Unmasking the information encoded as structural motifs of viral RNA genomes: a potential antiviral target

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ABSTRACT

RNA viruses show enormous capacity to evolve and adapt to new cellular and molecular contexts, a consequence of mutations arising from errors made by viral RNA-dependent RNA polymerase during replication. Sequence variation must occur, however, without compromising functions essential for the completion of the viral cycle. RNA viruses are safeguarded in this respect by their genome carrying conserved information that does not code only for proteins, but also for the formation of structurally conserved RNA domains that directly perform these critical functions. Functional RNA domains can interact with other regions of the viral genome and/or proteins to direct viral translation, replication and encapsidation. They are therefore potential targets for novel therapeutic strategies. This review summarizes our knowledge of the functional RNA domains of human RNA viruses, and examines the achievements made in the design of antiviral compounds that interfere with their folding, and therefore their function.

ABBREVIATIONS

AFM, atomic force microscopy.

CRE, *cis*-acting replicating element.

DENV, Dengue virus.

EMCV, encephalomyocardhitis virus.

FMDV, foot and mouth disease virus.

FRD, functional RNA domain.

GORS, genome-scale ordered RNA structures.

IF, initiation factor.

hCoV, human coronavirus.

HV, highly variable region.

HVR, hypervariable region.

IBV, infectious bronchitis virus.

IGR, intergenic region.

IRES, internal ribosome entry site.

ITAF, initiator *trans*-acting factor.

JEV, Japanese encephalitis virus.

PABP, polyA binding protein.

PCBP, poly-rC binding protein.

PK, pseudoknot.

PTB, polypyrimidine-tract binding protein.

SELEX, systematic evolution of ligands by exponential enrichment.

sp, specie.

UTR, untranslatable region.

YFV, yellow fever virus.

INTRODUCTION

The appearance of viral mutants resistant to antiviral compounds is a major public health concern. The continuous generation of such mutants is a characteristic of RNA viruses, many of which, e.g., hepatitis C virus (HCV), human immunodeficiency virus (HIV), and dengue virus (DENV) (all with a single–stranded, positive RNA genome) cause serious disease. The genome of RNA viruses acts as both the template for replication and as mRNA. During replication, mutations are introduced into the genome via errors in the action of viral RNA-dependent RNA polymerase; this can generate mutants that may greatly contribute to viral proliferation – although it can also lead to the disappearance of the virus if such changes leave it non-viable or induce acute pathogenesis in the host, the so-called error catastrophe (for reviews, see [1,2]). Viruses must, therefore, strive to reach an equilibrium that allows for adaptive fitness without compromising their persistence. To accomplish this, RNA viruses have developed an information storage system that does not code for proteins, but for specific, complex and highly conserved, folded genomic regions that play direct, functional roles in the viral cycle. These viral regions present an adaptive behaviour that has evolved with the selective pressure to achieve high genetic robustness [3-5]. As a result, mutations in viral RNA genomes present minor incidence in the functional elements [4].

RNA molecules show two levels of compaction: their secondary and their tertiary structures. The secondary structure is the result of double and single-stranded regions arrangements, e. g., helices closed by loops. The tertiary structure is determined by the relationships established between secondary structure elements. The combination of these two folding levels establishes the

final shape of the RNA, the so-called RNA structurome [6]. This conformational view of RNA molecules has prompted the search of structured RNA motifs as functional RNA domains (FRDs), not only in the viral genomes [7], but also in the transcriptome of all living organisms (for a review, see [6]).

A main feature of FRDs included in viral genomes is their high genetic robustness, likely derived from their essentiality for the consecution of the infective cycle [3-5]. FRDs are *cis*-acting elements typically involved in RNA-RNA and RNA-protein interactions, required for the correct execution and regulation of essential viral processes [8-10]. One of the FRDs that has attracted most attention is the internal ribosome entry site (IRES). IRES regions direct the recruitment of the cellular translation machinery in a cap-independent manner [11]. Similarly, viral replication and maturation are regulated by the direct binding of viral polymerases and related cofactors to FRDs in the 3' end of the genomic RNA, as well as by direct RNA-RNA interactions between distant regions of the virus genome [12].

The functional importance of structural elements in the viral cycle and their high conservation rate [8,9,13-15], suggests they might make good therapeutic targets, and the idea of developing novel antiviral tools (mostly RNA-based) to attack them has gained currency in recent years. This review briefly summarizes what is known about the FRDs of a number of viruses, paying special attention to those infecting humans, and recapitulates the state of the art in the development of therapeutic compounds that might interfere with FRD folding and, therefore, function.

VIRAL IRESs: RNA TAGS FOR EFFICIENT TRANSLATION

Many viral genomes achieve the efficient translation initiation by the use of IRESs, which replace initiation factors (IFs) by the combined use of several FRDs. These regions present high sequence and structural diversity across different viral groups, which define the mechanisms by which they promote translation. IRESs are therefore divided into four groups [16] according to common IF requirements (Figure 1):

- *Packed IRESs*. This group encompasses the IRES elements located in the intergenic region (IGR) of the genome of the *Dicistroviridae* family (whose members infect insects). The folding of these elements represents a highly conserved, compact structure that, via the so-called "all RNA-based" mechanism, mediates the recruitment of the ribosome with no need of additional protein factors [17]. The IRES structure can be split into three domains (Figure 1a), each defined by a pseudoknot motif (PKI-PKIII). This conformation is considered the "prefolded" isoform of the IRES. Domain PKIII mimics the folding of the initiator tRNA allowing it to occupy the P and E sites of the 40S ribosomal subunit. This promotes structural changes in the IRES that finally allow the binding of the 60S particle and the efficient initiation of translation from a non-AUG codon [17-22]. Thus, the conformation of PKIII replaces the need for an initiator tRNA [23].

