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Non-Templated Functions of Viral RNA in Picornavirus Replication

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Abstract

The genomic RNA of poliovirus and closely related picornaviruses perform templated and non-templated functions during viral RNA replication. The non-templated functions are mediated by *cis*-active RNA sequences that bind viral and cellular proteins to form RNP complexes. The RNP complexes mediate temporally dynamic, long-range interactions in the viral genome and ensure the specificity of replication. The 5' cloverleaf (5' CL)-RNP complex serves as a key *cis*-active element in all of the non-templated functions of viral RNA. The 5'CL-RNP complex is proposed to interact with the *cre*-RNP complex during VPgpUpU synthesis, the 3'NTR-poly(A) RNP complex during negative-strand initiation and the 3' end negative-strand-RNP complex during positive-strand initiation. Coordinating these long-range interactions is important in regulating each step in the replication cycle.

Introduction

A large number of viruses that cause human, animal and plant diseases contain RNA as their genetic material. The genomic RNA performs both templated and non-templated functions during the viral replication cycle. In positive-strand RNA viruses, the genomic RNA serves as a mRNA and as template for viral RNA replication. Besides storing genetic information and functioning as the template for RNA replication, the viral genome contains RNA determinants which perform non-templated functions that are mediated by distinct RNA sequences and structures present in the noncoding and coding regions of the viral genome. These RNA sequences and structures function as *cis*-active elements that participate in both RNA-RNA and protein-RNA interactions and are essential for viral replication. These *cis*-active RNA elements recruit and assemble *trans*-acting viral and cellular proteins to form membrane bound viral replication complexes that are sites of viral RNA replication.

In this review, we have discussed some of the non-templated functions of viral RNA during RNA replication. We have limited the discussion primarily to the replication of human enteroviruses belonging to the Picornaviridae family of positive-strand RNA viruses. Poliovirus has served as the prototype virus for studying the assembly of viral replication complexes on membrane vesicles using cell-culture systems permissive to viral replication. An important advance in the study of the molecular mechanisms regulating viral RNA replication has been the development of experimental systems to investigate the individual

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steps in the replication cycle using cell-free reactions [1–6]. Membrane-bound replication complexes isolated from cell-free reactions have been used to follow the synchronous initiation and sequential synthesis of authentic negative-and positive-strand RNAs [7]. This is in contrast to the situation in infected cells where a significant overlap develops between translation and RNA replication because of their mutual interdependence [8]. In addition, *trans*-replication assays have been developed to separate viral protein synthesis and RNA replication in the cell-free system [9–11]. This assay makes it possible to identify and characterize *cis*-active elements that are required for the replication of viral RNA templates. [9–11].

The 5’ end of the picornavirus genome is covalently linked to a viral protein, VPg, and is polyadenylated at the 3’ end. The RNA genome contains a large open reading frame flanked by 5’ and 3’ nontranslated regions (NTRs) (Fig. 1A). Conserved RNA sequences and structures present in the 5’ and 3’ NTRs and in the coding region of the genome participate in non-templated RNA functions that regulate the stability, translation and replication of the viral genome [12;13]. The 5’ cloverleaf (5’CL), the *cre* hairpin, the 3’NTR-poly(A) tail in positive-strand RNA and the 3’ and 5’ terminal sequences in negative-strand RNA are *cis*-active elements that are functionally important for picornavirus RNA replication. In this review, the non-templated functions of these RNA elements during viral RNA replication will be discussed.

Cre-dependent VPgpUpU synthesis

Cre hairpin

VPg is covalently linked to the 5’ end of both negative- and positive-strand RNAs and the uridylylated form of VPg (VPgpUpU) functions as the primer to initiate RNA synthesis [14–18]. A highly conserved hairpin structure, originally identified as a *cis*-replication element (*cre*) in the RNA coding region of picornaviruses [19–22], is used as the template for VPgpUpU synthesis in reconstituted assays containing purified viral proteins, VPg, the polymerase ($3D^{pol}$) and $3CD^{pro}$ [12;13;23;24]. The *cre* hairpin has been identified in the 2C region of poliovirus and Coxsackievirus [6;22;23], the VP1 region of human rhinovirus 14 (HRV14) [19], the 2A region of HRV2 [24], the VP2 region of cardioviruses [20] and in the 5’ NTR of foot and mouth disease virus (FMDV) [25]. Based on the above findings, it is not surprising that the *cre* hairpin has been shown to function in a position-independent manner [22;25–27].

