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Poly(rC) binding proteins mediate poliovirus mRNA stability

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ABSTRACT

The 5′-terminal 88 nt of poliovirus RNA fold into a cloverleaf RNA structure and form ribonucleoprotein complexes with poly(rC) binding proteins (PCBPs; AV Gamarnik, R Andino, *RNA*, 1997, *3*:882–892; TB Parsley, JS Towner, LB Blyn, E Ehrenfeld, BL Semler, *RNA*, 1997, *3*:1124–1134). To determine the functional role of these ribonucleoprotein complexes in poliovirus replication, HeLa S10 translation-replication reactions were used to quantitatively assay poliovirus mRNA stability, poliovirus mRNA translation, and poliovirus negative-strand RNA synthesis. Ribohomopoly(C) RNA competitor rendered wild-type poliovirus mRNA unstable in these reactions. A 5′-terminal 7-methylguanosine cap prevented the degradation of wild-type poliovirus mRNA in the presence of ribohomopoly(C) competitor. Ribohomopoly(A), -(G), and -(U) did not adversely affect poliovirus mRNA stability. Ribohomopoly(C) competitor RNA inhibited the translation of poliovirus mRNA but did not inhibit poliovirus negative-strand RNA synthesis when poliovirus replication proteins were provided *in trans* using a chimeric helper mRNA possessing the hepatitis C virus IRES. A C24A mutation prevented UV crosslinking of PCBPs to 5′ cloverleaf RNA and rendered poliovirus mRNA unstable. A 5′-terminal 7-methylguanosine cap blocked the degradation of C24A mutant poliovirus mRNA. The C24A mutation did not inhibit the translation of poliovirus mRNA nor diminish viral negative-strand RNA synthesis relative to wild-type RNA. These data support the conclusion that poly(rC) binding protein(s) mediate the stability of poliovirus mRNA by binding to the 5′-terminal cloverleaf structure of poliovirus mRNA. Because of the general conservation of 5′ cloverleaf RNA sequences among picornaviruses, including C24 in loop b of the cloverleaf, we suggest that viral mRNA stability of polioviruses, coxsackieviruses, echoviruses, and rhinoviruses is mediated by interactions between PCBPs and 5′ cloverleaf RNA.

Keywords: cloverleaf; mRNA stability; picornavirus; poliovirus; poly(rC) binding proteins

INTRODUCTION

Poliovirus is the prototypic member of the enterovirus genus of the Picornaviridae family of positive-strand RNA animal viruses. The genome of poliovirus is a single-stranded positive-sense RNA molecule approximately 7,500 nt in length. The 5′-terminus of poliovirus RNA is covalently linked to VPg, a 22 amino acid long viral protein (Lee et al., 1977; Nomoto et al., 1977; Ambros & Baltimore, 1978). The viral RNA possesses a 742-base 5′ nontranslated region, an open reading frame encoding the viral polyprotein, a 3′ nontranslated region, and a 3′-terminal poly(A) tail. Poliovirus RNA serves two temporally sequential roles in the cytoplasm of an infected cell; first, as a mRNA for viral protein synthesis, and second, as a template RNA for viral negative-strand RNA synthesis during RNA replication (Novak & Kirkegaard, 1994). As with other positive-strand RNA viruses, the genome RNA of poliovirus is sufficient to initiate a complete round of viral replication in the cytoplasm of a susceptible cell. Upon the delivery of poliovirus RNA into the cytoplasm of an infected cell, VPg is removed by a cellular enzyme (Ambros & Baltimore, 1980). Therefore, poliovirus mRNA does not possess a 5′-terminal cap structure typical of eukaryotic mRNAs but rather a 5′-terminal pUpU (Hewlett et al., 1976; Nomoto et al., 1976). Normally, uncapped mRNAs are rapidly degraded by 5′ exonuclease (reviewed in Decker & Parker, 1994). Poliovirus mRNA is very stable despite the absence of a 5′-terminal cap structure.

The 5′-terminal 88 nt of poliovirus mRNA forms a cloverleaf RNA structure (Andino et al., 1990). This cloverleaf RNA structure is highly conserved among enteroviruses, parechoviruses, and rhinoviruses; suggesting a common function (Zell & Stelzner, 1997). For

