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Replication of Poliovirus RNA with Complete Internal Ribosome Entry Site Deletions

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***cis*-acting RNA sequences and structures in the 5′ and 3′ nontranslated regions of poliovirus RNA interact with host translation machinery and viral replication proteins to coordinately regulate the sequential translation and replication of poliovirus RNA. The poliovirus internal ribosome entry site (IRES) in the 5′ nontranslated region (NTR) has been implicated as a *cis*-active RNA required for both viral mRNA translation and viral RNA replication. To evaluate the role of the IRES in poliovirus RNA replication, we exploited the advantages of cell-free translation-replication reactions and preinitiation RNA replication complexes. Genetic complementation with helper mRNAs allowed us to create preinitiation RNA replication complexes containing RNA templates with defined deletions in the viral open reading frame and the IRES. A series of deletions revealed that no RNA elements of either the viral open reading frame or the IRES were required in *cis* for negative-strand RNA synthesis. The IRES was dispensable for both negative- and positive-strand RNA syntheses. Intriguingly, although small viral RNAs lacking the IRES replicated efficiently, the replication of genome length viral RNAs was stimulated by the presence of the IRES. These results suggest that RNA replication is not directly dependent on a template RNA first functioning as an mRNA. These results further suggest that poliovirus RNA replication is not absolutely dependent on any protein-RNA interactions involving the IRES.**

Poliovirus (PV), the prototypic member of the viral family *Picornaviridae*, is a positive-polarity RNA virus 7,441 nucleotides (nt) in length (44). PV RNA is composed of a 5′ nontranslated region (NTR), an open reading frame (ORF) encoding the viral proteins, a 3′ NTR, and a 3′-terminal poly(A) tail. PV RNA is sequentially translated and replicated within the cytoplasm of an infected cell. The 5′ NTR of PV RNA is composed of two functionally discrete RNA elements. The first 88 nt of the 5′ NTR form a cloverleaf structure involved in RNA stability and RNA replication (31, 38). The 5′ NTR also contains the internal ribosome entry site (IRES). The IRES is composed of nt 124 to ~630 [PV type 1(M) nucleotide numbering] (21) and is known to interact with canonical and noncanonical translation factors to direct ribosomes to an internal translation initiation site at nt 743 (15). The 5′-terminal cloverleaf and 3′ NTR function coordinately to mediate viral negative-strand RNA synthesis (9, 24, 31), suggesting that viral RNA may assume a conformation involving direct interactions between ribonucleoprotein complexes containing the 5′ and 3′ NTRs for the initiation of RNA replication. It is possible that interactions of PV mRNA with the cellular translation machinery alter the conformation of viral RNA, bringing the 5′ and 3′ NTRs into a proximal orientation favorable for the subsequent formation of functional RNA replication complexes.

Previous investigations suggested that the IRES possesses signals required for both viral mRNA translation and viral RNA replication (12, 27, 47). Borman et al. (12) defined a nucleotide sequence at positions 500 to 502 [PV type 1(M) nucleotide numbers] of stem-loop V of the IRES as being required in *cis* for RNA replication. This group also found that a duplication of a 46-nt sequence of stem-loop IV (the duplication was inserted at nt 343) of the IRES prevented efficient RNA replication. Ishii et al. proposed that stem-loop II of the PV type 1(M) 5′ NTR functions during both viral mRNA translation (28) and viral RNA replication (27). By using dicistronic constructs, these authors concluded that a six-base deletion of stem-loop II [PV type 1(M) nt 143 to 148] induced a replication defect in the absence of a translation defect. Shiroki et al. (47) proposed that residue 133 [SLII of the PV type 1(M) IRES] is critical for the synthesis of positive-strand RNA, functioning either in the positive- or negative-strand RNA templates. Regardless of its mechanism, SLII is important for some aspect of viral replication and pathogenesis (17, 47).

In this study, we used cell-free translation-replication reactions and preinitiation RNA replication complexes containing mutant viral RNA templates to evaluate the role of the IRES and viral ORF in RNA replication. Cell-free translation-replication reactions and preinitiation RNA replication complexes are advantageous because they support authentic viral replication (5, 7, 35) and allow for *trans* complementation of mutant RNA templates (3). Preinitiation RNA replication complexes support synchronous, sequential, asymmetric replication of VPg-linked negative- and positive-strand RNA (6). Newly synthesized VPg-linked positive-strand RNA from preinitiation RNA replication complexes is packaged into infectious virus particles (4). The requirement for the PV 5′ cloverleaf RNA in negative-strand RNA synthesis (9) and VPg uridylylation (31)

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was established by using preinitiation RNA replication complexes. The precise role of CRE(2C) in VPg uridylylation and positive-strand RNA synthesis was recently established by using preinitiation RNA replication complexes (36, 37). These previous investigations have proven the validity and authenticity of PV RNA replication within cell-free translation-replication reactions. In this study, we used preinitiation RNA replication complexes containing PV RNA templates with deletions in the IRES and viral ORF to examine the requirement for these sequences in viral RNA replication. As shown herein, lethal IRES mutations that ablate translation can be complemented with PV replication proteins provided in *trans* from a helper virus mRNA. By using *trans*-replication experiments, we found that no elements of the IRES were required for either negative- or positive-strand RNA synthesis, although replication of genome length RNAs was modestly stimulated by the presence of a functional IRES.

MATERIALS AND METHODS

cDNA and cloning. Various mutations were engineered into viral cDNA clones as described below. Plasmids were transformed and grown in SURE cells (Stratagene, La Jolla, Calif.). All mutations were confirmed by restriction analyses and DNA sequencing.

(i) pDJB14. pDJB14 was kindly provided by James B. Flanegan (University of Florida College of Medicine, Gainesville). T7 transcription of *Mlu*I-linearized pDJB14 yields DJB14 RNA. DJB14 RNA consists of the 5′-terminal 629 nt of PV RNA, PV nt 6012 to 6056, and the 3′-terminal 1,007 nt of PV RNA with a poly(A) tail 83 bases in length.

(ii) pDNVR2. pDNVR2, as previously described (31), is a cDNA encoding a chimeric viral RNA composed of the 5′ NTR of hepatitis C virus (HCV) and the PV P2-P3 coding sequence, 3′ NTR-poly(A) tail.

(iii) pDNVR10. pDNVR10 was generated by cutting pDJB14 with *Bam*HI at nt 220 and with *Bsa*BI at nt 601. The large fragment was gel purified, treated with T4 DNA polymerase to fill the 5′ overhang generated by *Bam*HI, and ligated with T4 DNA ligase.

(iv) pDNVR17. pDNVR17 was generated by site-directed mutagenesis of pDNVR10. pDNVR17 has a deletion of PV type 1(M) nt 124 to 6517. pDNVR17 was generated with the Stratagene site-directed mutagenesis kit and primers KMp17A (5′ CCCGTAACTTAGACGCACAAAACCAAAGCTTCTAGTTTG AATGACTCAGTGGC 3′) and KMp17B (5′ GCCACTGAGTCATTCAAACT AGAAGCTTTGGTTTTGTGCGTCTAAGTTACGGG 3′).