- *Extended IRES with compact regions*. The prototype of this group, the HCV IRES, folds as an extended region with tightly compact domains. It is mostly contained in the 5'UTR of the RNA genome but also spans a short stretch of the coding sequence (Figure 1b) [24,25]. It can be divided into two major domains

6

(II and III) [26] and a short stem-loop containing the start codon (domain IV) [27]. The extended folding of the IRES allows for the proper coaxial alignment of domains II and III on either side of a complex pseudoknot structure [28]. This allows easy access for the 40S ribosomal particle; subsequent IRES conformational changes directly position the appropriate start codon in the P site [29]. Then, binding of eIF3 at the IIIabc junction aids the incorporation of the ternary complex eIF2-tRNA$_i^{Met}$ and the joining of the 60S subunit (for a review, see [30]). Interestingly, the requirement for eIF2-tRNA$_i^{Met}$ can be minimized under certain stress conditions [31], providing a selective advantage for efficient HCV propagation even in unfavourable physiological environments. This suggests that an IRES may change its mode of function depending on environmental conditions.

- *Flexible IRESs*. These are mainly represented by picornavirus type II IRESs (FMDV and encephalomyocarditis virus [EMCV] among others; Figure 1c) [9]. Their overall 3D structure reflects a greater degree of flexibility than that shown by IGR or HCV-like IRESs before ribosomal recruitment [32-35]. Nonetheless, they retain compact self-folded stem-loops and a GNRA sequence motif included within a tetraloop that directs the structural organization of central domain 3 [32]. Flexible IRESs cannot directly recruit the 40S ribosomal subunit; rather, they first recruit eIF4G, eIF4A, eIF4B and eIF3 at domains 4-5 [36,37] to finally induce conformational rearrangements that promote the recruitment of the 43S complex. The presence of domains 2 and 3 is crucial since they bind additional initiator *trans*-acting factors (ITAFs), such as polypyrimidine-tract binding protein (PTB), polyA binding protein (PABP) and ITAF45 [38-41]. ITAFs are thought to provide chaperone activity but may also help overcome cell

7

restrictions under stress conditions. Hence, the flexible folding provides specific biophysical properties that define a characteristic mode of action.

- *Inducible IRES*. In lentiviruses, the use of IRES elements is restricted to certain environmental conditions, the initiation of protein synthesis normally being accomplished by a cap-dependent mechanism. Two different IRES elements lie within the HIV genome: one in the 5'UTR, which only operates in the G2/M phase of the cell cycle [42,43], and one embedded within the coding sequence (Figure 1d) [42,44]. Enormous efforts have gone into fathoming the induction mechanism(s) of these elements, but without much success. It is well known that the 5'UTR of HIV-1 RNA can adopt two different conformations, but a direct translational regulation mediated by conformational exchanges in the genomic RNA has been ruled out [45]. Further, it seems likely that unknown ITAFs are differentially loaded by each conformer, providing a sophisticated and opportunistic viral strategy aimed at overcoming environmental difficulties. It is tempting to propose that the induction of the IRES within the coding sequence is further modulated by long distance RNA-RNA interactions with the 5'UTR, thus promoting alternative conformations that might regulate its functioning [46].

FUNCTIONAL DOMAINS LOCATED AT THE 3'UTR

FRDs are needed in the 3' end of viral genomes to promote the efficient binding of the polymerase and the regulation of the elongation step during RNA synthesis [7,12]. They are also important partners in the preservation of viral genomic stability and the control of processes mediated by the 5' end [47]. The latter is mediated by the establishment of a dynamic cross-talk between both termini of the genomic RNA. The sequence and 3D structure of the 3' *cis*-acting

domains are quite diverse, even among members of the same taxon (Figure 2), which must entail substantial differences in the mechanism of action if the same function is to be achieved. Such great complexity and variability precludes a detailed discussion of all of the 3' RNA structures present in different RNA viruses; only those most extensively studied are therefore mentioned below.

3'UTRs in the *Picornaviridae*

- Enterovirus sp.:

The genus *Enterovirus* includes human polioviruses, rhinoviruses and the human enteroviruses of groups B, C and D (which include different human coxsackieviruses). Their genomic RNA ends with the so-called *ori*R region, which is composed of a short, highly folded 3'UTR (75-100 nucleotides) capped by a polyA tail [48,49]. The *ori*R has been shown to play an important role in viral replication via its direct recruitment of viral RNA-dependent RNA polymerase and host cell factors such as PCBP (poly-rC binding protein), the La autoantigen and eEF1A [15]. Common architecture at the 3'UTR can be inferred for all the members of a same viral group (Figure 2a). Thus, rhinoviruses have a single stem-loop (named Y), polioviruses contain two stem-loops (X and Y), and coxsackieviruses have three consecutive stem-loops (X, Y and Z) [48]. The presence of two or more stem-loops defines a typical pseudoknot motif (PK) involving the apical loops of domains X and Y [50,51]. This pseudoknot may regulate dynamic spatial rearrangements in domains X and Y that fine-tune viral RNA synthesis [52,53]. Despite sequence diversity, enterovirus 3'UTR represents a good example of how RNA genome structure has diversified yet retains common essential functions.