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poliovirus, this 5′-terminal RNA sequence and structure has been shown to possess the ability to form various ribonucleoprotein complexes (Andino et al., 1990, 1993; Harris et al., 1994; Gamarnik & Andino, 1997; Parsley et al., 1997). In one of these ribonucleoprotein complexes, cellular poly(rC) binding proteins bind to the cloverleaf structure (Gamarnik & Andino, 1997). Poly(rC) binding proteins 1 and 2 form a UV-light induced covalent bond with nt 24 of the 5′-terminal cloverleaf of poliovirus RNA (Andino et al., 1993). In a second ribonucleoprotein complex, both poly(rC) binding protein(s) and viral protein 3CD bind to the 5′-terminal cloverleaf structure (Gamarnik & Andino, 1997; Parsley et al., 1997). A mutation of poliovirus nt 24 from C to A diminishes the ability of poly(rC) binding proteins to form these ribonucleoprotein complexes in vitro (Andino et al., 1993). A C24A mutation also diminishes viral replication in vivo, producing plaques 20% to 40% smaller than wild-type virus (Andino et al., 1993), suggesting that the 5′-terminal ribonucleoprotein complexes containing poly(rC) binding protein(s) are required for viral replication. The role(s) of these 5′-terminal ribonucleoprotein complexes in poliovirus replication are under active investigation (Xiang et al., 1995; Parsley et al., 1997; Gamarnik & Andino, 1998). Some possible functions for these 5′-terminal ribonucleoprotein complexes include the regulation of translation initiation (Gamarnik & Andino, 1998), the initiation of negative-strand RNA synthesis (Barton et al., 2001), and the initiation of positive-strand RNA synthesis (Andino et al., 1993).

Poly(rC) binding proteins 1 and 2 are related KH domain RNA binding proteins (Kiledjian et al., 1995; Leffers et al., 1995). Poly(rC) binding proteins 1 and 2 are expressed in most human tissues with especially high levels of poly(rC) binding protein 1 expressed in skeletal muscle (Aasheim et al., 1994; Leffers et al., 1995). The normal function of these proteins in cells may involve the metabolism of cellular mRNAs. Poly(rC) binding proteins 1 and 2 have been shown to be associated with α-globin messenger ribonucleoprotein complexes (Kiledjian et al., 1995; Wang et al., 1995) and erythropoietin messenger ribonucleoprotein complexes (Czyzyk-Krzeska & Bendixen, 1999). α-globin and erythropoietin mRNAs are very stable. The 3′ NTRs of these mRNAs mediate their stability via ribonucleoprotein complexes containing poly(rC) binding proteins 1 and 2 (Kiledjian et al., 1995, 1997; Wang et al., 1995; Russell et al., 1998; Czyzyk-Krzeska & Bendixen, 1999). Ribohomopoly(C) competitor RNA has been used to disrupt these ribonucleoprotein complexes in vitro (Wang et al., 1995; Czyzyk-Krzeska & Bendixen, 1999). In this study, the functional role of the poliovirus 5′-terminal ribonucleoprotein complexes containing poly(rC) binding proteins were studied. HeLa S10 translation-replication reactions were used to assay poliovirus mRNA stability, poliovirus mRNA translation, and poliovirus negative-strand RNA synthesis. HeLa S10 translation-replication reactions are advantageous because they support authentic translation and replication of the exogenous viral mRNA used to program the reaction (Molla et al., 1991; Barton & Flanegan, 1993, 1997; Barton et al., 1995). Furthermore, the stability of the exogenous viral mRNA incubated in these reactions can be easily assayed. The data in this report support the conclusion that poly(rC) binding proteins mediate the stability of poliovirus mRNA by binding to the 5′-terminal cloverleaf of poliovirus mRNA.

RESULTS

Ribohomopolymers [poly(A), poly(G), poly(C), and poly(U)] were added to HeLa S10 translation-replication reactions as competitor RNAs to determine whether cellular ribohomopolymer RNA binding proteins were essential for poliovirus mRNA stability, mRNA translation, or negative-strand RNA synthesis.

Ribohomopolymers and poliovirus mRNA stability

Poliovirus mRNA stability was assayed in HeLa S10 translation-replication reactions by programming the reactions with ^{32}P-labeled viral mRNA and following the integrity of the viral mRNA over time of incubation (Fig. 1). Poliovirus mRNA was synthesized by T7 transcription of a cDNA clone encoding RNA2, a subgenomic RNA replicon possessing an in-frame deletion of capsid genes (Collis et al., 1992). This RNA construct is advantageous because the capsid proteins are unnecessary for poliovirus RNA replication and the deletion of the capsid genes dramatically reduces the biosafety concerns relative to the use of infectious poliovirus RNA. Furthermore, RNA2 translates and replicates more robustly in HeLa S10 translation-replication reactions than full-length transcripts of poliovirus RNA. Thus, for the experiments described in this article, T7 transcripts of RNA2 were defined as "wild-type poliovirus mRNA." T7 transcripts of RNA2 possess two 5′-terminal nonviral G residues, an in-frame deletion of capsid genes, and a 3′-terminal poly(A) tail 80 bases in length. The following experiments do not address the effect, if any, of the 5′-terminal nonviral G residues on poliovirus mRNA stability.