The loop of the *cre* hairpin contains a conserved sequence ($R_1NNNA_5A_6R_7NNNNNNR_{14}$) in enteroviruses and rhinoviruses [12;13]. It was shown that the A_5 residue functions as the primary template for the addition of both U residues in VPgpUpU using a “slide-back” mechanism [28]. High resolution NMR structural studies together with mutational analysis of the *cre* hairpin provided evidence for the functional importance of the *cre* structure during VPgpUpU synthesis [21;27;29;30]. Biochemical and genetic studies suggest that the *cre* hairpin interacts with $3CD^{pro}$ and $3D^{pol}$ to form a *cre*-RNA complex that is important for VPgpUpU synthesis [27;31–37]. In summary, the *cre* hairpin is a position-independent, *cis*-active element that forms an RNP complex with $3CD^{pro}$ and $3D^{pol}$ and serves as the primary template for VPgpUpU synthesis by $3D^{pol}$.

5’ Cloverleaf (5’CL)

In membrane-bound viral replication complexes isolated from cell-free reactions, it was shown that in addition to the *cre* hairpin, the 5’CL is also required for VPgpUpU synthesis [38]. In recent studies, using a *trans*-uridylylation assay in which the replication proteins were provided by a helper RNA, it was shown that the *cre* and the 5’ CL were the only *cis*-active elements required for VPgpUpU synthesis. In addition, positioning the *cre* hairpin

adjacent to the 5'CL resulted in a large increase in VPgpUpU synthesis (Sharma and Flanegan, unpublished).

The 5′CL is organized into stem 'a' and stem-loops 'b', 'c' and 'd,' and an adjacent C-rich sequence (Fig. 1B). Stem-loop 'b' and the C-rich sequence are the binding sites for the cellular poly(C) binding protein (PCBP) and stem-loop 'd' is the binding site for $3CD^{pro}$[39–42]. Recent studies showed that 5'CL mutations which disrupt the binding of PCBP or $3CD^{pro}$ to the 5'CL strongly inhibit VPgpUpU synthesis in viral replication complexes indicating that the 5'CL bound to PCBP and $3CD^{pro}$ is required for VPgpUpU synthesis on the *cre* hairpin (Sharma and Flanegan, unpublished). This suggests a long-range interaction between the 5'CL-RNP complex and the *cre*-RNP complex is required for VPgpUpU synthesis as shown in Fig. 2. This idea appears to be consistent with the observation that VPgpUpU synthesis was significantly increased when the 5'CL and *cre* hairpin were positioned next to each other in the template RNA. It is possible that the 5'CL-RNP complex in concert with the *cre*-RNP complex provides the optimal conditions for VPgpUpU synthesis on the *cre* hairpin in the context of the membrane replication complex.

Negative-strand RNA synthesis

3' Nontranslated Region (NTR) and poly(A) tail

During RNA replication, negative-strand synthesis initiates at the 3' end of the RNA genome. A highly structured 3'NTR is present at the 3' end of the genome of picornaviruses [43–46]. Stem-loops X and Y are predicted to form a pseudoknot structure through base-pairing in a "kissing interaction" in the 3'NTR [46]. Mutations that disrupt the formation of the kissing interaction block viral RNA replication [47;48]. Alignment of the 3'NTR sequences of enteroviruses revealed an invariable region of five nucleotides (GUAAA) which forms a single-stranded region between stem Y and stem X [48;49]. Deleting this sequence from the 3'NTR results in a dramatic inhibition of negative-strand synthesis which suggests that it plays a critical role in viral replication [9;10;50]. Interestingly, a poliovirus mutant RNA in which the entire 3'NTR was deleted was still capable of replicating in infected cells suggesting that the 3'NTR is not absolutely required for negative-strand synthesis [51;52]. Finally, the binding of the cellular protein, nucleolin, to the 3' NTR of poliovirus RNA has been shown to be important for early viral replication [53]. In addition to nucleolin, the viral proteins 3AB and $3CD^{pro}$ together form an RNP complex with the 3'NTR [54]. These findings suggest that the 3'NTR serves as the platform for the assembly of an RNP complex containing viral and cellular proteins that facilitate the efficient initiation of negative-strand synthesis. Importantly, specific point mutations in the 3'NTR appear to have a more severe effect on viral RNA replication than deletions of the entire 3'NTR. It is possible that in contrast to the deletion mutant, the point mutations allow the mutant 3'NTR to form a nonfunctional RNP complex that interferes with negative-strand initiation.

The 3' terminal poly(A) tail is also important for several aspects of viral replication. Shortening the poly(A) tail reduced the infectivity of the genomic RNA indicating that the length of the poly(A) tail directly correlates with the infectivity of the genomic RNA [55;56]. Consistent with these findings is the observation that a poly(A) tail 20 nucleotides long or longer is needed for efficient negative-strand initiation [57;58]. It has been suggested that the poly(A) tail plays a role in the formation of a 5'-3' circular RNP complex [10;57;58]. In addition, VPgpUpU is reported to serve as the preferred primer for negative-strand initiation at internal sites on the poly(A) tail [59;60]. Taken together, this suggests that the poly(A) tail needs to be long enough to be both part of the circular RNP complex and to serve as an efficient template for VPgpUpU priming during negative-strand synthesis (Fig. 3).