- Aphtoviruses:

FMDV is the prototype of the aphtovirus group. Its genome contains an essential *ori*R region at the 3' end consisting of a well-defined 3'UTR of around 90 nucleotides, plus a genetically encoded polyA tract (Figure 2b). The 3'UTR adopts a quasi-globular conformation defined by two stem-loops, SL1 and SL2. It was recently shown that, while the deletion of SL2 completely interferes with virus viability, mutants defective for SL1 only show reduced replication capacity [54]. The *ori*R region in aphtoviruses is also involved in translational control by the establishment of molecular bridges with the IRES element at the 5' end of the viral genome [55].

cis-Acting elements within the 3' end of genomic *Flaviviridae* RNA

- *Hepacivirus* sp.:

The 3'UTR of the HCV genome is an evolutionarily conserved structural element of around 200 nucleotides with three different domains (Figure 2c): the highly variable region (HV), a polyU/UC tract of variable length and composition, and the 3'X tail consisting of three stem-loops (3'SLI-3'SLIII) [56,57]. Both the polyU/UC tract and the 3'X tail contain elements important in replication and infectivity in cell culture and a chimpanzee model [58-64]. The 3'UTR in hepaciviruses operates by the recruitment of viral proteins, such as RNA-dependent RNA polymerase and helicase [65], as well as numerous host factors [66-70]. Moreover, the formation of high order structures involving RNA motifs in the 3' end of the coding sequence provide additional regulation of 3'UTR folding and activity [71,72]. Together, the above elements constitute the complete functional scaffold required to achieve precise RNA replication.

- *Flavivirus* sp.:

This genus includes important human pathogens, such as DENV, yellow fever virus (YFV) and Japanese encephalitis virus (JEV). Their positive RNA genome contains a 3'UTR ranging from 350-700 nucleotides in length. Despite this heterogeneity in size, common secondary structure elements are found at the very 3' end of the genomic RNA, defining the so-called domain III, which is indispensable for replication [73] (Figure 2d). Domain III is composed of a short hairpin followed by a long and stable stem-loop (3'SL). It has been shown that the formation of a pseudoknot motif involving the apical loop of the short hairpin and an internal loop of the 3'SL is critical for the recognition of viral and cellular proteins during the initiation of RNA synthesis [74].

3' terminal domains in coronaviruses

The genome of the *Coronaviridae* family encodes for 5-7 overlapping, capped and polyadenylated subgenomic mRNAs [75]. The 3'UTR contains two highly conserved structural motifs: an essential bulge stem-loop (BSL) immediately followed by a hairpin (Figure 2e) [76,77]. Under certain conditions, this folding may be displaced towards the formation of a pseudoknot motif between the base of the first domain and the apical loop of the hairpin [78]. This leads to a conformational equilibrium that must be preserved for RNA replication to occur. At the 3' position of the pseudoknot, a hypervariable region (HVR) is found. It is highly divergent both in terms of primary and secondary structure, with the exception of the 5'-GGAAGAGG-3' motif. This octanucleotide is situated 70 to 80 nts from the 3′ end of all coronaviruses genomes, thus being considered a characteristic signature of these pathogenic agents [75]. Such stringent

conservation implies a critical, yet unknown function, in the coronaviruses biology [79].

A long list of viral and cellular proteins also bind the 3'UTR of different coronavirus and affect replication [75]. These include viral RNA polymerase, non-structural virus proteins, nuclear factors, and proteins involved in the translation process. Thus, once again, RNA-RNA and RNA-protein interactions provide a complex regulatory network that controls viral infection.

CIS-ACTING RNA ELEMENTS WITHIN THE CODING SEQUENCE

Recent work has shown a great variety of *cis*-acting RNA motifs embedded throughout the coding sequence [80-86]. These operate in a coordinated fashion with other functional regions and with viral and host proteins, providing complex control systems for replication.

Some of the best characterized *cis*-acting replicating elements (CREs) are found in the picornavirus genome [87-89]. All of them contain one or more short hairpins responsible for efficient priming during RNA synthesis [90]. Two adenine residues placed in an apical loop of one of these hairpins function as template for the uridylylation of the essential viral protein VPg, involved in picornaviral replication [90]. This mechanism has been shown to contribute to the overcoming of the rate-limiting effects of low nucleotide concentrations [91], thus providing a robust system for the initiation of replication.

A CRE element was identified within the 3' end of the coding sequence of the HCV genome [81]. It is a highly conserved structure composed of three stem-loops (5BSL3.1 to 5BSL3.3) (Figure 3a) [92], which was shown to be required for viral replication [64,93]. The establishment of long range RNA-RNA interactions between 5BSL3.2 and the 3'SLII within the 3'UTR [71,94-96], and

12

with the SL9110 hairpin motif in the coding sequence [72,95,96], is critical for the proper functioning of this region during viral RNA synthesis (Figure 3a) [71,72]. Importantly, the existence of an additional, direct, long-range RNA-RNA interaction involving the subdomain IIId of the IRES and the essential stem-loop 5BSL3.2 of the CRE region has important consequences for the regulation of HCV IRES function (Figure 3a) [96-98].