Wild-type poliovirus mRNA is remarkably stable in HeLa S10 translation-replication reactions despite the absence of a 5′-terminal cap structure (Fig. 1). In the absence of ribohomopolymer competitor, 49% of the ^{32}P-labeled viral mRNA programmed into the reaction remained intact after 3 h of incubation at 34 °C (Fig. 1B, lane 3 versus lane 2). Ribohomopoly(A) and -(U) had little effect on the stability of poliovirus mRNA whereas ribohomopoly(G) improved the stability of poliovirus mRNA slightly (Fig. 1B, lanes 4, 5, and 7 versus

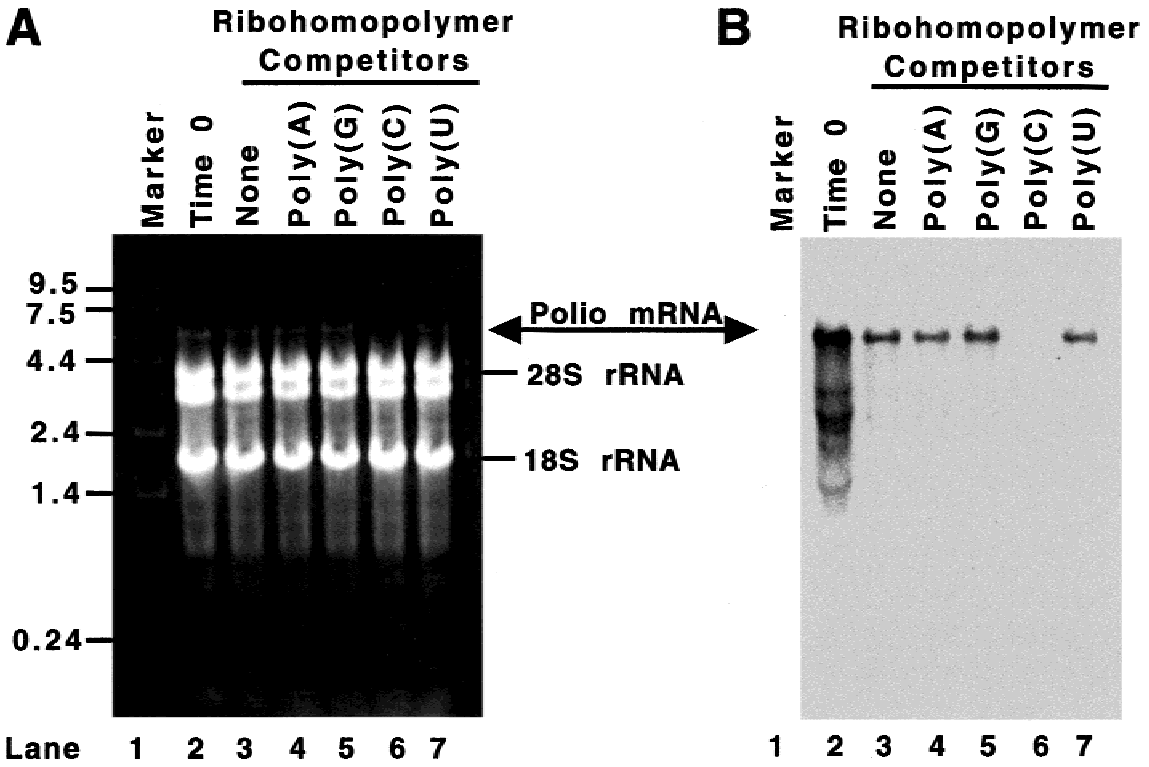

FIGURE 1. Ribohomopolymer RNA competitors and poliovirus mRNA stability. Poliovirus mRNA stability was assayed in HeLa S10 translation-replication reactions as described in Materials and Methods. ^{32}P-labeled wild-type poliovirus mRNA (77,000 CPM/μg) was incubated in 50 μL HeLa S10 translation-replication reactions at 34 °C for 0 min (lane 2) or for 3 h (lanes 3–7). The poliovirus mRNA was at a concentration of 50 μg/mL of reaction. Homopolymer RNAs (25 μg/mL) were added to the indicated reactions before incubation. **A:** The RNAs from the reactions were separated by electrophoresis in 1% agarose and visualized by ethidium-bromide staining and UV light. **B:** ^{32}P-labeled poliovirus mRNA was detected by autoradiography and quantitated using a phosphorimager.

lane 3). In contrast, ribohomopoly(C) dramatically diminished the stability of the poliovirus mRNA incubated in the reaction (Fig. 1B, lane 6 versus lane 3). Ethidium bromide staining of the gel was used to visualize RNA size markers and to ensure that each lane was loaded with equal portions of RNA from the reactions (Fig. 1A). Ribohomopoly(C) had no effect on the stability of ribosomal RNAs in the HeLa S10 translation-replication reaction (Fig. 1A, lane 6 versus lanes 2–5 and lane 7). Thus, of the four ribohomopolymers tested, only ribohomopoly(C) competed with the stability of wild-type poliovirus mRNA. Because poly(rC) binding proteins 1 and 2 bind tightly to ribohomopoly(C) in vitro (Kiledjian et al., 1995), these results suggest that poly(rC) binding protein(s) may be required for the stability of poliovirus mRNA.