5’ Cloverleaf (5’CL)

Mutational analysis of the 5’CL revealed its importance in the initiation of negative-strand synthesis [5;10;11;40;42;61;62]. The finding that the 5’CL is required for the initiation of negative-strand synthesis at its 3’ end suggests an interaction between the 5’ and 3’ ends to form a circular complex [10;58]. Mutations that disrupted the 5’CL-PCBP interaction exhibited dramatically reduced negative-strand synthesis [40–42;61;63]. Using the $(MS2)_2$ protein-RNA tethering system, PCBP could be artificially tethered to the 5’CL to partially restore negative-strand synthesis. This established that the 5’CL-PCBP interaction was required for efficient negative-strand synthesis and PCBP does not have to directly bind to the 5’CL to function in RNA replication [61]. Genetic evidence showed that the binding of PCBP to the C-rich sequence adjacent to the 5’CL was also important for RNA replication [5;39;64]. This suggests that PCBP binding to both stem-loop ‘b’ and the C-rich sequence is required for efficient negative-strand synthesis. Mutational analysis of stem-loop ‘d’ showed that the 5’CL-$3CD^{pro}$ interaction also plays a critical role in viral RNA replication and in negative-strand synthesis [5;10;40;41]. These findings clearly established the importance of the 5’CL-RNP complex during initiation of negative-strand synthesis. Interestingly, efficient negative-strand RNA synthesis and *cre*-dependent VPgpUpU synthesis requires the same 5’CL-RNP complex. Besides stem-loop ‘b’ and ‘d’, the duplex structure of stem ‘a’ is also required for efficient negative-strand synthesis [11]. This indicates that the duplex structure of stem ’a’ may play an important role in maintaining the overall structure of the 5’CL that is required to form the 5’CL-RNP complex. Therefore, the 5CL together with the 3’NTR-poly(A) tail may provide a scaffold to assemble a functional membrane-bound replication complex that is used to initiate negative-strand synthesis (Fig.3).

Cre hairpin

As discussed earlier, the *cre* hairpin serves as the template for the synthesis of VPgpUpU [19;22–24]. It was shown that disrupting the secondary structure of the *cre* hairpin by inserting multiple silent mutations resulted in the inhibition of VPgpUpU synthesis. These mutations totally inhibited positive-strand synthesis but had no significant effect on negative-strand synthesis [6;9;65]. In addition, in *trans*-replication assays, the complete deletion of the *cre* hairpin from the template RNA had no significant effect on negative-strand synthesis in reactions with helper RNAs containing either a wildtype *cre* or mutant *cre*. As expected, positive-strand synthesis was only observed with wildtype helper RNA [9]. These findings indicate that VPgpUpU synthesized on the *cre* hairpin is required for positive-strand synthesis but is dispensable for negative-strand synthesis. Therefore, VPgpUpU or VPg can serve as a primer to initiate negative-strand synthesis on the 3’ poly(A) tail. However, recent findings suggest that under conditions when the initiating nucleotide (UTP) is limiting, VPgpUpU synthesized on the *cre* hairpin is the preferred primer for negative-strand initiation [59]. In contrast to the mutations that completely disrupt the structure of the *cre* hairpin, point mutations in A_5 and A_6 nucleotides in the loop of the Coxsackievirus *cre* hairpin were found to completely inhibit VPgpUpU synthesis and to strongly inhibit negative-strand synthesis [6]. The question is why the effect on negative-strand synthesis was so different with the disrupted *cre* and the mutant *cre* containing the point mutations. This difference may be related to the observation that point mutations in the *cre* hairpin that inhibit VPgpUpU synthesis but do not completely disrupt its structure, have a *trans*-dominant negative effect on the replication of wildtype poliovirus [6;66]. This result was not observed with mutants that contained a disrupted *cre*. In contrast to the disrupted *cre*, point mutations in the *cre*, which disrupt its function, may allow the mutant *cre* to participate in protein-RNA interactions and form inhibitory RNP complexes by sequestering replication proteins that may be limiting and are required for negative-strand synthesis [66].