5'-3' COMMUNICATION IN VIRAL GENOMES

End-to-end communication in viral genomes is directly responsible for promoting and regulating different steps of the infectious cycle. Via the establishment of long distance contacts, differentially evolved functional regions at both ends of viral genomes can act in a coordinated fashion to generate molecular switch control systems between different steps of the viral cycle. Such cross-talk seems to provide important benefits, such as an increase in the local concentration of essential proteins and cofactors, and additional protection against the action of exonucleases. Genome circularisation provides new proof of the great versatility shown by compact viral RNA genomes.

The formation of the circular topology in picornaviruses is dependent on direct RNA contacts between the IRES and the 3'UTR (Figure 3b) [55]. Further, the RNA binding capacity of different factors, such as eIF4G to the IRES region, or PABP (polyA binding protein) to the 3' polyA tail, ensures the stability of the closed-loop conformation.

In hepacivirus genomes, circularisation was initially proposed to be mediated by protein factors alone [47], which would simultaneously bind to both ends of the viral genome. More recently, a long range contact between the IRES and the

CRE element was described to induce significant conformational changes in precise domains of the IRES (Figure 3a) [97,99], resulting in the regulation of IRES function [98]. Undoubtedly, these results do not exclude the participation of viral and cellular proteins in the stabilization of circular topology, nor the performance of important functional roles [100-103].

In other viruses, such as *Flavivirus* sp., the formation of the circular topology does not require the presence of proteins but the use of canonical and complementary sequences (CS, cyclization sequence; UAR, upstream AUG region; and DAR, downstream AUG region) at either end of the viral genome [104-106] (Figure 3c). These motifs achieve the association of the genomic termini by direct base-pairing. The closed-loop topology is essential for viral propagation and could be very advantageous for the regulation of viral translation and RNA synthesis, as well for the control of the switch between different steps of the viral cycle.

VIRAL RNA STRUCTURES AS NOVEL TARGETS FOR THERAPEUTIC AGENTS

Complex viral evolutionary dynamics are a great obstacle in the development of efficient therapeutic tools. The constant appearance of sequence mutants resistant to current treatments has prompted the search for alternative targets. Structurally conserved regions involved in the progression of the viral cycle are excellent candidates. The combining of different compounds targeting multiple viral regions and processes might contribute greatly to reducing the appearance of resistant variants. The following section summarizes the main advances

recently made in the development of molecules directed against different functional domains of viral RNA genomes.

Nucleic acids-based drugs

Nucleic acids, particularly RNA, are excellent candidates for the development of antiviral agents targeting functional regions of viral genomes. The success of these strategies relies on sequence complementarity and/or structural recognition between the inhibitor and the target. Therefore, highly conserved genomic domains are the most promising regions for targeting new nucleic acids-based antiviral compounds. Importantly, a number of aspects need to be taken into account in pursuing this goal [107], such as the cellular uptake of the inhibitor, target and inhibitor colocalization, the active inhibitor concentration inside the cell, the intracellular inhibitor stability and the access and binding to the target region. In recent years, great progress has been made in the field of nucleic acid treatment and some of the above problems have been now successfully solved. This has led to the design of new generations of antiviral RNA and DNA drugs that are currently being extensively developed [108].

- *Antisense oligonucleotides*:

  Antisense oligonucleotides are short nucleic acid molecules whose sequence is complementary to an existing motif in a target RNA. The molecular mechanisms of action of antisense oligonucleotides are diverse (Figure 4a) [109]. They act by hiding essential sites in the viral genomic RNA, such as those required for protein synthesis, replication and packaging; or if DNA-based, by inducing target degradation mediated by RNase H [108].

Antisense oligonucleotides can be administered either directly, by subcutaneous injection, or as pharmacological compositions. In this context, great advances in the combinatorial chemistry have allowed for the development of oligonucleotides with chemical substitutions at precise positions [108]. These modifications confer important features, such as significant half-life increase in serum and higher thermal stability of the duplex [110]. This strategy was followed by McHutchinson *et al*. for the design of an anti-HCV compound [111], but with low success in Phase I clinical trials.

A different administration strategy is based on the intracellular production of unmodified antisense oligonucleotides. It requires gene therapy protocols to deliver the construct inside the cell. Its main advantage is that chimeric constructs encompassing different antisense sequences (or even different nucleic-acid based inhibitors) can be produced, thus targeting multiple sites simultaneously. The use of this strategy may greatly contribute to diminish the appearance of viral resistant variants. This method has been successfully used for the inhibition of the HIV-1 replication [112-114].

- *Small interfering RNAs (siRNAs)*:

The cellular RNAi pathway can be triggered by synthetic double-stranded small interfering RNA molecules (siRNAs). These compounds are usually 21 nucleotides long, with a two-nucleotide 3' overhang. The siRNAs are loaded into the RISC (RNA-induced silencing complex), where the sense, guide strand is selected to target the complementary sequence within the genomic viral RNA for further degradation (Figure 4a) [115].

To date, numerous studies have been conducted using siRNAs as antiviral drugs [115]. Importantly, previous advances made for the therapeutic use of

16

antisense oligonucleotides have served as starting point for the development of this new strategy. Thus, the incorporation of stabilizing chemical modifications in the passenger (antisense) strand may greatly increase the potency of the siRNA without toxicity [116].

The application of gene therapy strategies to promote long-term decrease of viral loads has been also studied for the siRNA technology. One of the main drawbacks of this alternative is the appearance of escape mutants [117]. To overcome this problem, several strategies have been developed based in the generation of constructs combining multiple siRNAs targeting different regions of the viral genomes [118].