Ribohomopolymers and poliovirus mRNA translation/polyprotein processing

Poliovirus mRNA translation was assayed in HeLa S10 translation-replication reactions by the incorporation of [^{35}S]methionine into acid-precipitable material (Fig. 2A). Poliovirus polyprotein processing was examined using SDS-PAGE and fluorography (Fig. 2B). Ribohomopoly(A) and -(U) had no effect on poliovirus mRNA translation or viral polyprotein processing (Fig. 2). Ribohomopoly(G) increased the amount of poliovirus mRNA translation modestly (Fig. 2A) and had no effect on viral polyprotein processing (Fig. 2B, lanes 7–9 versus lanes 1–3). In contrast, ribohomopoly(C) completely blocked viral mRNA translation (Fig. 2). These results suggested that poly(rC) binding protein(s) were required for viral mRNA translation (as previously reported; Blyn et al., 1996, 1997) or that the viral mRNA was degraded before it could be translated.

Ribohomopolymers and poliovirus negative-strand RNA synthesis

Viral negative-strand RNA synthesis was assayed by the incorporation of [^{32}P]CTP into viral negative-strand RNA synthesized by preinitiation RNA replication complexes formed in HeLa S10 translation-replication reactions (Barton & Flanegan, 1997). Two nonviral G residues at the 5′ terminus of poliovirus RNA2 T7 RNA transcripts inhibit positive-strand RNA synthesis (Barton et al., 1999); therefore, the preinitiation

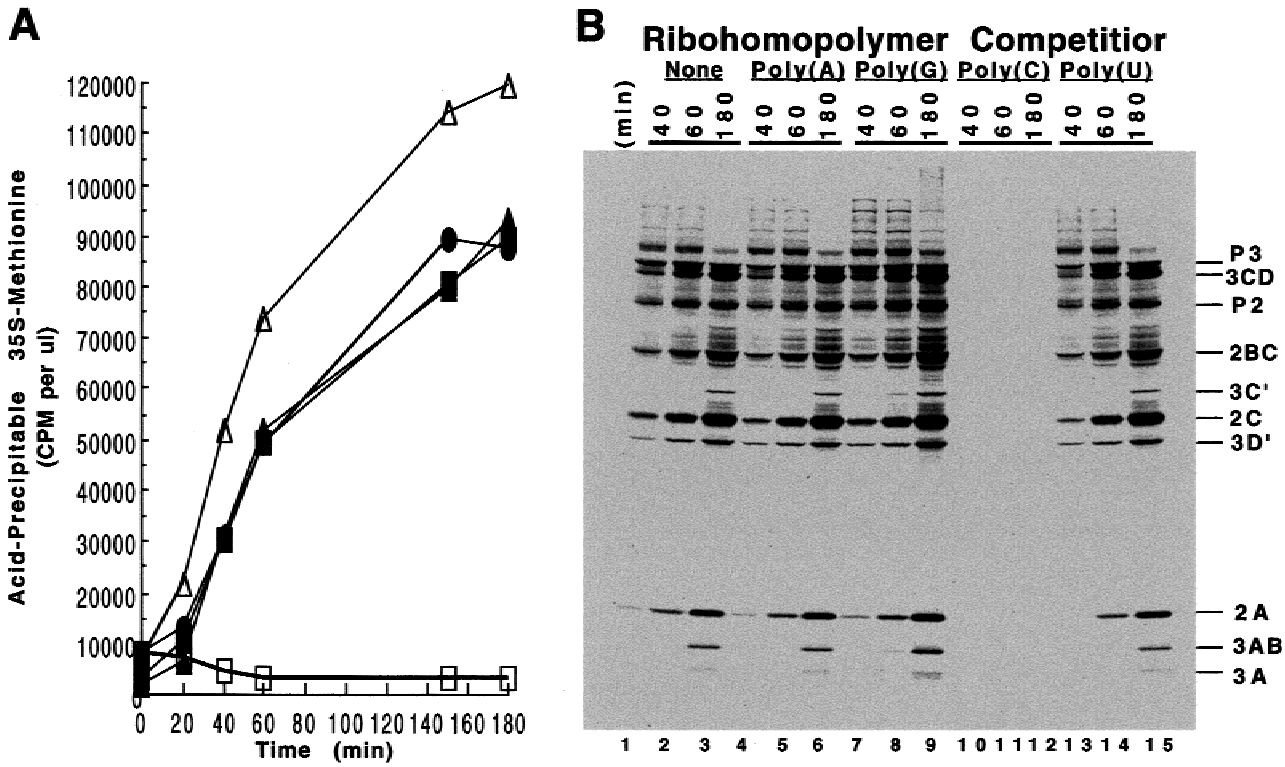

FIGURE 2. Ribohomopolymer RNA competitors and poliovirus mRNA translation. Poliovirus mRNA translation was assayed in HeLa S10 translation-replication reactions containing [^{35}S]methionine and 50 μg/mL of poliovirus mRNA as described in Materials and Methods. The reactions also contained ribohomopolymer RNAs (25 μg/mL) as indicated. **A:** The kinetics and magnitude of poliovirus mRNA translation were assayed by determining the incorporation of [^{35}S]methionine into acid precipitable material. Reactions were incubated without ribohomopolymer competitor (■), with ribohomopoly(U) (▲), ribohomopoly(A) (●), ribohomopoly(G) (△), and ribohomopoly(C) (□). **B:** [^{35}S]methionine-labeled poliovirus proteins were detected by SDS-PAGE and fluorography.