(v) pDNVR19. pDNVR19 was generated by site-directed mutagenesis of pDNVR10. pDNVR19 possesses a deletion of PV type 1(M) nt 220 to 7357. pDNVR19 was generated with a Stratagene site-directed mutagenesis kit and primers DNVR19A (5′ GCGTGGTTGAAAGCGACGGATCCTAGCTAAAA TCAGGAGTGTCTTGAC 3′) and DNVR19B (5′ GTCAAGACACTCCTGA TTTTAGCTAGGATCCGTCGCTTTCAACCACGC 3′).

(vi) pDNVR22. pDNVR22 was generated by site-directed mutagenesis of pDNVR19. pDNVR22 possesses a deletion of PV type 1(M) nt 124 to 7357. pDNVR22 was generated with a Stratagene site-directed mutagenesis kit and primers DNVR22A (5′ GACGCACAAAACCAACTAGCTAAAATCAGG 3′) and DNVR22B (5′ CCTGATTTTAGCTAGTTGGTTTTGTGCGTC 3′).

(vii) pDJB1. Plasmid pT7-PV1(A)80 encodes an infectious cDNA clone of PV RNA. pT7-PV1(A)80 has been previously described (31). pDJB1 was generated by insertion of the sequence CTAG at nt 2474.

(viii) pDNVR38. pMO-3, a plasmid encoding wild-type PV, was generously provided by Craig E. Cameron (Pennsylvania State University, University Park). pMO-3 encodes a 5′-terminal hammerhead ribozyme such that in vitro T7 transcription produces an RNA possessing an authentic PV 5′ terminus. pDNVR38 was generated by cutting pMO3 and pDJB1 with *Blp*I and *Mlu*I. Appropriate fragments were gel purified, phosphatase treated, and ligated with T4 DNA ligase.

(ix) pDNVR36. pDNVR36 was generated by site-directed mutagenesis of pDNVR38. pDNVR36 is pDNVR38 with a deletion of PV type 1(M) nt 124 to 742. pDNVR36 was generated with a Stratagene site-directed mutagenesis kit and primers DNVR35(for) (5′ CCCGTAACTTAGACGCACAAAACCAAAT GGGTGCTCAGGTTTCATCACAG 3′) and DNVR35(rev) (5′ CTGTGATG AAACCTGAGCACCCATTTGGTTTTGTGCGTCTAAGTTACGGG 3′).

Viral RNA-S10 translation reactions. PV RNAs were generated by T7 transcription of *Mlu*I-linearized plasmids with a commercially available kit (Epicentre, Madison, Wis.). With the exception of the pDNVR36 and pDNVR38 RNAs, all RNAs were produced with two 5′-terminal nonviral guanosine residues. PV mRNA translation was assayed by including [^{35}S]methionine (1.2 mCi/ml; Amersham) in HeLa S10 translation-replication reaction mixtures. HeLa cell S10 extracts (S10) and HeLa cell translation initiation factors were prepared as previously described (7). [^{35}S]methionine-labeled proteins were solubilized in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (2% SDS [Sigma], 62.5 mM Tris-HCl [pH 6.8], 0.5% 2-mercaptoethanol, 0.1% bromophenol blue, 20% glycerol). The samples were heated at 100°C for 5 min and separated by gel electrophoresis in SDS–9 to 18% polyacrylamide gels as previously described (7). [^{35}S]methionine-labeled proteins were detected by phosphorimaging (Bio-Rad, Hercules, Calif.).

Negative-strand RNA synthesis. PV negative-strand RNA synthesis was assayed with preinitiation RNA replication complexes containing PV RNA templates as previously described (6). Following 4 h of incubation at 34°C, preinitiation RNA replication complexes were isolated from HeLa S10 translation-replication reaction mixtures by centrifugation at 13,000 × *g*. Pellets containing preinitiation RNA replication complexes were resuspended in 50-μl labeling reaction mixtures containing [^{32}P]CTP and incubated at 37°C for 1 h. Under these conditions, radiolabel is incorporated into nascent negative-strand RNA as it is synthesized by the viral RNA replication complexes. Two 5′-terminal nonviral guanosine residues on the T7 RNA transcripts prevent initiation of positive-strand RNA synthesis within the preinitiation complexes. The products of the reactions were resuspended in 0.5% SDS buffer (0.5% SDS, 10 mM Tris-HCl [pH 7.5], 1.25 mM EDTA, 100 mM NaCl), phenol-chloroform extracted, ethanol precipitated, denatured with 50 mM methyl mercury hydroxide, and separated by electrophoresis in 1% agarose. ^{32}P-labeled RNAs were detected by phosphorimaging (Bio-Rad).

Positive-strand RNA synthesis. PV positive-strand RNA synthesis was assayed with preinitiation RNA replication complexes containing PV RNA templates with authentic 5′ termini as previously described (4, 6). Following 4 h of incubation at 34°C, preinitiation RNA replication complexes were isolated from HeLa S10 translation-replication reaction mixtures by centrifugation at 13,000 × *g*. Pellets containing preinitiation RNA replication complexes were resuspended in 50-μl labeling reaction mixtures containing [^{32}P]CTP and incubated at 37°C for 1 h. Under these conditions, radiolabel is incorporated sequentially into nascent negative-strand RNA and then into positive-strand RNA. The products of the reactions were resuspended in 0.5% SDS buffer (0.5% SDS, 10 mM Tris-HCl [pH 7.5], 1.25 mM EDTA, 100 mM NaCl), phenol-chloroform extracted, and ethanol precipitated. Reaction products were resuspended in a nondenaturing Tris-borate-EDTA (TBE) gel loading buffer and separated by electrophoresis in a 1% agarose–TBE gel. ^{32}P-labeled RNAs were detected by phosphorimaging (Bio-Rad).

RESULTS

Deletions within the IRES inhibited viral mRNA translation without inhibiting template activity for negative-strand RNA synthesis. To evaluate the role of the PV IRES in RNA replication, *trans*-complementation experiments were used (3, 38). DNVR2 helper mRNA (Fig. 1A; see Materials and Methods) was used to provide PV replication proteins in *trans* to subgenomic PV template RNAs (Fig. 1B). DJB14 (Fig. 1B) is a translationally competent subgenomic template RNA. DJB14 RNA possesses a wild-type PV IRES, a small ORF encoding a COOH-terminal fragment of 3D^{Pol} (Δ3D^{Pol}), and the PV 3′ NTR-poly(A) tail. Translation of DJB14 produces a COOH-terminal fragment of 3D^{Pol} denoted Δ3D^{Pol} (Fig. 1C, lane 1). DNVR10 RNA (Fig. 1B) possesses an IRES deletion spanning nt 220 to 601. This deletion eliminates stem-loops IV, V, and VI of the PV IRES. DNVR10 was translationally incompetent (Fig. 1C, lane 2; note the loss of the Δ3D^{Pol} protein fragment). Cotranslation of DNVR2 helper mRNA with the DJB14 and DNVR10 PV template RNAs resulted in production of the PV P2 and P3 replication proteins with and without the expression of Δ3D^{Pol} (Fig. 1C, lanes 1 and 2).