The great potential of siRNA-based therapeutics has prompted the development of novel internalization strategies that should allow for a better sustained antiviral response. Important contributions have been made in the field for the HCV infection. For example, the use of nanosomes (lipidic nanoparticles) achieves the encapsulation of multiple siRNAs with a 100% of delivery yield in a liver tumor-xenotransplant mouse model of HCV [119]. Alternatively, multilayered polyelectrolyte films (MPFs) have been also shown to be an efficient way of nucleic acids intracellular delivery, providing dose-dependent, specific, and long-term inhibition of HCV replication in hepatocyte-derived cells [120].

- Aptamers:

Aptamers are nucleic acids that bind to a specific ligand with high affinity. They are isolated by SELEX (systematic evolution of ligands by exponential enrichment), an *in vitro* selection strategy [121,122]. This process consists of iterative series of the synthesis, binding, positive selection and amplification of a

randomised oligonucleotide pool to yield a population enriched in those molecules that can bind to a desired target molecule (Figure 4b). This strategy has identified RNA and DNA molecules of potential therapeutic use in a great variety of diseases, including many viral infections [123]. In addition, aptamers offer useful information about the 3D architecture of the target molecule. They are therefore a valuable alternative to complementary oligonucleotides (antisense and siRNAs). An aptamer's mode of action is dependent on its folding and mediated by direct loop-loop interactions (Figure 4b), which block the function of the RNA target domain, either by interfering with the recruitment of essential factors, or by affecting the folding of the surrounding area and thus impeding RNA-RNA interactions, or both. This may contribute to diminish the chance of appearance of resistant viral mutants.

Among the preferred viral targets, the most extensively investigated has been the 5'UTR, including that of the genomic RNA of HIV [124-129] and HCV [130-133]. Other functional RNA regions have been used as targets for aptamers, such as the 3'UTR [130] and the CRE region of HCV [134]. These inhibitors have returned promising results both in cell culture and *in vitro*, and have been used to produce antiviral compounds efficient in the nanomolar range [135,136]. Further, this research has served as a starting point for the development of novel strategies combining two or more inhibitor RNA molecules [137-142].

The emergence of SELEX methodologies has provided novel therapeutic and diagnostic agents with improved pharmacokinetic and pharmacodynamic properties and low immunogenicity. These compounds are already being investigated by several companies for possible clinical use (reviewed in [143]). The conjugation of the 5' end of the aptamer sequence with stabilizing agents

such as polyethylene glycol (PEG) has been shown very effective in pharmacological formulations [144]. The additional incorporation of ligands for specific cell surface receptors helps in the efficient and precise delivery of agents to target cell types or tissues. Given the great progress being made in this field, aptamers may be in widespread use as antiviral agents in the future.

Antibiotics against RNA functional domains

The use of antibiotics for targeting RNA structural elements was firstly described for the aminoglycoside paramomycin. This compound recognizes the aminoacyl A site of the bacterial ribosome by its interaction with an enlarged deep grove defined by two bulging and one unpaired adenine residues [145,146]. This 3D conformation was shown to be strongly similar to that acquired by the HIV-1 DIS (dimerization initiation site) kissing–loop complex [147]. Further modelling and crystal structure resolution provided strong basis for the development of modified, high affinity antivirals derived from aminoglycosides [148]. Even more, these studies have recently provided a more accurate view of the folding of the HIV DIS region [149]. These results will greatly prompt the design of small, improved ligands able to interfere with the folding of the essential DIS domain.

Branched peptides targeting the TAR element of HIV

Branched peptides are a common therapeutic tool used in the development of synthetic peptide vaccines and drug delivery vehicles [150]. Their usefulness as efficient ligands of RNA molecules is associated with the latter carrying so-called multivalent targets, i.e., functional RNA domains with multiple interacting sites. Additionally, amino acids may carry a radical of specific molecular architecture that increases the target selectivity. These molecules have been

19

isolated from random peptide libraries to generate efficient, non-toxic and cell-permeable antiviral compounds that target the HIV TAR element [151].

Benzimidazole derivatives

The benzimidazole nucleus is the main constituent of many therapeutic drugs, all of them based on the combination of heterocyclic compounds to yield isosters of naturally occurring nucleotides. The chemical composition of these isosters makes benzimidazole derivatives excellent candidates for interaction with natural biopolymers. One of these derivatives, ISIS-11, has attracted much attention in recent years as a potential anti-HCV molecule. This compound specifically interacts with the basal portion of the IRES domain II, the so-called domain IIa [152,153]. During the recruitment of the 40S ribosomal particle, domain II adopts an L-shape that directs the apical hairpin loop IIb toward the 40S subunit E site, thus inducing a conformational shift that promotes the positioning of the start codon close to the active site. In the presence of ISIS-11, domain IIa acquires the shape of an open helix that alters the proper folding of domain II, thus impeding the correct positioning of the viral RNA in the catalytic binding cleft. These findings confirm the important principle of interfering with RNA folding to achieve an inhibitory effect.

