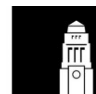

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Abstract

Positive-stranded RNA viruses include important human, animal and
20 plant pathogens. Their genomes are able to fold into complex structures stabilised by base pairing between individual nucleotides, many of which are highly conserved and have essential functions during virus replication. With new studies and technological advances the diversity of roles, mechanisms and interactions in which such structured viral RNA functions is becoming increasingly clear. It is also evident that many RNA structures do not function as discrete elements but through mechanisms involving multiple, long-range and often dynamic RNA-RNA interactions. Through a range of examples and recent advances, this review illustrates the diverse roles and mechanisms of structured viral RNA during the replication of
30 positive-stranded RNA viruses infecting humans and animals.

Introduction

Positive-stranded RNA viruses are a phylogenetically diverse grouping including families such as the *Flaviviridae*, *Coronaviridae*, and

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Picornaviridae. They possess single-stranded RNA genomes (~3-32 Kb) that function as both genetic material and mRNA template. With selection pressure to maximise their coding potential, their genomes have evolved a range of non-template functions involved in processes including virus translation, replication, sub-genomic mRNA transcript production, encapsidation and modulation of host antiviral responses. Such non-template functions can be mediated through nucleotide sequence composition (Atkinson *et al.*, 2014), specific motifs or more commonly via RNA structures interacting with host or viral *trans*-activating factors. RNA structures within virus genomes are stabilised by Watson/Crick base pairing and may comprise a simple stem-loop with duplexed stem and single-stranded terminal-loop, or complex higher-order structures such as pseudoknots and dynamic long-range RNA-RNA interactions.

While their host range, structure and life cycle are extremely diverse the requirement for positive-strand RNA virus genomes to act as both genetic material and mRNA remain in common, Fig. 1. Following release into the cytoplasm of a permissive cell, infecting genomes initially template translation of viral proteins including RNA-dependent RNA polymerase (RdRp), which catalyses synthesis of RNA negative strand intermediates that in turn template synthesis of positive-strand RNA molecules. Daughter positive-strand RNA is multifunctional and templates further rounds of genome replication and translation or can be packaged into new virion particles. In many instances mechanisms for temporal and spatial regulation of these key events depend on structured RNA. Such functional RNA elements act through an extremely diverse range of mechanism involving interaction with host/viral *trans*-activating factors and complex, often dynamic, RNA-RNA interactions. Our understanding of how such mechanisms function has until recently been limited, however with new structural mapping methods such as SHAPE (Merino *et al.*, 2005; Wilkinson *et al.*, 2006) and powerful *in silico* prediction algorithms, we are starting to develop a clearer picture of the interactions involved. Structured RNA and its dynamic interactions play an essential role in the replication cycle of positive-stranded RNA viruses infecting the full range of prokaryotic and eukaryotic hosts. However, due to their large number

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and the range of mechanisms in which they are involved, this review cannot be an exhaustive discussion of all functional RNA elements (for reviews of structured RNA in positive-strand RNA viruses of plants and prokaryotes see (Pathak *et al.*, 2011) and (Harvey *et al.*, 2013; Olsthoorn, 2011) respectively). Consequently, using a range of examples and recent advances, this review illustrates the mechanistic and structural diversity of viral RNA elements involved in the replication of positive-strand RNA viruses that infect animal and human hosts.

80 **Translation**

Positive-strand RNA viruses compete with endogenous cellular mRNAs for host translational machinery. In many instances these viruses have evolved functional RNA structures that impart a competitive advantage in recruiting translation initiation factors. Alternatively, such structures may provide temporal control to translation events by modulating the efficiency of initiation or reprograming translational extensions.