Positive-strand RNA synthesis

3’ and 5’ terminal sequences in negative-strand RNA

Once negative-strand synthesis is complete, positive-strand synthesis initiates at the 3’ end of the negative strand RNA. The synthesis of negative-strand RNA may result in the formation of a double-stranded RNA (dsRNA) intermediate known as replicative form RNA (RF). Partial denaturation of the duplex RNA would be necessary to allow VPgpUpU to anneal to the AA sequence at the 3’ end of the negative-strand and function as a primer for positive-strand synthesis. However, the exact mechanism by which the two strands in the RF RNA separate to initiate positive-strand synthesis is not known.

The primary sequence and structure of stem ‘a’ in the 5’CL are highly conserved and the duplex structure of stem ‘a’, as part of the 5’CL is required for negative-strand synthesis [11]. Interestingly, the conserved 5’ terminal sequence $^{5'}$UUAAAACAG$^{3'}$ in stem ‘a’, or more appropriately, the 3’ terminal sequence ($^{3'}$AAUUUUGUC^{5}) in the negative-strand is a *cis*-active sequence required for positive-strand synthesis [11]. In addition, it was shown that deleting either one or both of the 3’ terminal A nucleotides dramatically reduced positive-strand synthesis [11]. This finding is consistent with a model in which preformed VPgpUpU base-pairs with the 3’ terminal AA sequence to initiate positive-strand synthesis. Supporting this model is the observation that wildtype virus was recovered from cells transfected with RNAs in which either one or both 3’ terminal A nucleotides were deleted [11;67;68]. These findings demonstrate that even in the absence of the terminal A nucleotides, preformed VPgpUpU can prime and initiate positive-strand synthesis thereby restoring the 3’ terminal A nucleotides.

The cellular protein, hnRNPC, is a cofactor that is required for efficient synthesis of positive-strand RNA and has been shown to bind near the 3’ end of the negative-strand RNA [69;70]. Interestingly, the 3’ terminal sequence $^{3'}$AAUUUUGUC^{5} in the negative-strand RNA appears to be similar to the RNA sequence recognized by the hnRNPC proteins and may explain why this sequence is important for positive-strand initiation (Fig. 4). A second hnRNPC binding site has been recently identified at the 5’ end of the negative strand [71]. The authors of this study propose that binding of hnRNPC to both ends of the negative-strand RNA may stabilize the interaction between the 5’ and 3’ ends. This interaction may in turn assist in the formation of the replication complex required for efficient initiation of positive-strand synthesis.

5’ Cloverleaf (5’CL)

As discussed earlier, the 5’CL is an important *cis*-active RNA element that is required for *cre*-dependent VPgpUpU synthesis and negative-strand synthesis. Since positive-strand synthesis is dependent on both preformed VPgpUpU and negative-strand synthesis, it has been difficult to dissect the role of the 5’CL during positive-strand synthesis. To overcome this problem, a novel experimental approach was used in which a second CL was inserted near the 5’ end of the positive-strand RNA [72]. Using this approach, the authors confirmed the earlier finding that the sequence of stem ‘a’ is important for positive-strand initiation. In addition, they showed that the 5’ CL-RNP complex formed at the 5’ end of the positive-strand RNA, which includes PCBP and 3CD^{pro}, is required for positive-strand synthesis. The authors suggest that after negative-strand synthesis is complete the RF RNA partially unwinds to allow the 5’CL in the positive-strand RNA to reform and assemble the 5’CL-RNP complex. According to this model, the 5’CL-RNP complex formed at the 5’ end of the positive-strand RNA functions in *trans* at the 3’ end of the negative-strand RNA allowing VPgpUpU to initiate positive-strand RNA synthesis [72] (Fig.4).

Conclusions

C*is*-active RNA elements in the viral genome help regulate viral replication and ensure that this process is both efficient and specific for viral RNA templates. A major conclusion of this review is that the 5'CL serves as a key *cis*-active element in all of the non-templated functions of the viral RNA genome (Figs. 2–4). It is required for each step in the viral RNA replication cycle and serves as a scaffold for the assembly of the multi-protein viral RNA replication complex.

The *cis*-active elements in the viral genome contain conserved sequences and structures that provide specific binding sites for viral and cellular proteins. This results in the formation of RNP-complexes that serve as the functional form of the *cis*-active elements during the RNA replication cycle. Interestingly, point mutations in a *cis*-active RNA element sometime inhibit viral RNA replication at levels significantly higher than those observed with mutations that delete the element or disrupt its structure. With point mutations, it is possible that the formation of a non-functional RNP complex may further inhibit replication by sequestering replication proteins present in limiting concentrations. Examples of this point were discussed for the *cre* hairpin and the 3' NTR in this review. In some cases, the formation of non-functional complexes may increase the fidelity of viral RNA replication by blocking the replication of defective viral genomes.