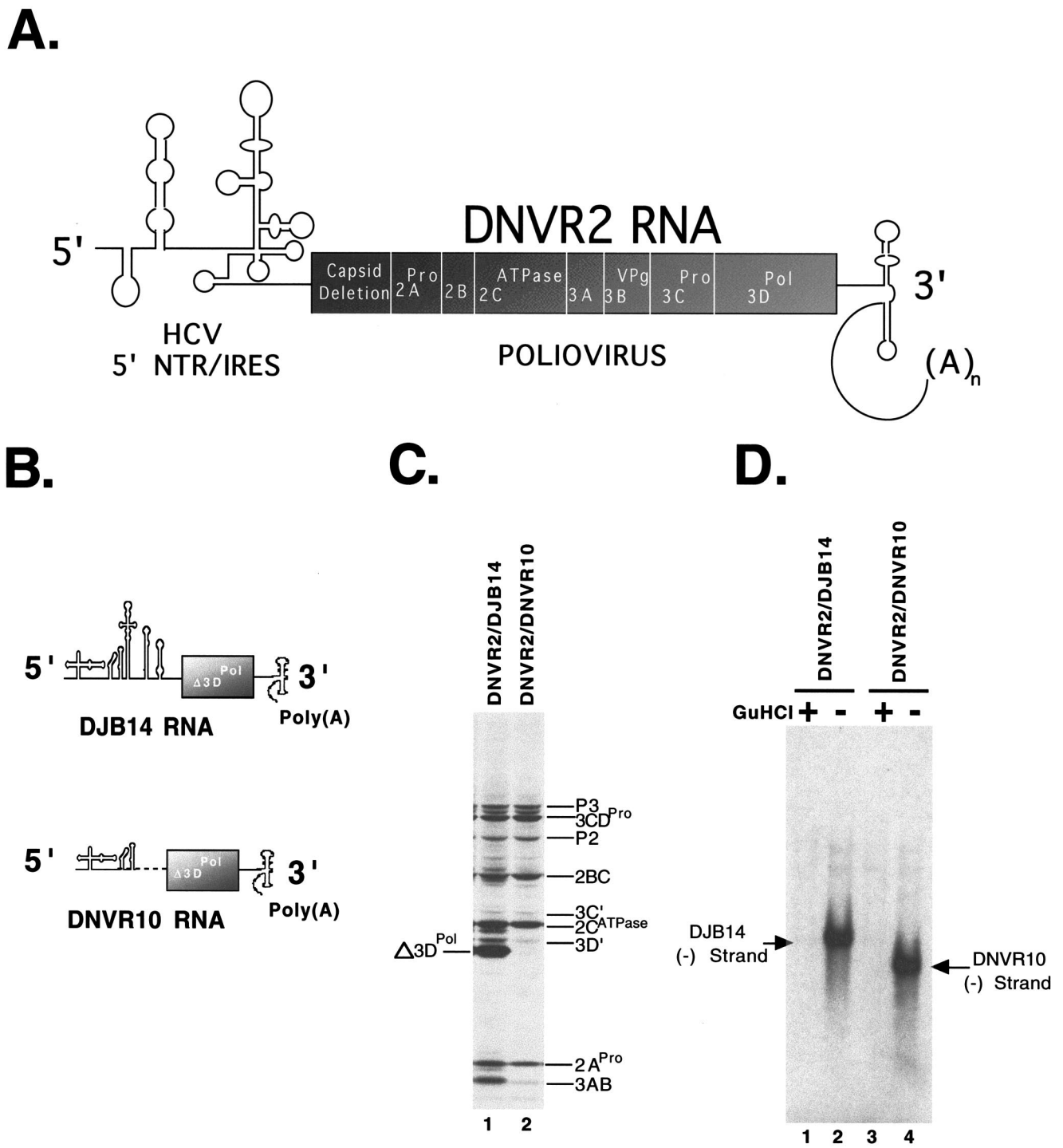

FIG. 1. IRES stem-loops IV, V, and VI are dispensable for negative-strand RNA synthesis. (A) DNVR2 helper mRNA. DNVR2 is a chimeric HCV-PV helper mRNA. This mRNA possesses the 5′ NTR of HCV, an ORF encoding the PV replication proteins, and the 3′ NTR-poly(A) tail of PV. (B) PV template RNAs. Diagrams of DJB14 and DNVR10 RNAs. DJB14 RNA possesses a wild-type PV IRES, a small ORF encoding a COOH-terminal fragment of $3D^{Pol}$ ($\Delta 3D^{Pol}$), and the PV 3′ NTR-poly(A) tail. DNVR10 RNA possesses a deletion of IRES nt 220 to 742. (C) Cotranslation of DNVR2 helper mRNA with DJB14 and DNVR10 PV template RNAs. Proteins were synthesized in reaction mixtures containing [^{35}S]methionine, DNVR2 RNA, and DJB14 RNA (lane 1) or DNVR10 RNA (lane 2). (D) Negative-strand RNA synthesis. Preinitiation RNA replication complexes formed during cotranslation reactions were assayed for the ability to synthesize negative-strand RNA as described in Materials and Methods. Preinitiation RNA replication complexes contained either DJB14 (lanes 1 and 2) or DNVR10 (lanes 3 and 4) RNA templates. Negative-strand RNA synthesis was assayed in the presence (lanes 1 and 3) and absence (lanes 2 and 4) of 2 mM guanidine HCl. Radiolabeled negative-strand RNAs fractionated by electrophoresis in a denaturing methyl mercury hydroxide agarose gel were detected by phosphorimaging. Mobilities of DJB14 and DNVR10 negative-strand RNAs are indicated.