90 *Initiation*

Cellular translation is generally initiated by recognition of an m^7G cap by eukaryotic initiation factor 4F (eIF4F) - a complex of eIF4A, eIF4E and eIF4G - that recruits the 40S ribosome pre-initiation complex to the mRNA. Many viruses inhibit host cell translation through cleavage or modification of cap-dependent factors and may directly recruit canonical and non-canonical factors in a cap-independent process, via structured RNA designated internal ribosome entry sites (IRES). Based on structure, mechanism and initiation factor requirements, IRES elements can be
100 grouped into four categories. Type I elements include poliovirus, type II foot and mouth disease virus (FMDV), type III hepatitis C virus (HCV) and type IV the intragenic region (IGR) cricket paralysis virus (CrPV) IRES. The 40S preinitiation complex either binds at an upstream position and scans downstream to the authentic AUG start codon (type I) or interacts directly with the start codon (types II–IV). Depending on IRES type 40S

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subunit binding is initiated through different combinations of canonical/non-canonical translation factors. However, type IV elements such as the CrPv IGR-IRES, interact directly with the 40S subunit, independent of cellular initiation factors (Jan & Sarnow, 2002).

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Modulation

IRES elements are generally located within 5′ non-coding regions (NCRs), however their initiation function may be modulated by RNA structures or long-range interactions within other regions of the virus genome. FMDV translation is up regulated by a long-range interaction between its IRES and a region of the 3′NCR containing RNA structures SL1, SL2 and a poly(A) tract, Fig. 2(a) (Lopez de Quinto *et al.*, 2002; Serrano *et al.*, 2006). Rather than specific stem-loops, enhancement is
120 dependent on the overall higher-order RNA folding structure adopted by the entire IRES and 3′NCR (Serrano *et al.*, 2006). Interestingly, this structure is competitive with an alternative interaction between SL1-SL2 and the S-fragment stem-loop in the 5′NCR. It has been postulated that the S-fragment is involved in genome replication and that the alternative mutually exclusive long-range interactions represent a mechanism modulating FMDV translation and replication (Serrano *et al.*, 2006).

Similarly, regulation of HCV translation involves various complex (and potentially competing) higher order RNA interactions, Fig. 2(b).
130 Stem-loop SL9266 (alternatively 5BSL3.2 (You *et al.*, 2004) or SLV (Lee *et al.*, 2004)) in the RdRp encoding region of the open reading frame (ORF), is the core of a dynamic pseudoknot (SL9266/PK), undergoing multiple RNA-RNA interactions involved in regulation of virus translation and replication. The sub-terminal bulge of SL9266 forms RNA-RNA interactions with both the IIId loop of the IRES (Romero-Lopez & Berzal-Herranz, 2012) and an upstream region of the RdRp encoding region centred on nucleotide 9110 (Diviney *et al.*, 2008). The two potentially competing interactions are mutually exclusive, with the IIId loop interaction inhibiting HCV translation (Romero-Lopez & Berzal-Herranz,
140 2012) and that with the 9110 region essential for virus replication

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(Diviney *et al.*, 2008). The terminal-loop of SL9266 forms a 'kissing-loop' interaction with stem-loop SL9571 (alternatively SL2) in the 3'NCR (Friebe *et al.*, 2005). The 'kissing-loop' is a dynamic structure forming open and closed conformations, both of which are essential for efficient virus replication (Tuplin *et al.*, 2012). We have shown that the closed conformation stimulates HCV IRES initiated translation (Tuplin *et al*., unpublished results) and the open structure preferentially binds cellular protein EWSR1 up-regulating genome replication (Oakland *et al.*, 2013). It has been suggested that SL9266/PK functions in the temporal control of
150 early translation and replication events (Diviney *et al.*, 2008; Oakland *et al.*, 2013). However, much remains to be understood regarding this complex mechanism of dynamic RNA-RNA and RNA-protein interactions, in particular how alternative conformations are stabilised and interact with different *trans*-activating factors.