RNA replication complexes described in this report were incapable of synthesizing positive-strand RNA. ^{32}P-labeled negative-strand RNA was detected by autoradiography following denaturing agarose gel electrophoresis (Fig. 3). HeLa S10 translation-replication reactions containing poliovirus mRNA and 2 mM guanidine HCl were incubated at 34 °C for 3 h in the absence (Fig. 3, lane 1) and presence (Fig. 3, lanes 2–5) of the indicated ribohomopolymers. Preinitiation RNA replication complexes formed in these reactions were isolated and incubated in labeling reactions containing [α-^{32}P]CTP under conditions permissive for the initiation and synthesis of negative-strand RNA. Preinitiation RNA replication complexes formed in the absence of ribohomopolymer synthesized viral negative-strand RNA (Fig. 3, lane 1). Likewise, preinitiation RNA replication complexes were formed in the presence of ribohomopoly(A), -(G), and -(U) and were capable of synthesizing negative-strand RNA (Fig. 3, lanes 2, 3, and 5). Poly(U) partially inhibited poliovirus negative-strand RNA synthesis (Fig. 3, lane 5), potentially due to its complementarity to the 3′ poly(A) tail on poliovirus mRNA. Preinitiation RNA replication complexes were not formed in the presence of ribohomopoly(C) (Fig. 3, lane 4); primarily due to the lack of viral protein synthesis in the presence of ribohomopoly(C) (Fig. 2).

To more precisely determine the role, if any, for poly(rC) binding proteins in poliovirus negative-strand RNA synthesis, we assayed poliovirus negative-strand RNA synthesis in a *trans*-replication experiment (Fig. 4). In this experiment, DNVR2 RNA was used as a helper mRNA to provide the poliovirus replication proteins *in trans* to DJB14 RNA. DNVR2 mRNA encodes the poliovirus replication proteins downstream of the hepatitis C virus internal ribosome entry site (Fig. 4A). DJB14 mRNA represents poliovirus mRNA with deletions of poliovirus nt 629–6011 and nt 6057–6516. DJB14 mRNA does not express any functional proteins required for replication, but does express a protein fragment corresponding to the COOH-terminus of 3D^{Pol}, which we call Δ3D (Fig. 4B). In this experiment, DJB14 mRNA was made with a 5′ 7-methylguanosine cap to prevent its degradation in the presence of ribohomopoly(C) (see Fig. 8). When cotranslated with DNVR2 mRNA in the absence of ribohomopoly(C), both DNVR2 and DJB14 mRNAs were translated efficiently (Fig. 4B, lanes 1 and 2). Ribohomopoly(C) inhibited the

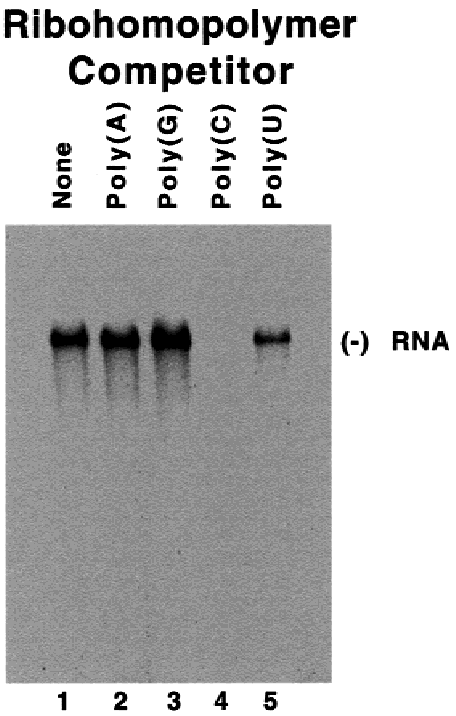

FIGURE 3. Ribohomopolymer RNAs and poliovirus negative-strand RNA synthesis. Poliovirus negative-strand RNA synthesis was assayed using preinitiation RNA replication complexes formed in HeLa S10 translation-replication reactions. Preinitiation RNA replication complexes were formed in HeLa S10 translation-replication reactions containing poliovirus mRNA (50 μg/mL) and the indicated ribohomopolymer RNAs (25 μg/mL) by incubation at 34 °C for 3 h. Preinitiation RNA replication complexes were isolated from the HeLa S10 translation-replication reactions, resuspended in labeling reaction mix containing [^{32}P]CTP, and incubated at 37 °C to allow for the synthesis of negative-strand RNA and the corresponding incorporation of [^{32}P]CTP into the newly synthesized negative strands. The RNA from the reactions was denatured with 50 mM methylmercury hydroxide and separated by electrophoresis in 1% agarose. ^{32}P-labeled negative-strand RNA synthesized by the preinitiation RNA replication complexes was detected by autoradiography.