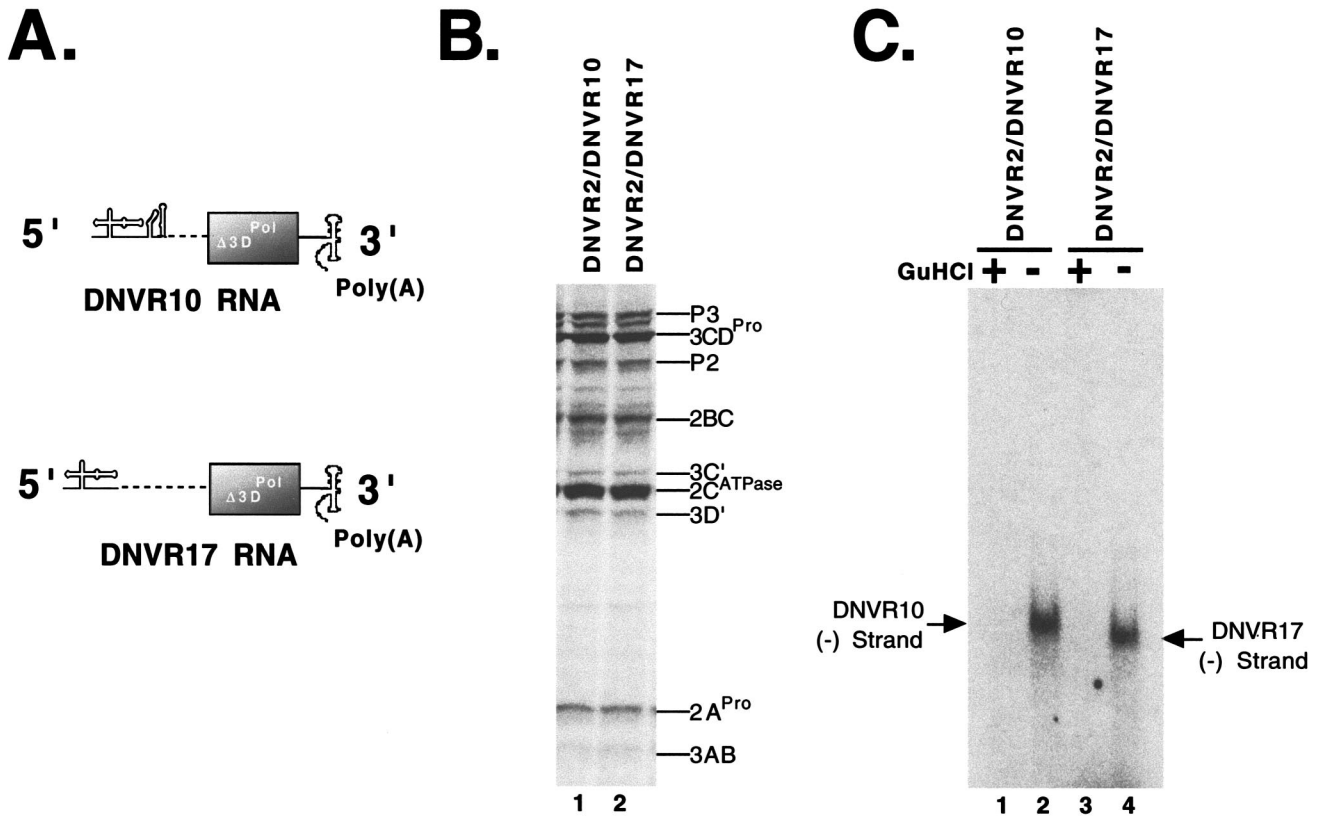

FIG. 2. Negative-strand RNA synthesis with a template with a complete IRES deletion. (A) PV template RNAs. Diagrams of DNVR10 and DNVR17 RNAs. DNVR10 possesses a deletion of IRES nt 220 to 742. DNVR17 possesses a deletion of IRES nt 124 to 742. (B) Cotranslation of DNVR2 helper mRNA with PV template RNAs. Proteins were synthesized in reaction mixtures containing [^{35}S]methionine, DNVR2 RNA, and DNVR10 RNA (lane 1) or DNVR17 RNA (lane 2). (C) Negative-strand RNA synthesis. Preinitiation RNA replication complexes formed during cotranslation reactions were assayed for the ability to synthesize negative-strand RNA as described in Materials and Methods. Preinitiation RNA replication complexes contained either DNVR10 RNA templates (lanes 1 and 2) or DNVR17 RNA templates (lanes 3 and 4). Negative-strand RNA synthesis was assayed in the presence (lanes 1 and 3) and absence (lanes 2 and 4) of 2 mM guanidine HCl (GuHCl). Radiolabeled negative-strand RNAs were fractionated by electrophoresis in a denaturing methyl mercury hydroxide agarose gel and detected by phosphorimaging. The mobilities of DNVR10 and DNVR17 negative-strand RNAs are indicated.

When we assayed negative-strand RNA synthesis, we found that, as previously shown (31), the PV replication proteins produced by the DNVR2 mRNA were able to function in *trans* to support the synthesis of negative-strand RNA on DJB14 RNA templates (Fig. 1D, lane 2). A 2 mM concentration of guanidine HCl inhibited the synthesis of negative-strand RNA (Fig. 1D, lane 1) (6). Despite the deletion of stem-loops IV, V, and VI of the PV IRES, DNVR10 RNA was a functional template for PV negative-strand RNA synthesis (Fig. 1D, lane 4). These data suggest that stem-loops IV, V, and VI of the PV IRES do not possess RNA elements required for negative-strand RNA synthesis. Additionally, it is unlikely that a replication signal residing in the PV IRES could be supplied in *trans* from the helper RNA, as DNVR2 possesses the HCV 5′ NTR (Fig. 1A).

Complete deletion of the IRES inhibits viral mRNA translation but has no deleterious effects on negative-strand RNA synthesis. Ishii et al. (27) proposed that structural elements found within PV IRES stem-loop II are multifunctional, possessing roles in both viral mRNA translation and viral RNA replication. To evaluate the role of stem-loop II (and stem-loop III) in RNA synthesis, we created DNVR17 (Fig. 2A). DNVR17 possesses an IRES deletion spanning nt 124 to 742 (this constitutes the entire sequence of the PV IRES). Like DNVR10, DNVR17 was translationally incompetent (Fig. 2B, lane 2; note the absence of the Δ3D^{Pol} protein fragment). However, as with the DJB14 and DNVR10 RNAs, the DNVR17 RNA was a competent template for negative-strand RNA synthesis (Fig. 2C, lane 4). These data indicate that the entire IRES is dispensable for negative-strand RNA synthesis.

Structural elements of the viral ORF are dispensable for negative-strand RNA synthesis. Previously, it has been shown that the PV CRE(2C)-dependent VPg uridylylation is required only for the synthesis of positive-strand RNA (36, 37). Data shown herein (Fig. 1 and 2) and elsewhere (31, 38) suggest that PV RNAs possessing partial deletions of the ORF are competent templates for negative-strand RNA synthesis in *trans*-replication assays (DJB14; Fig. 1D, lane 2). Computational analyses of predicted folds of the 3′ region of the PV 3D^{Pol} gene, however, suggest the possibility of functional RNA structures (41). To determine if the portion of the 3D^{Pol} gene encoding Δ3D^{Pol} contains any *cis*-active RNA elements required for negative-strand RNA synthesis, we engineered DNVR19 RNA (Fig. 3A). With *trans*-replication experiments,