Many positive-strand RNA viruses that use capped, rather than IRES initiated translation, also contain RNA structures that confer regulatory control and a competitive advantage over cellular mRNA initiation. The 3'NCR of flaviviruses such as Dengue virus (DENV) contain
160 two dumbbell-like stem-loops (DB1 and DB2) that form local pseudoknots (DB/PK) necessary for optimal translation, Fig. 2(c) (Manzano *et al.*, 2011). Eukaryotic m^7G cap dependent translation is regulated by interaction between the mRNA 3' poly(A) tract and poly(A) binding protein (PABP) that recruits eIF4G, triggering initiation complex formation and cyclisation. DENV lacks a poly(A) tract, however the DB/PKs independently bind PABP, enabling efficient initiation; even under conditions of reduced eiF4G (Polacek *et al.*, 2009).

Reprogramming

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Many positive-strand RNA viruses increase the coding capacity of their genome through translation reprograming mechanisms such as stop codon read-through or programmed -1 ribosomal frameshifting. Reprograming enables translation of C-terminal extended polypeptides and control over levels of translation products. Alphavirus non-structural

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proteins are translated as a polyprotein from a single ORF but in many instances (for example Sindbis virus) an in frame UGA stop codon is located upstream of the RdRp coding region (Strauss *et al.*, 1983). In a proportion of ribosomes the stop signal is read through in a mechanism
180 stimulated by (but not dependent on) a large 3′ RNA structural element (Firth *et al.*, 2011). Alternatively, ribosomal frameshifting generally depends on signals incorporating structured RNA and 'slippery' nucleotide motifs. Coronaviruses (CoV) contain a signal in ORF1a that enables a proportion of elongating ribosomes to switch into the -1 reading frame (ORF1b), bypassing a stop codon and translating c-terminal extended ORF1a/b products (Giedroc & Cornish, 2009; Plant & Dinman, 2008). In SARS-CoV the signal incorporates a pseudoknot containing three internal stems at which a proportion of ribosomes pause and stutter, shifting into the -1 reading frame at an upstream 'slippery' motif.

190

Genome replication

Temporal and spatial regulation of negative-strand synthesis is frequently provided by structured RNA elements in the positive-strand 3′NCR, whilst growing evidence suggests that RNA structures in the negative strand 3′NCR influence positive-strand synthesis. However, it is becoming increasingly clear that regulation also often involves RNA interactions and structure in both the 5′NCR and ORF. Such mechanisms can function via direct RNA-RNA complementarity or RNA-binding protein
200 intermediates and frequently initiate genome cyclisation between the 5′ and 3′ NCRs.

Flaviviruses

Flaviviruses possess structurally dynamic genomes that switch between linear and circular conformations, both of which are essential for virus replication. Genome cyclisation is stabilised by direct RNA-RNA interactions between inverted complementary repeats (UAR, DAR and CS motifs) at either end of the genome, Fig. 2(c) (Gebhard *et al.*, 2011;
210 Villordo & Gamarnik, 2009). In DENV cyclisation is essential for negative-

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strand synthesis (Alvarez *et al.*, 2005a; Khromykh *et al.*, 2001; Lo *et al.*, 2003) and a balance between both conformations is critical for virus replication (Villordo *et al.*, 2010). DENV genome replication is dependent on activation of viral RdRp via binding 5′NCR stem-loop SLA before genome cyclisation repositions the activated replication complex at the 3′NCR transcription initiation site (Filomatori *et al.*, 2011; Filomatori *et al.*, 2006). Although SLA and genome cyclisation are essential for DENV negative-strand synthesis they are not sufficient. Evidence from various laboratories indicates the involvement of further RNA rearrangements and
220 structures in both NCRs and the ORF. For example, the 3′NCR DB/PKs enhance transcription and the base of stem-loop 3′SL represses it due to overlapping with the 3′UAR cyclisation signal (Filomatori *et al.*, 2011; Manzano *et al.*, 2011); consequently the base of 3′SL must open to allow 5′-3′UAR binding and subsequent negative strand synthesis.