It is important to note that the *cis*-active RNA elements form multi-functional and temporally dynamic RNP complexes, which facilitate long-range interactions in the viral genome. The RNP complexes formed with the individual *cis*-active RNA elements interact with each other to form larger RNP complexes that are apparently stabilized by protein-protein interactions. These interactions change with each step in the replication cycle. For example, the models shown in Figs. 2–3 suggest that 5'CL-RNP complex interacts with 1) the *cre*-RNP complex during VPgpUpU synthesis, 2) the 3'NTR-poly(A) RNP complex during negative-strand synthesis and 3) the negative-strand 3' end-RNP complex during positive-strand synthesis. Coordinating these long-range interactions is important in regulating the sequential steps in the viral replication cycle. Developing a better understanding of what promotes or inhibits these interactions is a challenge for the future. Clarifying the details of the protein-RNA and protein-protein interactions in the RNP complexes will be very important in further defining the molecular mechanisms that regulate viral RNA replication and in the development of new antiviral agents.

Highlights

C*is*-active elements regulate non-templated functions of viral RNA genome

C*is*-active elements form RNP complexes that regulate viral RNA replication

RNP complexes mediate long-range interactions in viral RNA

5'CL-RNP complex is required in *trans* for VPgpUpU-primed (+) strand initiation

5'–3' circular RNP complex regulates initiation of (−) strand synthesis

5'CL-RNP and *cre*-RNP interaction is required for VPgpUpU synthesis

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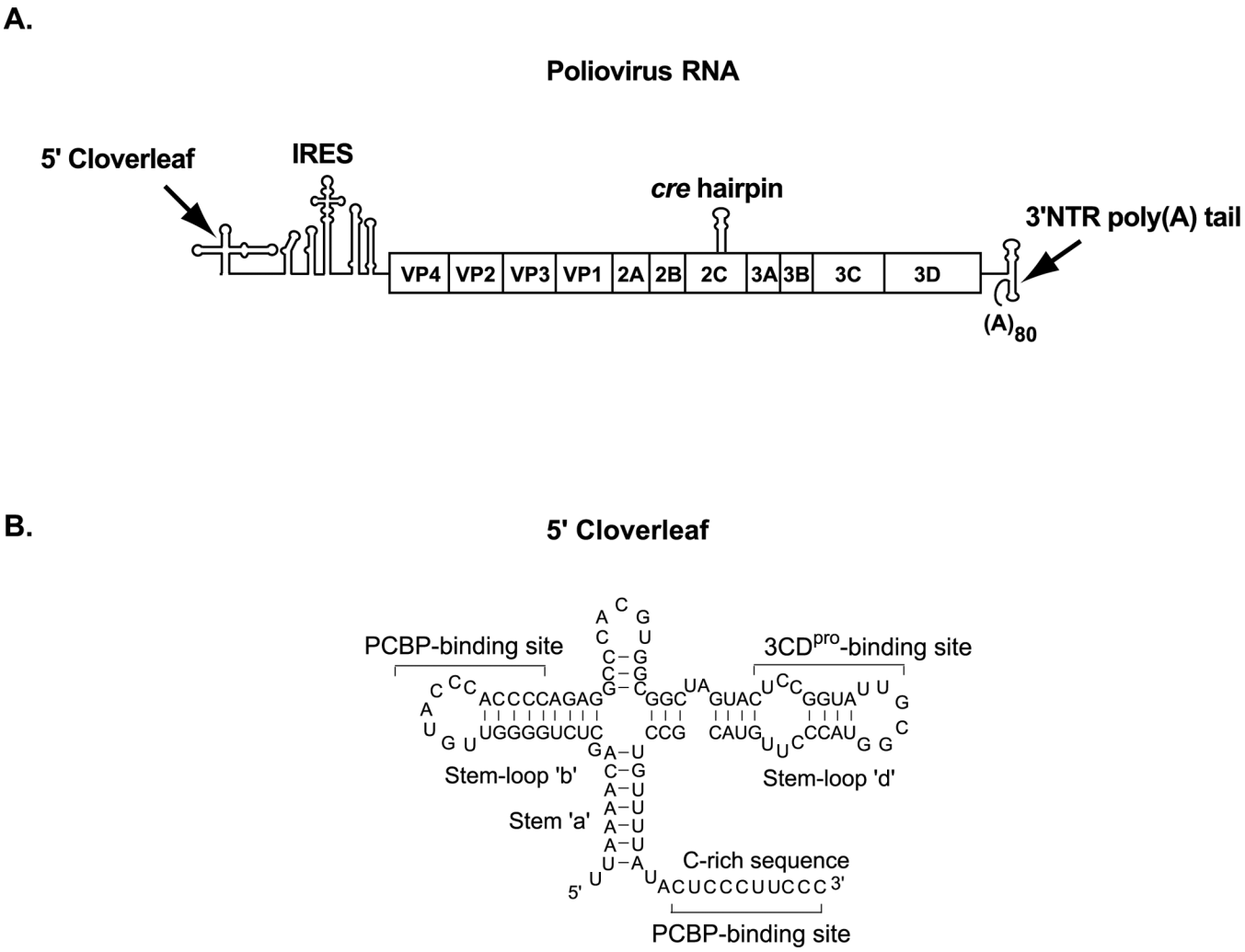

Figure 1.