CONCLUDING REMARKS

The compactness of RNA viral genomes provides important advantages in terms of propagation efficiency. Such compactness, however, requires the intervention of a supracoding system (i.e., information in the genome beyond that of the nucleotide sequence) to direct the folding required; without such a supracoding system, no such compactness would exist and essential functions

of the genome in transcription, replication and encapsidation associated with this folding would be lost. Structurally conserved functional RNA elements interact with protein factors and other RNA domains to direct and regulate these functions as well as switching between different steps of the viral cycle. Interfering with the functioning of these structural domains offers a potential means of treating viral infections. The great advances made in the field of chemical synthesis and nucleic acid production has prompted novel initiatives involving small molecules that can alter the 3D conformation of viral genomic RNA domains. This might be used to complement classical antiviral therapies, such as the use of neutralizing antibodies or interferon treatment. The combination of clinical strategies is currently under extensive investigation with the aim of improving therapeutic responses and long-term outcomes while minimizing toxicity and secondary effects.

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FIGURE LEGENDS

Figure 1. Diagram showing the secondary structure of different types of viral IRESs. a) *Dicistroviridae* IGR (intergenic region) IRES. b) HCV, with the minimum IRES region boxed. c) FMDV IRES. d) HIV-1 IRES elements. Functional domains are shadowed or indicated as noted. Start translation codons are marked by arrows. PK, pseudoknots.

Figure 2. Diagram showing the structural diversity of the 3'UTRs of different viruses. a) Several examples of 3'UTRs of enterovirus, b) aphtoviruses, c) HCV, d) *Flavivirus* sp., and e) the *Coronaviridae* family. Proposed pseudoknot structures (PK) are indicated with a line. Structural motifs defining functional RNA elements are noted. Stop codons are marked by an arrow. BSL, bulge stem-loop; HV, highly variable region; HVR, hypervariable region; N, any nucleotide; R, purine.

Figure 3. Circularisation mechanisms in different viral genomic RNA molecules. Detailed diagrams of the secondary structure proposed for the 5' and 3' ends, as well as for the described CREs, are shown. a) HCV. The region required for IRES activity is noted. The 3' end of the viral genomic RNA is organized into two structural elements: the CRE region and the 3'UTR. b) FMDV RNA genome with the VPg viral protein bound to its 5' end. The IRES region is indicated. Binding sites for eIF4G and polyA binding protein (PABP) are shadowed. The circular form is stabilized by protein-protein (eIF4G and PABP, among others) and RNA-RNA interactions. c) *Flavivirus* genome capped at its 5' end (indicated by m7G) to ensure viral protein synthesis. The locations of the complementary sequences CS, UAR and DAR are marked by grey solid lines. The black solid

lines indicate long-distance interactions (direct RNA-RNA and protein-protein contacts). Arrows denote the position of the start and stop translation codons. Pseudoknots (PK) are noted as described in Figure 1.

Figure 4. Nucleic acid-based antiviral compounds. a) Mechanism of action for antisense oligonucleotides and siRNAs. RNase H mediates the cleavage of the heteroduplex antisense deoxyoligonucleotide:target RNA. For non-DNA oligomers, inhibition is achieved by directing the oligonucleotides against functional regions of the RNA, such as those involved in the recruitment of the translational or replicative machinery. siRNAs bind to the macromolecular complex RISC, which selects the guide strand that recognizes the target sequence in the RNA of interest. This promotes the RNA degradation by specific endonucleases. b) Diagram of a SELEX process to identify RNA aptamers targeting a specific RNA domain. Suppression of transcription and reverse transcription allows for the isolation of active DNA aptamers.
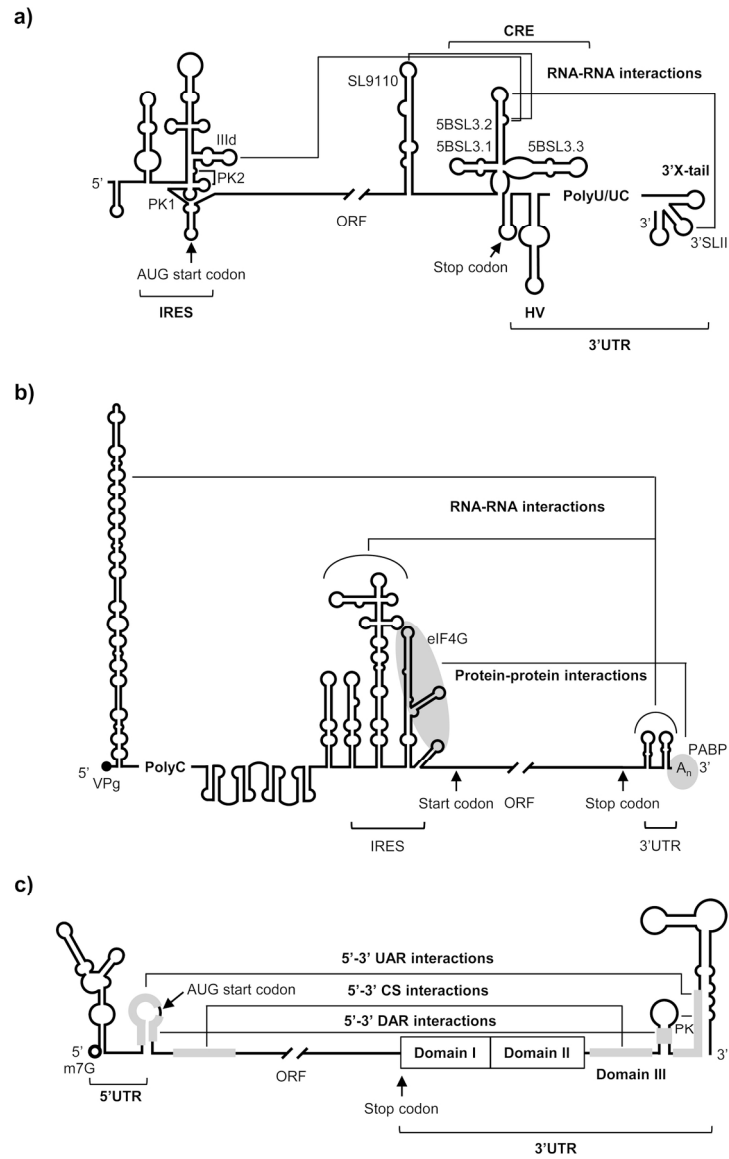
Figure 1