Flavivirus cyclisation is dependent on direct RNA-RNA complementarity rather than protein factors (Alvarez *et al.*, 2005b). However, various cellular and viral proteins, essential to efficient virus replication, bind in structured regions of the genome and have been
230 postulated to control or stabilise conformational rearrangement. In arboviruses such as DENV and alphaviruses, stem-loop mutants have been observed with different phenotypes in mammalian and mosquito cells (Fayzulin & Frolov, 2004; Gorchakov *et al.*, 2004; Groat-Carmona *et al.*, 2012; Villordo & Gamarnik, 2013). Such differences most likely reflect differences in *trans*-activating protein factor availabilities, although differences in intracellular environments such as temperature and cation concentrations may also play a role.

Picornaviruses

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The genome of poliovirus contains various functional RNA structures such as *cis* replicating elements (cre) responsible for uridylylation of the small protein-nucleotide primer of replication, VPg. The genome also includes a highly structured 5′NCR containing a 5′ cloverleaf (5′CL) and IRES and two stem-loops in the 3′NCR (SL-x and SL-y) that

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form a 'kissing-loop', Fig. 2(d) (Ogram & Flanegan, 2011). Cellular poly(rC) binding protein 2 (PCBP2) interacts with the poliovirus IRES during translation initiation and with stem-loop 5′CL, as part of a ribonucleoprotein complex with viral protein $3CD^{pro}$ (5′CL-RNP), during
250 genome replication. Formation of the 5′CL-RNP is essential for initiation of negative strand synthesis, with genome cyclisation occurring through interaction between PCBP2 in the 5′CL-RNP and PABP bound to the 3′NCR. PCBP2 is cleaved by poliovirus proteinase 3CD rendering it unable to bind the IRES and preventing translation initiation, while binding to 5′CL and thus replication is unaffected. Indeed, cleavage of PCBP2 by 3CD is necessary for poliovirus replication and may constitute a switching mechanism between translation and RNA replication (Chase *et al.*, 2014). The 3′NCR 'kissing-interaction' is also required for efficient replication and surprisingly its disruption has a much greater inhibitory effect than
260 deleting the entire 3′NCR, possibly due to faulty positioning of the 5′CL-RNP in 'kissing-loop' mutants blocking successful initiation. Structures within the poliovirus genome are relatively well documented. However, the dynamics of their interactions within host cells and the role of other regions of structured RNA remain to be fully characterised. For example, two recent studies revealed a number of novel structures within the ORF (Burrill *et al.*, 2013; Song *et al.*, 2012), one of which - element 3D-7000 – is implicated in RNA replication through an as yet unknown mechanism (Burrill *et al.*, 2013).

270 **Evasion of innate immune responses**

Antiviral innate responses generally depend on recognition of viral RNA through pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs). Recognition of viral RNA PAMPs (such as double-stranded molecules, length, sequence, structure, and location), trigger a range of antiviral responses. Consequently, viruses have evolved numerous countermeasures, often involving structured RNA, to block recognition by PRRs or overcome innate responses. For example, an RNA structure in the $3C^{pro}$ coding region of poliovirus (ciRNA) binds RNase L,
280 competitively inhibiting its antiviral activity (Han *et al.*, 2007; Keel *et al.*,

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2012). Many positive-stranded RNA viruses, such as HCV, exhibit extensive RNA structure designated genome-scale ordered RNA structure (GORS) (Simmonds *et al.*, 2004). Distinct from discrete structural elements, GORS inhibits detection by cellular PRRs with its presence correlating to persistent viruses (Witteveldt *et al.*, 2014). A number of PRR interferon induced proteins bind 5′ caps lacking 2′-O methylation, in alphaviruses secondary structures in their 5′NCR alter this binding and block their antiviral effect (Hyde *et al.*, 2014). Alternatively, highly structured sub-genomic flavivirus RNAs (sfRNA) - produced during
290 flavivirus infection as the product of host Xrn1 endonuclease activity terminating at a pseudoknot resistant to Xrn1 helicase activity (Chapman *et al.*, 2014) - increase viral pathogenicity in mice, play a role in inhibition of a type I interferon responses and are involved in silencing RNAi in insect cells (Schnettler *et al.*, 2012). Such varied mechanisms by which structured RNA interacts with or manipulates antiviral responses, illustrates its key role in the evolutionary arms race between viruses and host innate immunity.