(A). A schematic diagram showing the RNA structures in the poliovirus RNA genome which perform non-templated RNA functions during viral replication. The 5' cloverleaf (5'CL) and the internal ribosome entry site (IRES) are present in the 5' non-translated region (NTR) of the genome. The open reading frame in the viral genome encodes a polyprotein which is cleaved to generate the structural and replication proteins. The *cre* hairpin is present in the 2C coding region and the 3' NTR and poly(A) tail is at 3' end of the genome. (B). The secondary structure and nucleotide sequence of the 5'CL showing the binding sites for the cellular poly(C) binding protein (PCBP) and viral protein, $3CD^{pro}$.

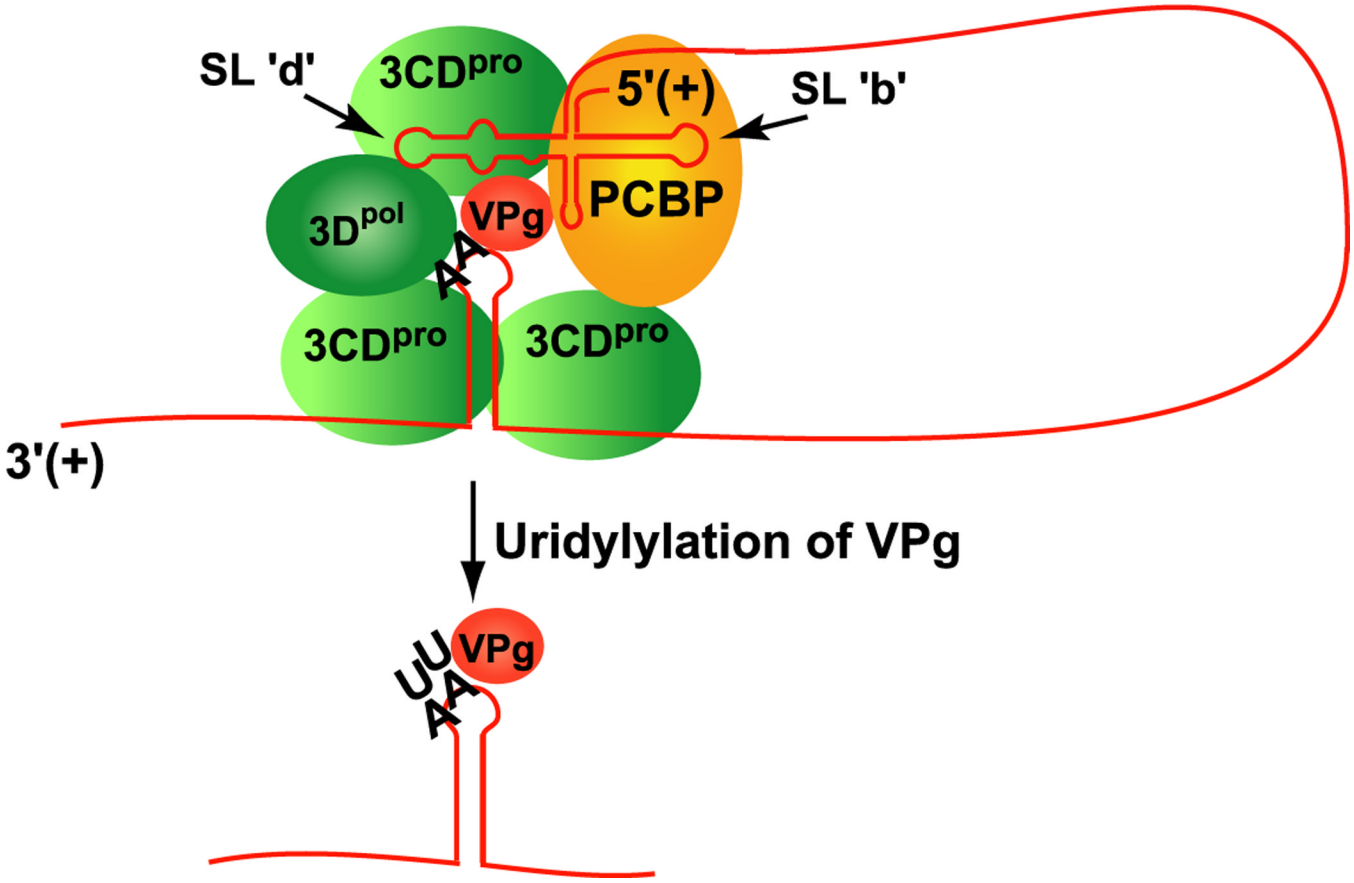

Figure 2.

