The poliovirus 2C cis-acting replication element-mediated uridylylation of VPg is not required for synthesis of negative-sense genomes

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Nucleotides in the terminal loop of the poliovirus 2C cis-acting replication element (2C CRE), a 61 nt structured RNA, function as the template for the addition of two uridylate (U) residues to the viral protein VPg. This uridylylation reaction leads to the formation of VPgpUpU, which is used by the viral RNA polymerase as a nucleotide–peptide primer for genome replication. Although VPg primes both positive- and negative-strand replication, the specific requirement for 2C CRE-mediated uridylylation for one or both events has not been demonstrated. We have used a cell-free in vitro translation and replication reaction to demonstrate that 2C CRE is not required for the initiation of the negative-sense strand, which is synthesized in the absence of 2C CRE-mediated VPgpUpU formation. We propose that the 3′ poly(A) tail could serve as the template for the formation of a VPg–poly(U) primer that functions in the initiation of negative-sense strands.
However, the sensitivity of this in vivo assay was unlikely to detect the first round of genome replication during which the input RNA is copied into a negative-sense genome forming a doubled-stranded RNA replicative form (RF; see Fig. 1C). Therefore, the formal possibility that 2C CRE was required for positive- but not negative-strand initiation could not be excluded. Recent studies from D. J. Barton and colleagues imply that there may be differences in the initiation of opposing genome strands (Barton et al., 2001; Lyons et al., 2001) which prompted us to more precisely define the role of 2C CRE-mediated uridylylated VPg in poliovirus replication. We have studied the replication of 2C CRE-defective replicons using in vitro translation and replication reactions (IVTR) containing HeLa S10 extracts prepared as previously described (Molla et al., 1991). The HeLa S10 and Xenopus oocyte (Gamarnik & Andino, 1996) replication systems are currently the only reproducible methods of detecting the first round of genome replication. Efficient formation of positive-sense genomes in IVTR reactions requires an authentic (i.e. identical to viral RNA) 5′ end as the two G nucleotides added to the 5′ end of in vitro-synthesized RNA transcripts generated using T7 RNA polymerase block positive-sense genome synthesis (Barton et al., 1996, 1999; Herold & Andino, 2000). To overcome this block, the efficient removal of the additional nucleotides is required; this is achieved using a cis-acting hammerhead ribozyme (Herold & Andino, 2000).

Pre-initiation replication complexes, programmed with 1 μg of in vitro-transcribed RNA derived from wild-type poliovirus type 3 (PV3) replicons (pT7Rep3 and pT7Rep3+R) or from 2C CRE-defective replicons (pT7Rep3/SL3 and pT7Rep3/SL3+R), with (indicated by the + R suffix) or without a hammerhead ribozyme at their 5′ ends (Fig. 1A and Goodfellow et al., 2000), were formed in the presence of 2 mM guanidine hydrochloride to allow translation, but prevent replication (as described by Barton et al., 1995). Genome replication was subsequently monitored using [32P]UTP to label newly synthesized RNA in a guanidine hydrochloride-free environment, as previously described (Barton et al., 1995). RNA from pT7Rep3/SL3 contains eight synonymous mutations that disrupt 2C CRE structure and prevent detectable VPgpUpU formation in an in vitro uridylylation assay (Goodfellow et al., 2000). We have never succeeded in selecting revertant viruses when these mutations are present in a full-length genome, confirming a completely null phenotype (Goodfellow et al., 2000). In Fig. 1(B), lanes 1 and 2, we demonstrate for the first time that PV3-derived replicons replicate in IVTR reactions and that, as for PV1, additional nucleotides at the 5′ end of the transcript prevent efficient replication (compare the formation of SS form). The 2C CRE-defective replicons Rep3/SL3 and Rep3/SL3 + R both failed to produce positive-sense SS progeny, though the double-stranded RF was readily detectable in IVTR reactions primed with these RNAs.