Summary and perspectives

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Using a range of examples and recent advances this review illustrates the diversity of mechanisms by which structured RNA influences different stages of positive-RNA virus replication. As our understanding of these mechanisms develops, in part through new methodologies enabling structural mapping of longer RNA molecules at higher resolutions, the importance and diversity of – often long-range and dynamic - higher order RNA structures is becoming increasingly clear. For example, various studies have clearly demonstrated that flavivirus genomes switch between alternative RNA conformations and that a balance between the two is
310 critical to virus replication. However, our understanding of the mechanisms and *trans*-activating factors involved in controlling or sensing such complex and often dynamic interactions is limited. Indeed, elucidation of transient RNA-RNA and RNA-protein interactions remains challenging and we have little comprehension of how essential RNA structural changes and dynamic interactions are regulated within different

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intracellular environments and compartments. In summary, recent advances have made a significant impact on our understanding of viral RNA structure and its diverse roles across a range of mechanisms essential to positive-strand RNA virus replication. With future studies
320 refining and extending our understanding, it can be expected that the potential of structured RNA as an antiviral target will be further investigated and that a clearer understanding of their dynamics during virus replication will inform the study of RNA structures in other systems, such as eukaryotic mRNA and long non-coding RNA function.

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Figure legends

Figure 1

Replication of positive-strand RNA virus genomes

Generalised schematic representation of single ORF, positive-strand RNA virus, replication. After release into permissive cell cytoplasm the infecting positive-strand RNA molecule acts as an mRNA and is translated by host cell machinery. Translation initiates at the 5′ of the ORF, producing a polyprotein (blue arrow) that is cleaved into mature viral proteins. As viral protein levels build up synthesis of negative-strand RNA intermediates by viral RdRp is initiated from the 3′ end of the viral genome. Negative-strand RNA acts as a template for synthesis of daughter positive-strand RNA molecules, which in turn can act as template for further rounds of translation and replication or are packaged with viral structural proteins into new virion particles. As translation and negative strand synthesis initiate at opposing ends of the genome they are mutually exclusive processes that are tightly regulated, both specially and temporally, within the cell. Where known, regulation mechanisms often involve dynamic interactions between host and/or virally encoded proteins and viral RNA structures.

Figure 2

RNA secondary structures and higher-order interactions within positive-strand RNA virus genomes

Simplified cartoon representations of viral genomes showing functional local RNA structures and higher-order RNA-RNA interactions between genome regions as dashed red lines. Breaks in genomes and regions not shown are represented by diagonal lines (-//-) and black-dashed lines respectively. Relative genome positions are indicated below each RNA molecule by schematic genome maps showing NCRs and the ORF. **A**) FMDV showing 'kissing-loop' interactions between the terminal loops of SL-1 and SL-2 and alternative long-distance interactions between different regions of the 3′NCR and either the S-fragment or IRES. Pk represents a region of predicted pseudoknots. **B**) HCV showing a 'kissing-loop' interaction between the terminal loops of SL9266 and SL9571 and alternative long-range interactions between the sub-terminal bulge of SL9266 and either the IRES or upstream to a position centred on nucleotide position 9110. **C**) DENV showing alternative linear and circular conformations and DB pseudoknot interactions. Unbroken red, yellow and blue lines represent complementary UAR (upstream AUG region), DAR (downstream AUG region) and CS (cyclisation sequence) motifs respectively. **D**) Poliovirus showing pseudoknot interactions between regions of the crRNA element and between the terminal loops of SL-y and SL-x.