translation of DJB14 mRNA (Fig. 4B; note the absence of Δ3D in lanes 3 and 4) but did not inhibit the translation of DNVR2 mRNA (Fig. 4B, lanes 3 and 4). Preinitiation RNA replication complexes formed in these cotranslation reactions were assayed for negative-strand RNA synthesis in the absence and presence of ribohomopoly(C) (Fig. 4C). As previously published (Barton & Flanegan, 1997), 2 mM guanidine HCl inhibited poliovirus negative-strand RNA synthesis (Fig. 4C, lanes 1 and 3). In the absence of 2 mM guanidine, preinitiation RNA replication complexes containing DJB14 mRNA synthesized poliovirus negative-strand RNA coincident in size with the DJB14 mRNA template (Fig. 4C, lane 2). Although ribohomopoly(C) inhibited the translation of DJB14 mRNA (Fig. 4B, lanes 3 and 4), it did not prevent the formation of preinitiation RNA replication complexes containing DJB14 mRNA nor did ribohomopoly(C) inhibit the synthesis of negative-strand RNA within such complexes (Fig. 4C, lane 4).

These experiments demonstrated that ribohomopoly(C) inhibited the stability of poliovirus mRNA and prevented poliovirus mRNA translation but did not directly inhibit the ability of poliovirus mRNA to function as a template for viral negative-strand RNA synthesis. These results suggest that poly(rC) binding proteins are required for poliovirus mRNA stability and mRNA translation but not for negative-strand RNA synthesis.

C24A mutation rendered poliovirus mRNA unstable

A C24A mutation was engineered into poliovirus mRNA (RNA 2) as described in Materials and Methods. Previous studies established that a C24A mutation disrupted the interaction of poly(rC) binding proteins with the 5′-terminal cloverleaf RNA (Andino et al., 1993). Two independent clones containing the C24A mutation were used to make ^{32}P-labeled poliovirus mRNA. The stability of the ^{32}P-labeled mutant C24A mRNA was compared to the stability of ^{32}P-labeled wild-type mRNA by incubation in HeLa S10 translation-replication reactions at 34 °C. Wild-type poliovirus mRNA is remarkably stable in HeLa S10 translation-replication reactions (Fig. 5A, lanes 1–4, and Fig. 5B). In contrast, the viral mRNA with the C24A mutation was dramatically less stable than the wild-type mRNA (Fig. 5A,B). After 2 h of incubation at 34 °C, the mutant C24A mRNA was present at 20% the amount of wild-type mRNA (Fig. 5A, lanes 8 and 12 versus lane 4, and Fig. 5B). Thus, a C24A mutation rendered poliovirus mRNA unstable.

C24A mutation and viral mRNA translation

Next, the effect of the C24A mutation on viral mRNA translation and viral polyprotein processing was determined. Despite the instability of the C24A mRNA in HeLa S10 translation-replication reactions, viral protein synthesis was only marginally affected by the mutation (Fig. 6). The initial rate of viral protein synthesis was the same in the reaction containing C24A mRNA as that in the reaction containing wild-type mRNA (Fig. 6, 20-min and 40-min time points). After 40 min, the reaction containing C24A mRNA was observed to have a slight decrease in the accumulation viral proteins relative to the reaction containing wild-type mRNA (Fig. 6). This decrease only occurred after the time in which the degradation of C24A mRNA would lead to nonsaturating concentrations of C24A mRNA. The C24A mutation did not affect polyprotein processing (Fig. 6B). Thus, the C24A mutation did not affect the rate of translation initiation on the mutant mRNA and only slightly diminished the total yield of viral proteins in the reaction due to the eventual degradation of the mutant viral mRNA.