Model showing the non-templated functions of the 5’CL and *cre* hairpin during *cre*-dependent VPgpUpU synthesis. The model shows long-distance interaction between the 5’CL-RNP complex, formed with $3CD^{pro}$ and PCBP, and the *cre*-RNP complex, formed with $3CD^{pro}$, $3D^{pol}$ and VPg. The interaction between the 5’CL-RNP complex and the *cre*-RNP complex results in the efficient synthesis of VPgpUpU by $3D^{pol}$. For clarity, cellular membranes are not depicted and additional viral and cellular proteins that may be required are not shown in this model and in the other models in this review.

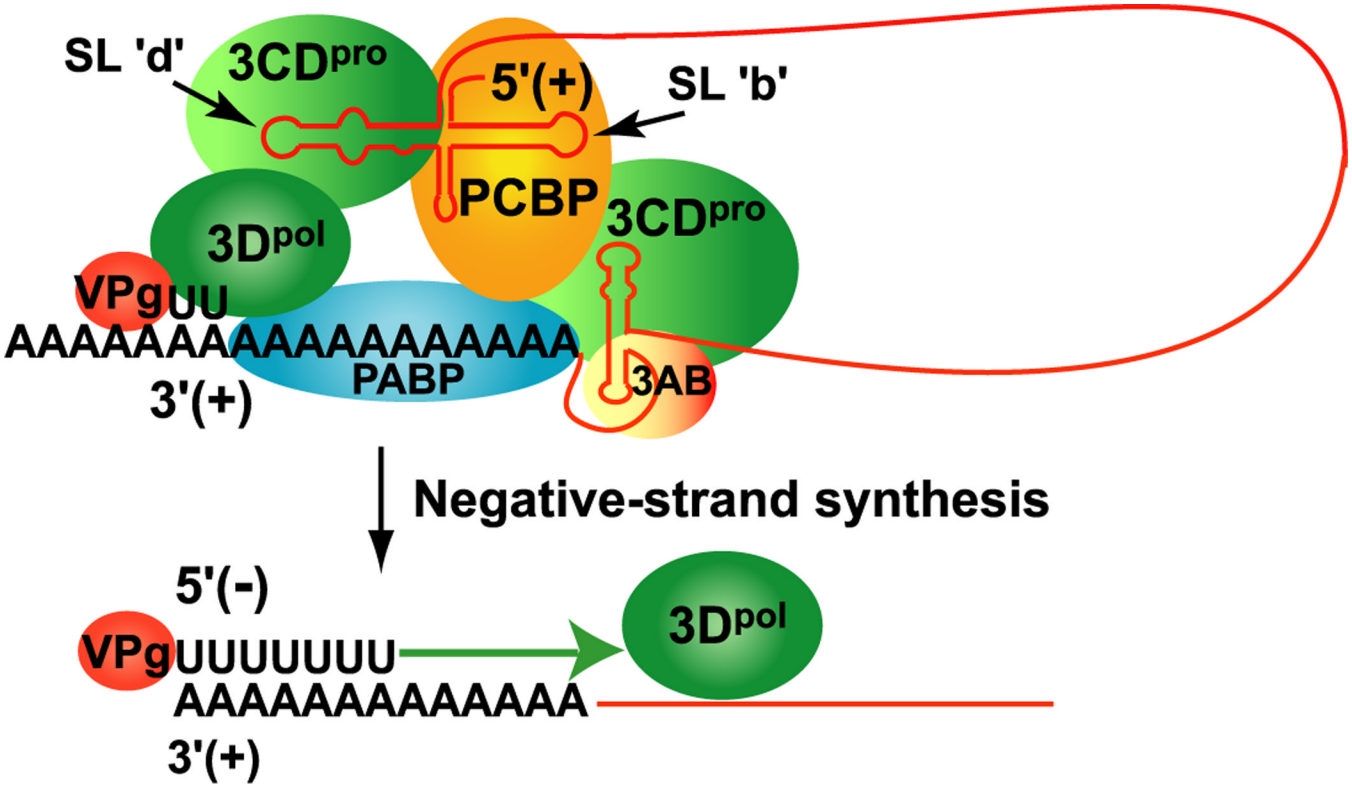

Figure 3.