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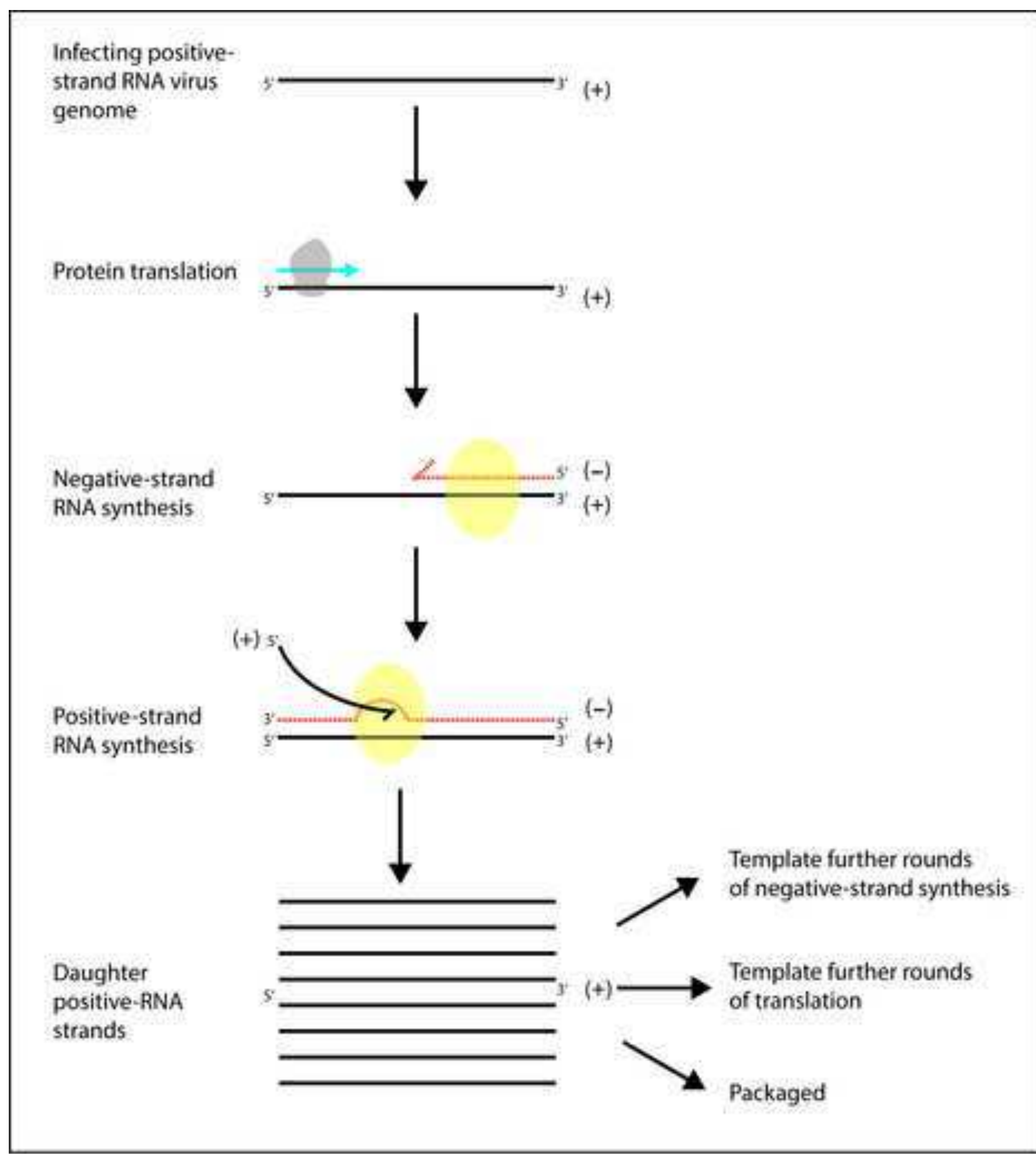