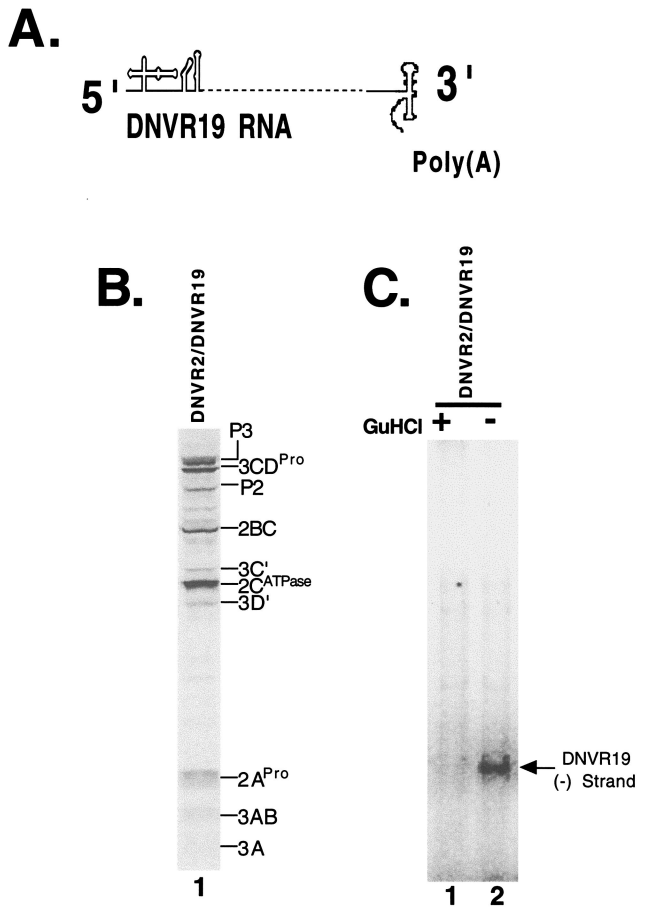

FIG. 3. Negative-strand RNA synthesis with a template with a complete ORF deletion. (A) Diagram of DNVR19 RNA. DNVR19 possesses a deletion of PV nt 220 to 7357. (B) Cotranslation of DNVR2 helper mRNA with DNVR19 template RNA. Proteins were synthesized in reaction mixtures containing [^{35}S]methionine, DNVR2 RNA, and DNVR19 RNA (lane 1). (C) Negative-strand RNA synthesis. Preinitiation RNA replication complexes formed during cotranslation reactions were assayed for the ability to synthesize negative-strand RNA as described in Materials and Methods. Preinitiation RNA replication complexes containing DNVR19 RNA templates were assayed in the presence (lane 1) and absence (lane 2) of 2 mM guanidine HCl (GuHCl). Radiolabeled DNVR19 negative-strand RNA was fractionated by electrophoresis in a denaturing methyl mercury hydroxide agarose gel and detected by phosphorimaging. The mobility of DNVR19 negative-strand RNA is indicated.

we found that deletion of the entire ORF had no adverse effect on the ability of the DNVR19 RNA to serve as a template for negative-strand RNA synthesis (Fig. 3C, lane 2). These and other (3) data indicate that there are no sequences or RNA structures in the ORF that are required in *cis* for negative-strand RNA synthesis.

A minimal template for negative-strand RNA synthesis is defined by as few as 309 nt. To extend our deletion analyses to their ultimate conclusion, we created DNVR22 RNA (Fig. 4A). DNVR22 RNA is 309 nt in length, possessing the 5′-terminal 123 nt of PV (the 5′ cloverleaf), the 3′NTR, and the 3′-terminal poly(A) tail (80 nt). Although a small portion of the sequence (~35 nt) of the 3D^{Pol} gene was retained in this construct to preserve the fold of the 3′NTR, this portion of the 3D^{Pol} sequence was previously evaluated by deletion mutagenesis and found to be dispensable for negative-strand RNA synthesis (data not shown). When cotranslated with DNVR2 helper mRNA (Fig. 4B), DNVR22 RNA was a competent template for negative-strand RNA synthesis (Fig. 4C, lane 4). These data (1, 2, 29, 34, 42, 45, 48, 53) indicate that the 5′ cloverleaf, 3′ NTR, and poly(A) tail constitute a sufficient set of *cis*-active RNA structures necessary for negative-strand RNA synthesis.

Positive-strand RNA synthesis in the absence of an IRES. As shown in Fig. 1 to 4, the IRES was not required in *cis* for negative-strand RNA synthesis. Those experiments, however, did not address the possibilities that sequences or structural elements of the IRES, or complementary sequences of the IRES in negative-strand RNA, may be required for the synthesis of positive-strand RNA. To address the potential role of IRES sequences or complementary sequences of the IRES in positive-strand RNA synthesis, we engineered DNVR36 RNA (Fig. 5A). DNVR36, DNVR38, and DJB1 RNAs (Fig. 5A) each possess a four-base insertion near the P1-P2 junction that induces a −1/+2 frameshift, preventing synthesis of the P2 and P3 replication proteins (Fig. 5B, lanes 2 and 3). Deleting the IRES prevented the expression of any proteins from DNVR36 RNA (Fig. 5B, lane 4).

DNVR2, the chimeric helper virus mRNA (Fig. 1A), was used to supply, in *trans*, equal concentrations of viral replication proteins to DJB1, DNVR36, and DNVR38 RNA templates (Fig. 6A, lanes 2 to 4) (31). DJB1 RNA templates supported negative-strand RNA synthesis, as evidenced by the presence of replicative-form RNA (Fig. 6B, lane 6). As previously reported (23, 31), DJB1 RNA templates (Fig. 5A) with two 5′-terminal nonviral G residues were incapable of synthesizing positive-strand RNA (Fig. 6B, lane 6). DNVR38 RNA, with an authentic 5′ terminus, was a competent template for both negative- and positive-strand RNA syntheses when replication proteins were provided by cotranslation with DNVR2 mRNA (Fig. 6B, lane 10). DNVR36 RNA, with a complete deletion of the PV IRES, was also a competent template for both negative- and positive-strand RNA syntheses when replication proteins were provided in *trans* by cotranslation with DNVR2 mRNA (Fig. 6B, lane 8). Quantification of the RNA synthesized from each construct revealed that DNVR36 RNA was impaired in both negative- and positive-strand RNA syntheses compared to DNVR38 RNA (Fig. 6B, compare lanes 8 and 10). Repeated experiments indicated that negative- and positive-strand RNA syntheses from the DNVR36 RNA templates were reduced to 20 to 60% of the levels of wild-type RNA possessing the IRES (DNVR38 RNA). Thus, while the IRES sequences were not absolutely required in *cis* for either negative- or positive-strand RNA synthesis, they stimulated RNA replication in genome length RNA templates (Fig. 6B). In contrast, the IRES did not stimulate RNA synthesis in small subgenomic-length templates (Fig. 1).

DISCUSSION

Two important conclusions can be drawn from this investigation: (i) translation of PV RNA is not an absolute prerequisite for RNA replication, and (ii) specific IRES-protein interactions are not absolutely required for PV RNA replication.