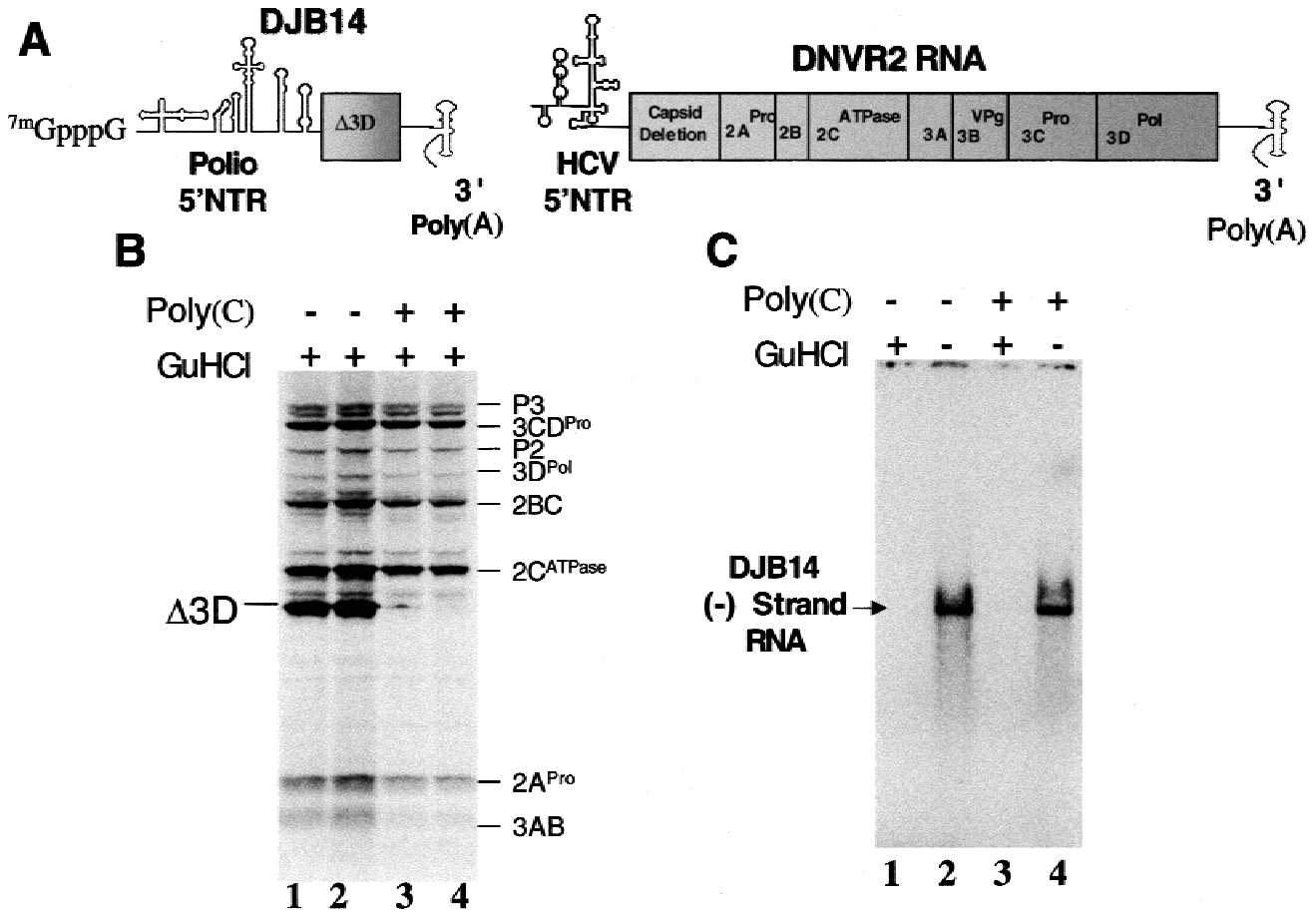

FIGURE 4. *Trans*-replication of poliovirus mRNA in the presence of ribohomopoly(C) competitor. DNVR2 mRNA and 5′ 7-methylguanosine capped DJB14 mRNA were cotranslated for 3 h at 34 °C in HeLa S10 translation-replication reactions containing 2 mM guanidine HCl in the absence (lanes 1 and 2) and presence (lanes 3 and 4) of 25 μg/mL ribohomopoly(C) competitor RNA. **A:** Diagrams of viral RNAs. **B:** SDS-PAGE. Proteins synthesized in the cotranslation reactions were radiolabeled with [^{35}S]methionine, fractionated by SDS-PAGE, and detected by autoradiography. **C:** Negative-strand RNA synthesis. Preinitiation RNA replication complexes formed during cotranslation reactions were assayed for their ability to synthesize poliovirus negative-strand RNA in the absence and presence of 25 μg/mL ribohomopoly(C) competitor and in the absence and presence of 2 mM guanidine HCl as indicated.