a) *Dicistroviridae* IGR

b) HCV

c) FMDV

d) HIV-1
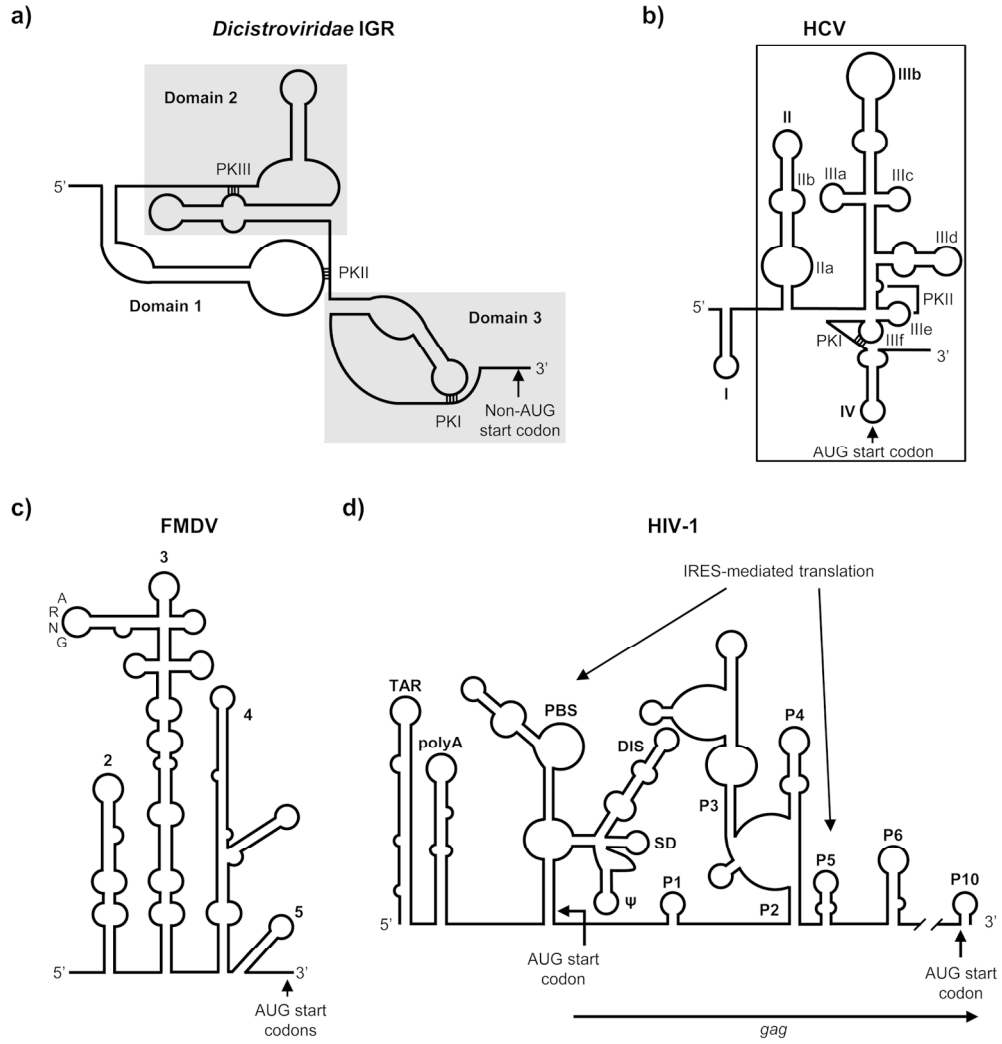
99x109mm (600 x 600 DPI)