![Fig. 1. IVTR analysis of poliovirus subgenomic replicons. (A) Schematic diagram illustrating the replicons used in this study indicating the nucleotides at the 5′ end of RNA synthesized in vitro using bacteriophage T7 polymerase. The pT7 prefix is omitted when indicating RNA produced from the cDNA. The cDNA for replicons with the +R suffix is preceded with a self-cleaving hammerhead ribozyme to leave a product with no non-viral nucleotides in the transcript. CAT and LUC indicate the location of the chloramphenicol acetyltransferase or luciferase reporter genes respectively. The defective 2C CRE in replicons bearing the SL3 mutations is indicated with an X. (B) Products from IVTR reactions primed with the indicated replicons were analysed by 0.8% native agarose gel electrophoresis. The replicative form (RF), present in all reactions, is indicated, together with the replicative intermediate (RI) and single strand (SS) products present only in the reaction primed with RNA from pT7Rep3+R. (C) Schematic diagram of the IVTR reaction in which the input RNA is converted to a double-stranded replicative form (RF), from which new single-stranded (SS) positive strands are generated via a replicative intermediate (RI). Solid lines, dashed lines and filled circles indicate positive-sense RNA, negative-sense RNA and VPg, respectively.](image-url)
Correct function of the hammerhead ribozyme present at the 5' end of transcripts derived from pT7Rep3+R and pT7Rep3/SL3+R was verified by monitoring the release of the ribozyme-containing RNA fragment from the full-length RNA transcript (data not shown). The formation of RF in extracts programmed with the $2^{\text{CRE}}$-defective replicon (Rep3/SL3+R) RNA requires the formation of negative-strand RNA. This implies that the $2^{\text{CRE}}$, and therefore $2^{\text{CRE}}$-mediated uridylylation of VPg, is not required for the initiation of negative-sense genome synthesis.

Prim ing of positive-sense genome synthesis occurs on the AA dinucleotide present at the 3' end of the negative-sense genome. To confirm that positive-sense RNA was not produced by Rep3/SL3+R due to lack of the nucleotide–peptide primer (VPgpUpU), we directly monitored the formation of VPgpUpU in IVTR reactions (as described by Lyons et al., 2001). VPgpUpU was readily detected in IVTR reactions programmed with wild-type PV3 replicon RNA (Rep3+R) but was absent in reactions programmed with the $2^{\text{CRE}}$-defective replicon (Rep3/SL3+R; Fig. 2, lanes 1 and 2 respectively). The labelled VPg observed in IVTR reactions was confirmed as VPgpUpU by comparison with in vitro uridylylated VPg (data not shown and Paul et al., 2000).

We have previously demonstrated that genomes containing a defective $2^{\text{CRE}}$ cannot be rescued by co-infection with a helper virus, implying that $2^{\text{CRE}}$ functions only in cis under these conditions (Goodfellow et al., 2000). To address whether the VPgpUpU primer formed from a ‘helper’ genome in IVTR reactions could function in trans, we studied the ability of luciferase-encoding replicons with functional $2^{\text{CRE}}$ structures (Rep3-L and Rep3-L+R) to restore the replication of a $2^{\text{CRE}}$-defective replicon (Rep3/SL3+R) by co-translation and replication in IVTR reactions (Fig. 3). Pre-initiation replication complexes were formed (as described above) containing $2^{\text{CRE}}$-defective replicon (Rep3/SL3+R; Fig. 3, lane 1), wild-type replicons (Rep3-L and Rep3-L+R; lanes 2 and 3) or an equal amount of $2^{\text{CRE}}$-defective replicon with wild-type replicon RNA (Fig. 3, lanes 4 and 5). RNA from the $2^{\text{CRE}}$-defective replicon (Rep3/SL3+R) is 750 nt smaller than the ‘helper’ replicons (Rep3-L and Rep3-L+R) because of the difference in the size of luciferase and chloramphenicol acetyltransferase (CAT) reporter genes present in the replicons, and so can be distinguished by gel electrophoresis.

In contrast to our previous cell-based assays (Goodfellow et al., 2000), functional $2^{\text{CRE}}$ in the luciferase-encoding replicons could rescue $2^{\text{CRE}}$-defective replicons in trans. An authentic 5' end on the ‘helper’ genome increased, but was not absolutely necessary for, the trans-rescue of the smaller CAT-encoding $2^{\text{CRE}}$-defective replicon (Fig. 3, lanes 4 and 5). The apparent difference between our previous results obtained in vivo (Goodfellow et al., 2000) and those generated in vitro are likely to be due to the lack of compartmentalization in IVTR reactions, which allows for enhanced mixing of replication complexes. The large amounts of RNA used to prime the IVTR also means that the available levels of VPgpUpU are likely to be higher than those in intact cells. Tiley et al. (2003) have recently reported the trans-rescue of an foot-and-mouth disease

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**Fig. 2.** VPgpUpU is produced only in IVTR reactions programmed with replicons containing a functional $2^{\text{CRE}}$. IVTR reactions containing P$_{32}$ UTP were primed with the indicated replicons and the products analysed by acrylamide gel electrophoresis (12-5% containing SDS/Tricine). The uridylylated VPg peptide is indicated.

