–viral protein complexes abolishes dengue virus and HSV-1 release.

Future perspective

- The role of hnRNPs in viral pathogenesis, described for murine norovirus, poliovirus and influenza virus infections, deserves further investigation extending the analysis to other viral infections.
- Better understanding of the relation between virus escape from innate immune response and viral modulation of hnRNP expression or function requires a deeper analysis.

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