Figure 2
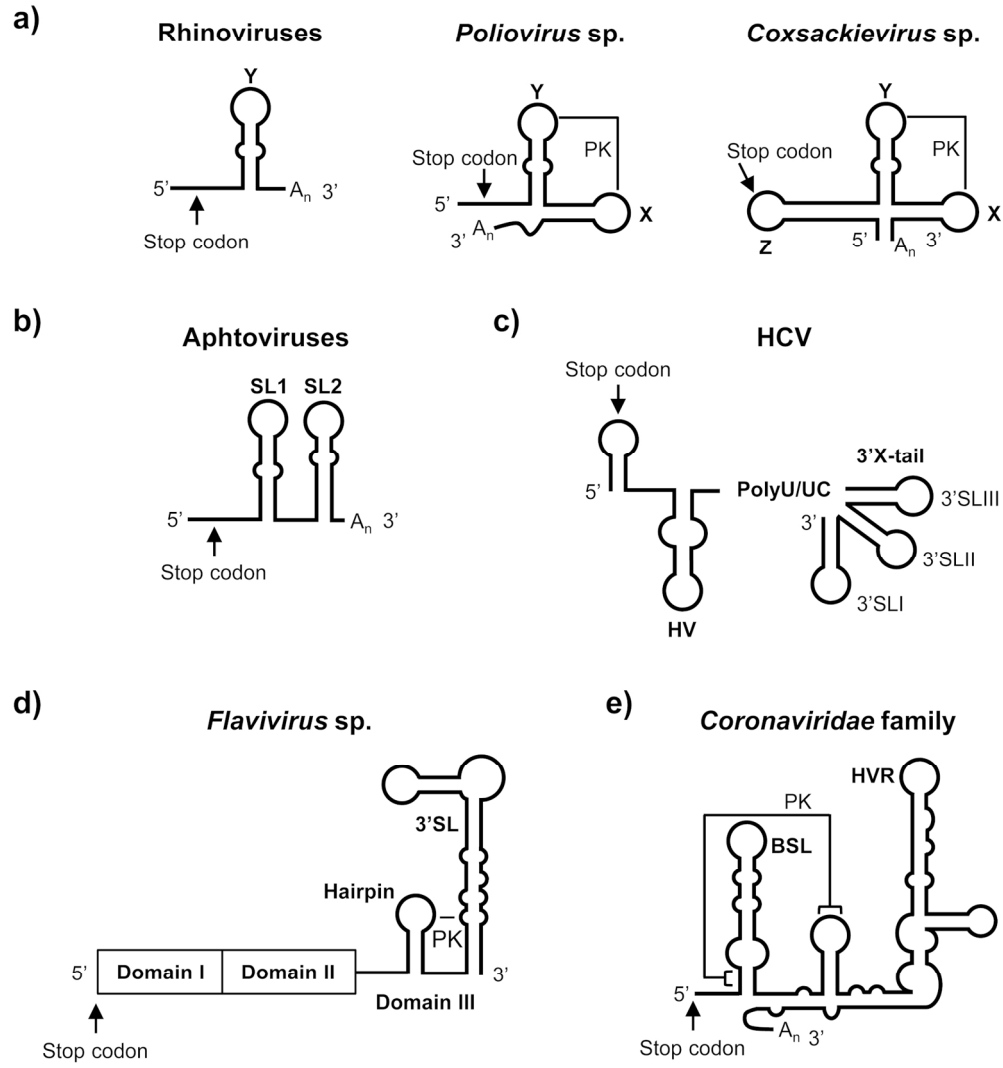
78x89mm (600 x 600 DPI)

Figure 3
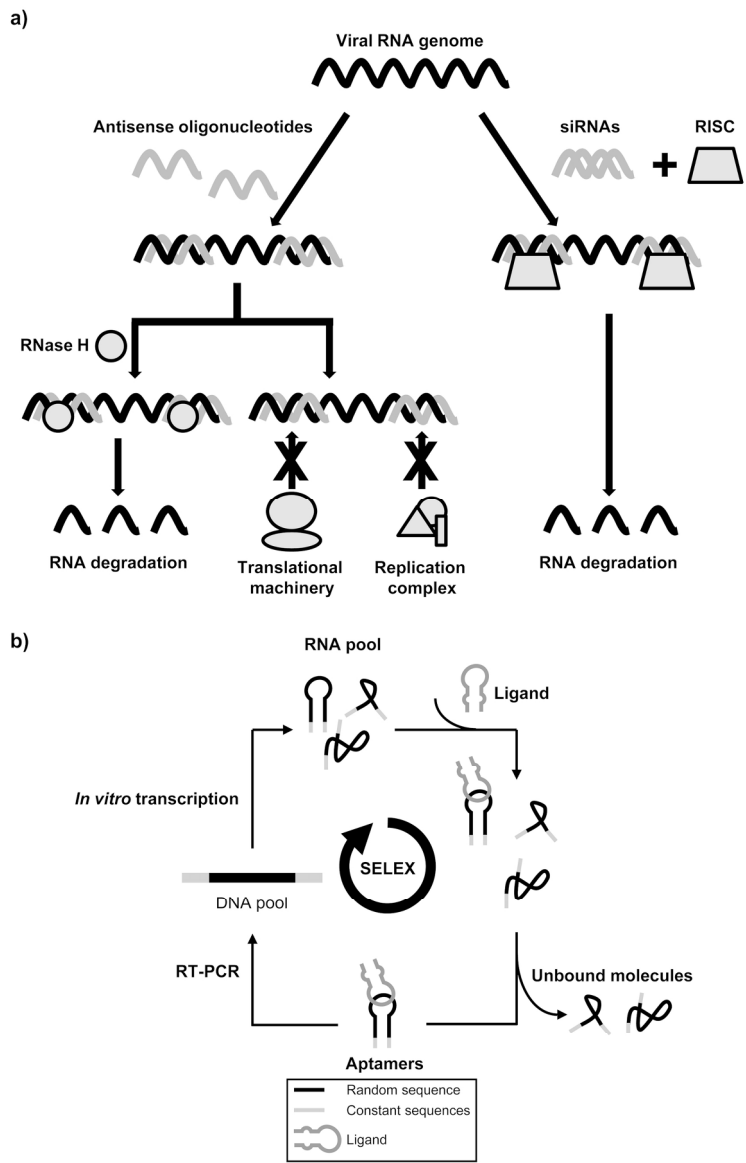

134x221mm (300 x 300 DPI)

Figure 4

a)

b)

146x236mm (300 x 300 DPI)