**Fig. 3.** $2^{\text{CRE}}$ functions in trans in IVTR reactions. IVTR reactions were programmed with the replicon RNA (1 μg) indicated in the table and the products were analysed using native agarose/low melting point agarose (SeaPlaque; 3:1, w/w) gel electrophoresis. The single-stranded (SS) and replicative forms (RF) of CAT- and luciferase-encoding replicons (see Fig. 1A) can be distinguished by size in this analysis. Incorporation of [$^{32}$P]JUTP into ribosomal RNA (rRNA) also occurs as indicated.
virus cre mutant in vivo using a ‘helper’ virus with reduced replication.

E. Wimmer and colleagues have previously reported the formation of VPgpUpU and VPg–poly(U) in vitro using poly(A) RNA as a template (Paul et al., 1998, 2003). The efficiency of this in vitro reaction is very poor compared with VPgpUpU formation using 2C CRE as a template, and works optimally with manganese as a cofactor (Paul et al., 2000, 2003), conditions that are known to reduce the specificity of polymerase-template recognition (Arnold et al., 1999). Paul et al. (2000) suggest that the 2C CRE is the in vivo template for uridylylation of VPg, and that 2C CRE-mediated uridylylated VPg translocates to the 3’ poly(A) tail of the virus to prime the initiation of the negative-sense genome. Our results demonstrate that 2C CRE is not required for negative-strand synthesis. Furthermore, the double-stranded RF (Fig. 1C) is generated in the absence of detectable VPgpUpU. We therefore propose a new model in which 2C CRE functions to form a pool of uridylylated VPg (VPgpUpU) which is retained in the replication complex until positive-strand initiation begins. We further suggest that the 3’ poly(A) tail is likely to be the template for the uridylylation of VPg, and probably acts to form VPg–poly(U). Although apparently relatively inefficient in vitro (Paul et al., 1998), the yield of VPg–poly(U) could be marked by the length of the 3’ poly(A) tail which has been shown by Herold & Andino (2001) to have a marked effect on the efficiency of genome replication, or by the adjacent 3’ NCR sequences of the virus. Our previous analysis of VPg uridylylation using an RNA transcript encompassing the poliovirus 3’ NCR with a poly(A) tail of 9 nt indicated that the majority of the product formed in this reaction is VPg–poly(U) and not VPgpUpU (Goodfellow et al., 2003). These reactions were done in the presence of manganese; more recent studies using magnesium as a cofactor—which increases the specificity of polymerase–template interaction (Arnold et al., 1999) have suggested that the structured 3’ NCR specifically recruits 3D P0, thereby increasing the efficiency of VPg–poly(U) formation from the poly(A) tail (I. G. Goodfellow and others, unpublished). Numerous other studies also support a sequence-specific interaction of the 3’ NCR and the virus polymerase. It has been reported that poliovirus 3D P0 exhibits 5-fold greater affinity for RNA substrates containing the virus 3’ NCR (Oberste & Flanegan, 1988), an interaction also observed in the cardioviruses, in which a requirement for both the structured elements of the 3’ NCR and the poly(A) tail has been shown (Cui & Porter, 1995; Cui et al., 1993). Revertants of viruses containing debilitating mutations in the 3’ NCR have been mapped to residues in 3D P0, further supporting a direct and specific interaction of the polymerase and the 3’ NCR (Duque & Palmenberg, 2001; Meredith et al., 1999). Additional studies will be needed to define the 3’ sequences that could function in the formation of VPg–poly(U), and to confirm the role of this nucleotide–peptide primer in negative-strand synthesis.

It is interesting to note that several other products appear to be uridylylated in the IVTR reactions (Fig. 2). We speculate that these labelled products are uridylylated VPg-containing precursor proteins, though this interpretation will need verification. There are recent data to suggest that 2C CRE can function in vitro to uridylylate VPg-containing precursors (Pathak et al., 2002). Given the defect in the 2C CRE present in the RNA derived from the replicon pT7Rep3/SL3 + R, it is likely that the radiolabelled products observed are derived from poly(A)-templated VPg uridylylation. The identity of the VPg-containing precursor(s) uridylylated in vivo during poliovirus replication is the subject of ongoing analysis.

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