Model showing non-templated functions of the 5’CL and 3’ NTR-poly(A) tail during initiation of negative-strand synthesis. The model shows the circular RNP complex used to initiate negative-strand synthesis. The long-range interaction between the 5’CL-RNP complex and the 3’ NTR-poly(A) RNP complex results in the formation of a 5’-3’ circular complex. This circular complex is thought to facilitate the initiation of negative-strand synthesis by using VPgpUpU as a primer on the 3’ poly(A) tail. This results in the synthesis of negative-strand RNA containing a 5’ terminal poly(U) sequence.

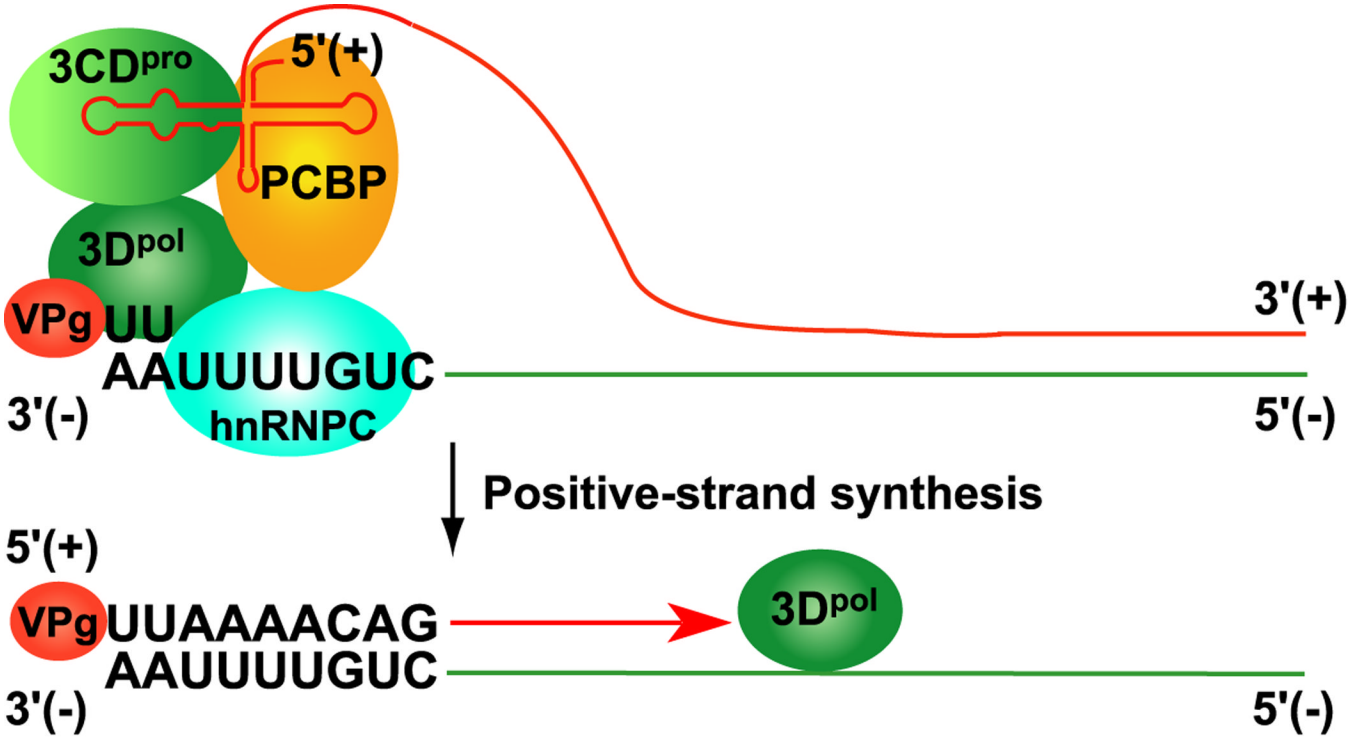

Figure 4.

Model showing non-templated functions of the 5’ CL and the 3’ terminal sequence of the negative-strand RNA during initiation of positive-strand synthesis. The model suggests that partial unwinding of the duplex RF RNA allows the cellular protein, hnRNPC, and VPgpUpU to bind at the 3’ end of the negative-strand RNA [69]. This would also facilitate the formation of the 5’CL-RNP complex in positive-strand RNA as shown in the model. It is proposed that the 5’CL-RNP complex functions in *trans* to promote the VPgpUpU primed initiation of positive-strand synthesis by $3D^{pol}$ [72]. Although not shown, it is possible that hnRNPC bound to the 5’ end of the negative-strand RNA may also be part of the positive-strand initiation complex [71].