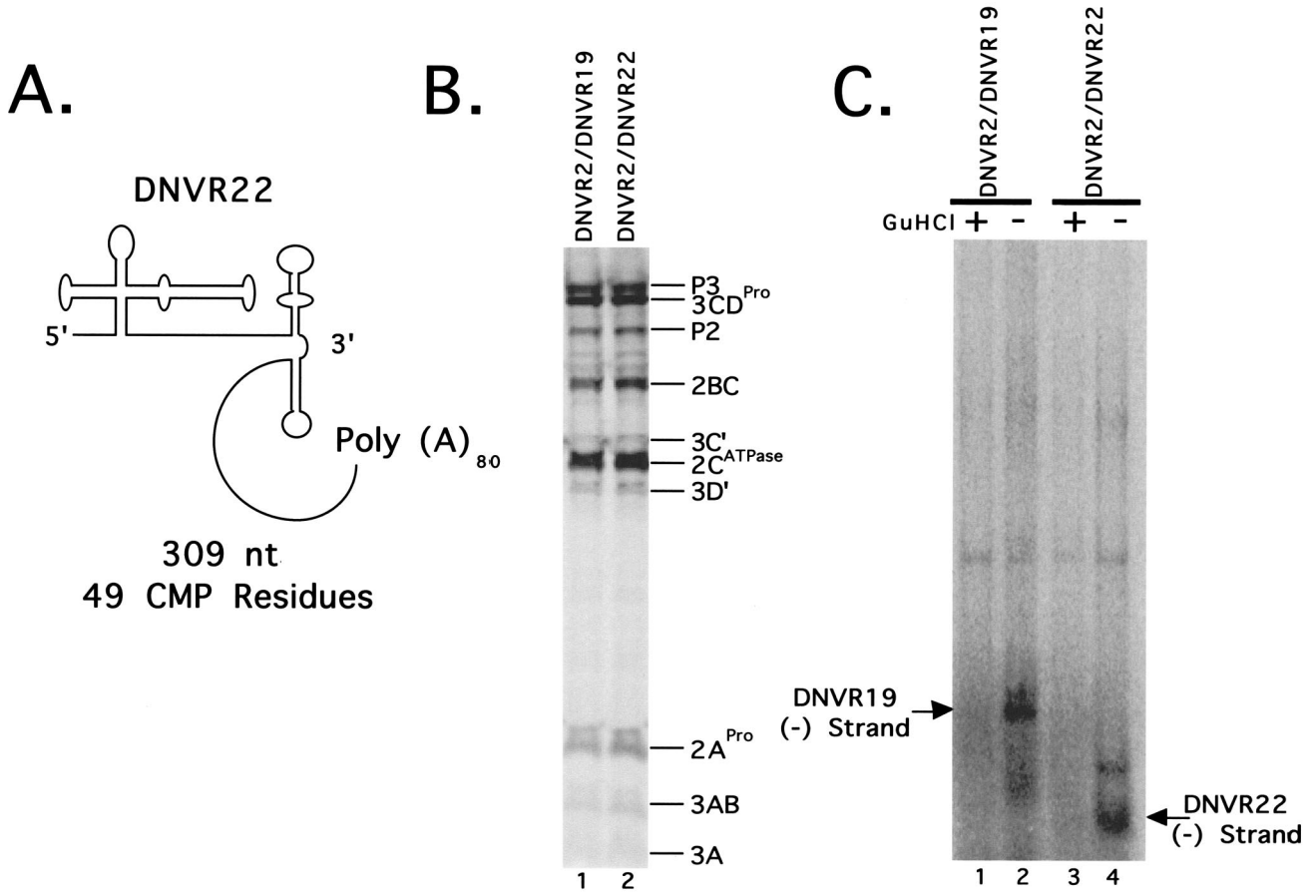

FIG. 4. Negative-strand RNA synthesis with a minimally sufficient RNA template. (A) Diagram of DNVR22 RNA. DNVR22 RNA possesses the 5′-terminal 123 nt (cloverleaf) and the 3′ NTR-poly(A) tail of PV. (B) Cotranslation of DNVR2 helper mRNA with PV template RNAs. Proteins were synthesized in reaction mixtures containing [^{35}S]methionine, DNVR2 RNA, and DNVR19 RNA (lane 1) or DNVR22 RNA (lane 2). (C) Negative-strand RNA synthesis. Preinitiation RNA replication complexes formed during cotranslation reactions were assayed for the ability to synthesize negative-strand RNA as described in Materials and Methods. Preinitiation RNA replication complexes contained either DNVR19 (lanes 1 and 2) or DNVR22 (lanes 3 and 4) RNA templates. Negative-strand RNA synthesis was assayed in the presence (lanes 1 and 3) and absence (lanes 2 and 4) of 2 mM guanidine HCl (GuHCl). Radiolabeled negative-strand RNAs were fractionated by electrophoresis in a denaturing methyl mercury hydroxide agarose gel and detected by phosphorimaging. The mobilities of DNVR19 and DNVR22 negative-strand RNAs are indicated.

Previous investigations implied that sequences and/or structures within the IRES are directly required for both viral mRNA translation and viral RNA replication (47). In this investigation, we found that the PV IRES is not directly involved in RNA replication, as it was dispensable for both negative- and positive-strand RNA syntheses. Thus, while the IRES is clearly required for viral mRNA translation (14, 32, 39, 49, 52), it does not appear to be involved directly in viral RNA replication. A PV RNA template composed of the 5′ cloverleaf RNA, 3′ NTR, and poly(A) tail was sufficient for negative-strand RNA synthesis within preinitiation RNA replication complexes. This minimal RNA template contains the 5′- and 3′-terminal *cis*-active RNA elements of PV RNA characterized in previous investigations (16). Although some investigations suggest that the 3′ NTR is required for viral RNA replication (42, 45), PV containing complete 3′ NTR deletions replicates relatively well (51). Additional studies may help reveal the role(s) of the 3′ NTR in PV replication. CRE(2C), a *cis*-active RNA element in the viral ORF, is required for positive-strand RNA synthesis but is not required for negative-strand RNA synthesis (36, 37).

It is important to note that our study has been done in vitro with HeLa cell extracts and that the IRES could be needed for efficient RNA replication in vivo, in addition to its role in viral mRNA translation. Although cytoplasmic HeLa cell extracts appear to support all of the metabolic steps of PV replication (3, 35, 37), such reactions may not faithfully mimic all circumstances of natural virus replication. Neural polypyrimidine tract-binding protein affects PV IRES-dependent translation in a tissue-specific manner (20, 22, 43). Furthermore, when the PV IRES is replaced with the HCV IRES, the chimeric PV fails to replicate in neurons (56) but such chimeric constructs replicate well in other tissues and in tissue culture cells (30, 56–58). Thus, various tissues could contain specific translation and/or RNA replication factors that are unnecessary for translation and RNA replication in HeLa cell extracts. Also, as discussed in more detail below, the PV IRES did potentiate PV RNA replication in genome length RNA constructs in our in