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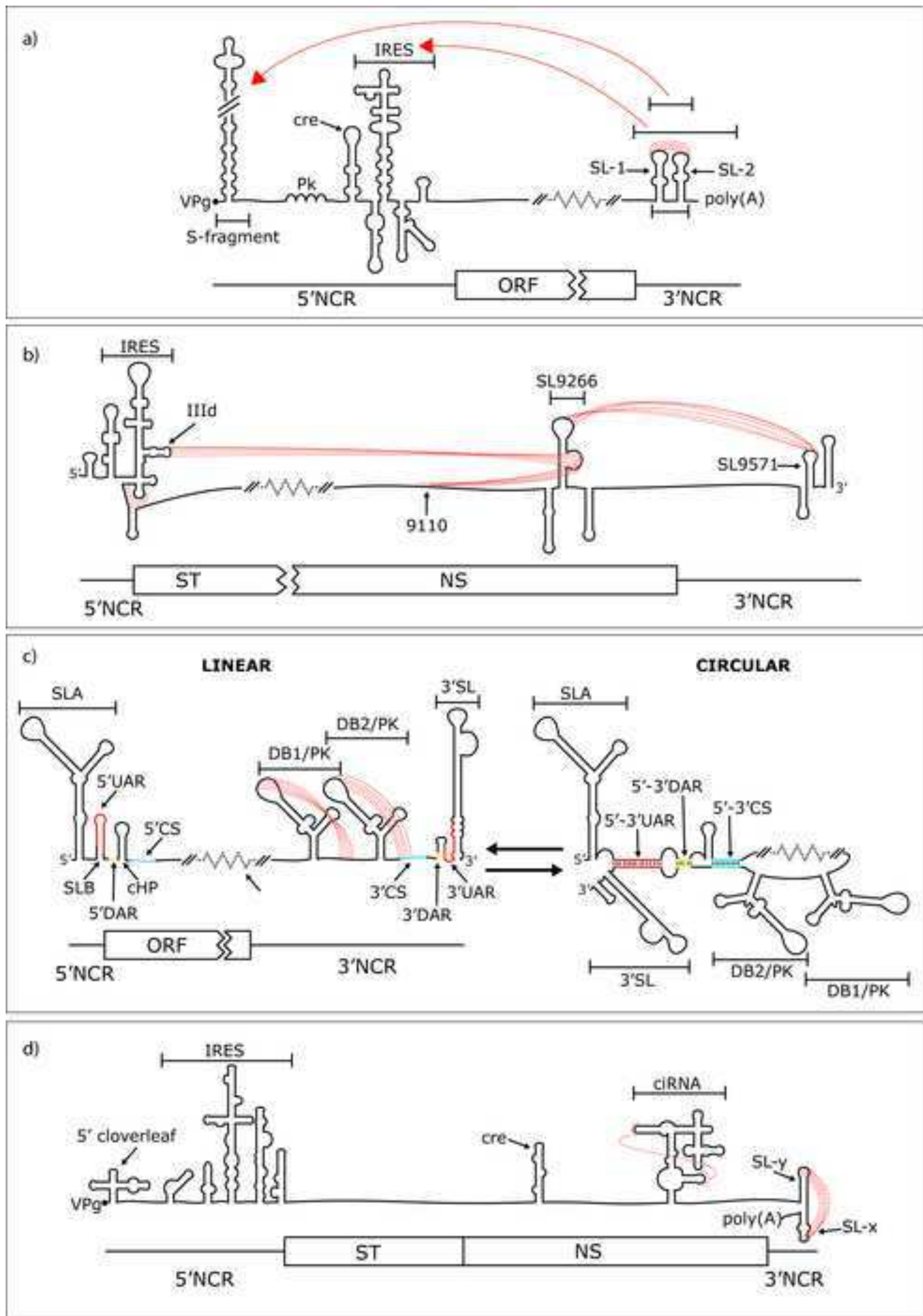

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