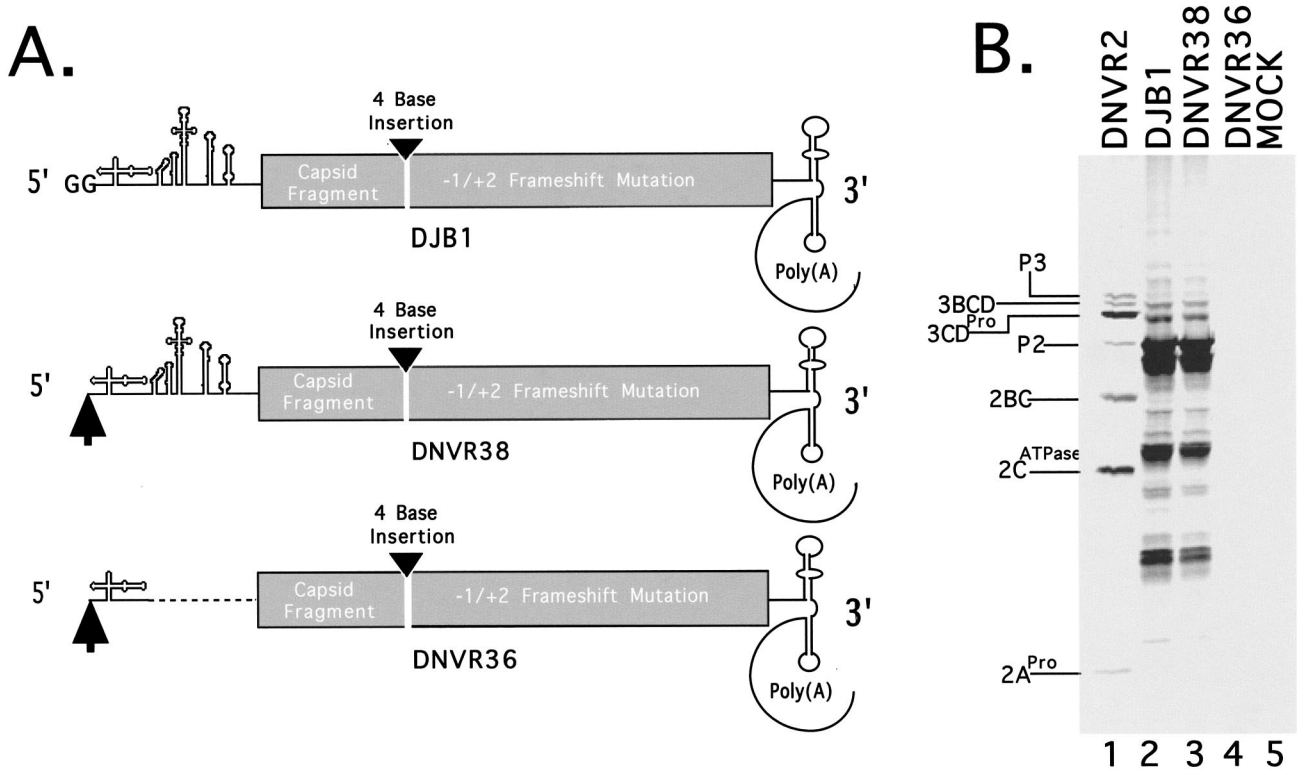

FIG. 5. Deletion of the IRES from a full-length RNA template. (A) Diagrams of genome length PV RNA templates. DJB1 and DNVR38 possess a wild-type IRES, while DNVR36 possesses a complete deletion of the IRES. Each of these template RNAs possesses a four-base insertion (CTAG) at PV nt 2474 to prevent translation of the P2 and P3 replication proteins. DJB1 possesses two 5′-terminal G residues that inhibit the initiation of positive-strand RNA synthesis (3, 23). DNVR38 and DNVR36 possess authentic 5′ termini. (B) Translation of PV RNAs. Proteins were synthesized in reaction mixtures containing [^{35}S]methionine and DNVR2 RNA (lane 1), DJB1 RNA (lane 2), DNVR38 RNA (lane 3), DNVR36 RNA (lane 4), or no RNA (lane 5). Radiolabeled proteins fractionated by SDS-PAGE were detected by phosphorimaging.

vitro experiments, potentially owing to its influence on the topology of PV RNA in conjunction with host cell translation machinery.

Template circularization. Previous investigations (9, 24, 37) indicated that PV RNA replication requires the interaction of the 5′-terminal cloverleaf and the 3′ NTR via an RNA-protein-protein-RNA bridge. Within mRNP complexes, viral mRNA, like cellular mRNA, may assume a conformation in which the 5′ and 3′ termini are proximally oriented (10, 46). This circularized conformation, imposed by translation factors, may be maintained as viral mRNA transforms into a template for RNA replication (9, 24). Because viral mRNA is normally translated before serving as a template for RNA replication (40), it was possible that circularization of the RNA by the translation machinery was a prerequisite for RNA replication. As shown herein, the IRES was dispensable for the formation of RNA replication complexes and the synthesis of negative-strand RNA. Small subgenomic PV constructs lacking the entirety of the IRES (DNVR17 and DNVR22) were functional, efficient templates for negative-strand RNA synthesis. These constructs, however, may possess "de facto" 5′-3′ interactions owing to their relatively small size. De facto 5′-3′ interactions may obviate the requirement for mRNA translation to circularize the template for RNA replication as postulated above. To address this issue, we examined the replication of a nearly full-length PV RNA with a complete IRES deletion. DNVR36 RNA, a nearly genome length PV RNA without an IRES, was a functional template for both negative- and positive-strand RNA syntheses (Fig. 6B, lane 8). RNA synthesis from DNVR36 RNA templates, however, was reduced to 20 to 60% of that from a control template with an IRES (Fig. 6B, compare lane 8 to lane 10). These data suggest that while the IRES does not possess any replication signals that are directly required for RNA replication, the interaction of the IRES with the translational machinery may induce tertiary interactions that facilitate or enhance the initiation of RNA replication. We speculate that interactions between the viral mRNA and translation factors may potentiate 5′-3′ RNA interactions that are favorable for the subsequent formation of functional preinitiation RNA replication complexes.

Dicistronic constructs. Previous investigations concerning potential RNA replication signals within the PV IRES used dicistronic RNA constructs (12, 27, 47, 55). Dicistronic RNAs are advantageous because translation of the nonstructural or replication proteins is mediated by a second, heterologous IRES located upstream of the replication protein genes. With dicistronic constructs, mutations can be engineered into the PV IRES without disrupting the expression of the P2 and P3 replication proteins. When the PV IRES was mutated in such dicistronic RNAs, RNA replication was profoundly inhibited in transfected cells (12, 27, 47, 55). The data presented herein demonstrated that comparable IRES mutations in monocis-

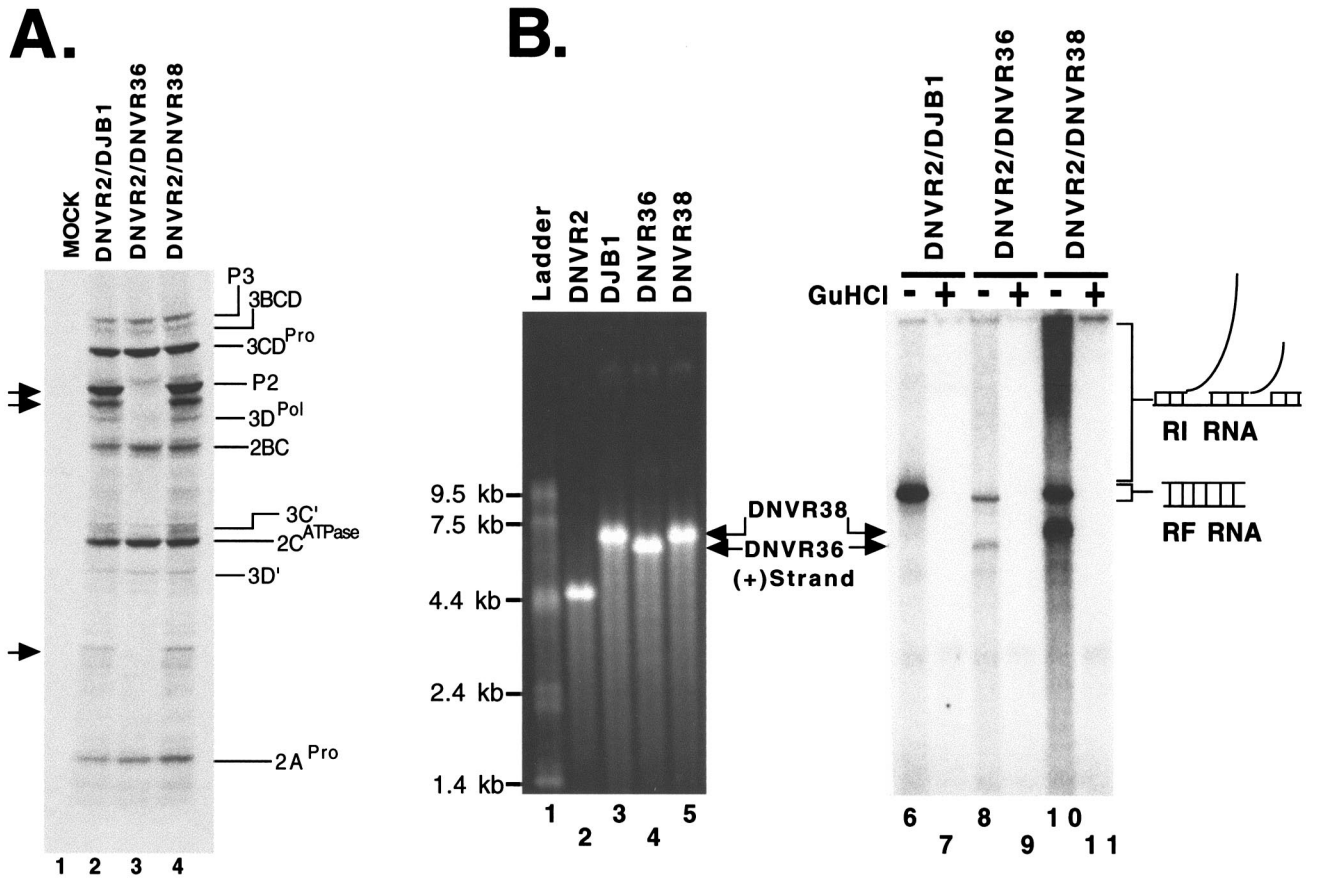

FIG. 6. The IRES is dispensable for positive-strand RNA synthesis. (A) Cotranslation of helper mRNA and template RNAs. PV proteins were synthesized in reaction mixtures containing [^{35}S]methionine, DNVR2, and either DJB1 RNA (lane 2), DNVR36 RNA (lane 3), or DNVR38 RNA (lane 4). Radiolabeled proteins fractionated by SDS-PAGE were detected by phosphorimaging. Arrows mark the mobility of frameshifted protein fragments from DJB1 and DNVR38 RNAs. (B) Positive-strand RNA synthesis. PV RNA was synthesized in reaction mixtures containing [α-^{32}P]CTP by preinitiation RNA replication complexes containing either a DJB1 template (lanes 6 and 7), a DNVR36 template (lanes 8 and 9), or a DNVR38 template (lanes 10 and 11). Reactions were performed in the presence (lanes 7, 9, and 11) and absence (lanes 6, 8, and 10) of 2 mM guanidine HCl (GuHCl). Products from the reactions were fractionated by electrophoresis in a nondenaturing 1% agarose–TBE gel. Radiolabeled RNA was detected by phosphorimaging. An RNA ladder (lane 1), DNVR2 helper mRNA (lane 2), and positive-strand template RNAs were used as markers in the gel. Lanes: 3, DJB1 RNA; 4, DNVR36 RNA; 5, DNVR38 RNA. The mobilities of positive-strand, replicative-form (RF), and replicative-intermediate (RI) RNAs are indicated.

tronic mRNAs only modestly diminished PV RNA replication by preinitiation RNA replication complexes. If translation machinery can modify the conformation of viral mRNA (10), then mutations within the PV IRES of a dicistronic construct might allow the translation machinery to alter the conformation of viral mRNA in a manner unfavorable for RNA replication. In particular, mutation of the PV IRES within dicistronic templates may allow the cellular translation machinery to remodel the mRNP, realigning the 3′ NTR to be proximal to the internal encephalomyocarditis virus IRES. Such a lariat conformation of viral mRNA within mRNPs could be disadvantageous for subsequent RNA replication because it could prevent the 5′-3′ interactions necessary for the initiation of negative-strand RNA synthesis (9, 24, 37). Deleting the IRES from monocistronic constructs, such as those in this investigation, would not lead to the formation of translation-induced lariat structures disadvantageous to RNA replication. In addition, because multiple rounds of RNA translation and amplification are required to detect RNA replication in transfected cells, modest defects in replication may be more profound within transfected cells than within preinitiation RNA replication complexes, where RNA replication is restricted to one round (8).

Protein-RNA interactions of the IRES and RNA replication. The PV IRES interacts with a number of cellular RNA binding proteins in addition to the canonical translation initiation factors. These proteins include poly(rC) binding protein 2 (PCBP2) (1, 2, 11, 19, 54, 55), the autoantigen La (33, 50), polypyrimidine tract-binding protein (25), and Unr (a cold shock domain family member) (13, 26). The exact role(s) of each of these protein-IRES interactions during translation is still debated. Although various IRES-protein interactions could be involved in RNA replication, only the PCBP–IRES-cloverleaf interaction has been implicated directly in RNA replication (55). The PCBP2-IRES interaction may regulate the switch between translation and viral RNA replication (18, 19, 55). Previously (38), we found that PV negative-strand RNA synthesis was unaffected by ribohomopoly(C) competitor RNA even though such conditions functionally inhibited

PCBP-RNA interactions. Data presented herein, generated with PV RNA templates with complete IRES deletions, support the conclusion that specific IRES-protein interactions are completely dispensable for both negative- and positive-strand RNA syntheses. Nonetheless, because we use puromycin to remove ribosomes from viral RNA templates within preinitiation RNA replication complexes (8), our results do not preclude the indirect role(s) of specific IRES-protein interactions in regulating the switch between translation and replication. Furthermore, as mentioned above, our results do not exclude the indirect role(s) of specific IRES-protein interactions in modifying the topology of viral RNA to facilitate the formation of functional replication complexes.

Other potential indirect roles of the IRES. PV replication proteins function efficiently in *trans* in the context of cell-free translation-replication reactions (Fig. 1 to 6) (31, 38). In contrast, during the infection of a susceptible cell by PV, replication proteins cannot be provided efficiently in *trans* (40). The inability to complement PV RNA replication in *trans* in vivo led Novak and Kirkegaard (40) to suggest a potential indirect role for the IRES during RNA replication. These investigators proposed that RNA synthesis may be subordinate to viral mRNA translation because ribosomes may be required to traverse the viral ORF to ablate certain secondary or tertiary structures, which would otherwise prevent the successful transit of the viral replicase. Work presented herein indicates that there is no requirement for a ribosome to traverse the ORF encoding viral replication proteins prior to RNA synthesis. DNVR38 RNA possesses the complete viral ORF, however, a −1/+2 frameshift mutation prevented the generation of the P2 and P3 replication proteins. As ribosomes were unable to traverse the entirety of the PV ORF in DNVR38 RNA and this RNA was an efficient template for viral RNA replication, it is unlikely that the coupling of translation and RNA replication is dependent on the ability of ribosomes to traverse the ORF. A more likely explanation for the inability to provide PV replication proteins in *trans* in vivo may simply involve diffusion barriers. Our data suggest that the *cis*-acting nature of the viral replication proteins for RNA synthesis in vivo may result from an inability of proteins generated in discrete locations within the cell to diffuse to distant sites in concentrations or stoichiometries sufficient to support RNA replication in *trans*. In the context of cell-free translation-replication reactions, homogenized cytoplasmic extracts containing saturating concentrations of helper mRNA overcome potential diffusion barriers, allowing efficient complementation.

In summary, our data indicate that the IRES is dispensable for RNA replication when PV replication proteins are provided efficiently in *trans*. These results indicate that specific IRES-protein interactions are not absolutely required for PV RNA replication. Nonetheless, the IRES and cellular translation machinery may influence the conformation of viral RNA to facilitate the 5′-3′ interactions necessary for the initiation of RNA replication.

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