C24A mutation and viral negative-strand RNA synthesis

Poliovirus preinitiation RNA replication complexes formed in HeLa S10 translation-replication reactions were used to determine if the C24A mutation affected viral negative-strand RNA synthesis. Preinitiation RNA replication complexes were isolated from HeLa S10 translation-replication reactions containing wild-type RNA (Fig. 7, lanes 1 and 2) and C24A mRNA (Fig. 7, lanes 4 and 5). The preinitiation RNA replication complexes were then incubated in labeling reactions containing [α-^{32}P]CTP in the presence (Fig. 7, lanes 1 and 4) and absence (Fig. 7, lanes 2 and 5) of 2 mM guanidine HCl. The products of the reactions were denatured with 50 mM methylmercury hydroxide and separated by electrophoresis in 1% agarose. The gel was stained with ethidium bromide to visualize the RNA in each lane (Fig. 7A). Then, the gel was dried and ^{32}P-labeled negative-strand RNA was detected by autoradiography (Fig. 7B). The instability of the C24A mRNA was apparent in the ethidium stained gel (Fig. 7A, lanes 4 and 5 versus lanes 1 and 2). Despite the presence of less template RNA within the isolated preinitiation complexes from the reactions containing C24A mRNA, normal amounts of negative-strand RNA were made (Fig. 7B, lane 5 versus lane 2). Only small amounts of the input template RNA are copied into negative-strand RNA in this experimental system; therefore, the reduced C24A template concentration was sufficient to support normal negative-strand RNA synthesis. Thus, the C24A mutation did not prevent the formation of preinitiation RNA replication complexes or the synthesis of viral negative-strand RNA.

The experiments above demonstrated that the only significant phenotype associated with the C24A mutation was the dramatic defect in viral mRNA stability.

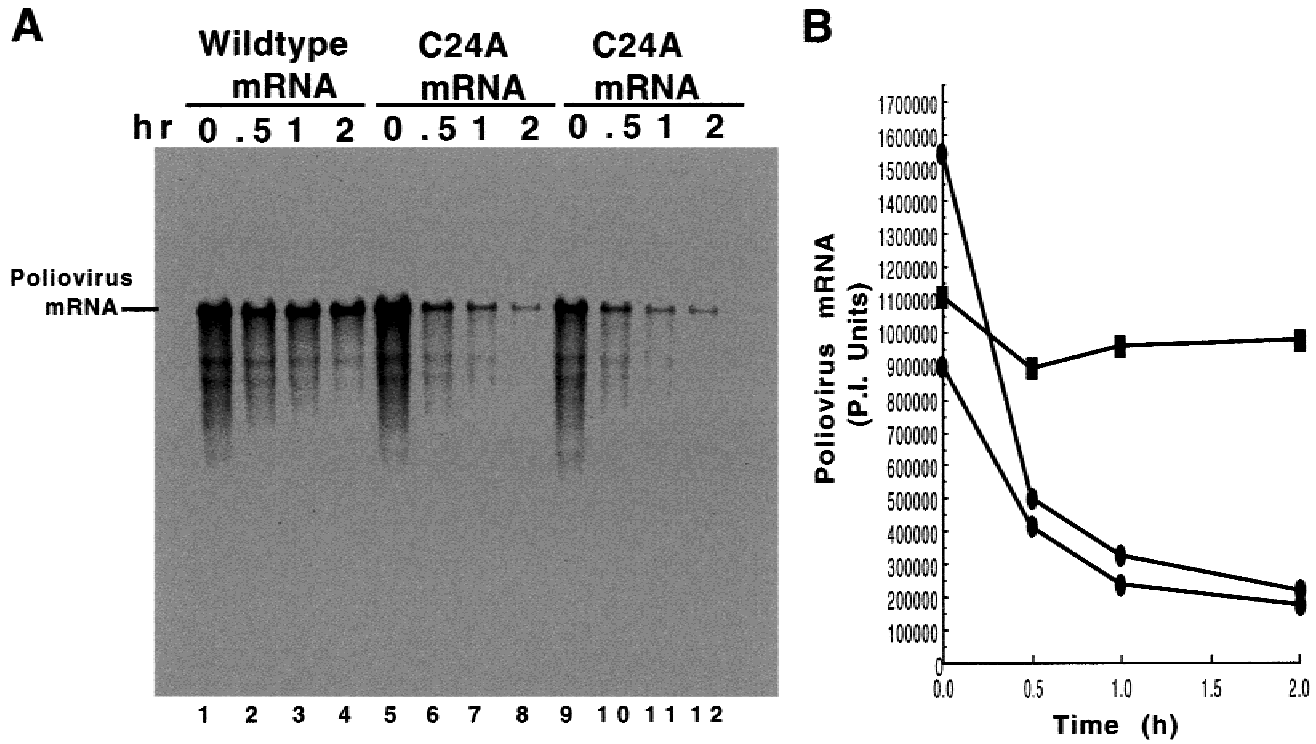

FIGURE 5. C24A mutation and poliovirus mRNA stability. ^{32}P-labeled wild-type poliovirus mRNA and ^{32}P-labeled C24A mutant poliovirus mRNAs from two independent cDNA clones (125,000 CPM/μg) were incubated in 100 μL HeLa S10 translation-replication reactions at 34 °C. The poliovirus mRNAs were at a concentration of 25 μg/mL of reaction. Samples (20 μL) of each reaction were solubilized in 0.5% SDS buffer after 0, 0.5, 1, and 2 h of incubation. RNA from the samples were denatured with 50 mM methylmercury hydroxide and separated by electrophoresis in 1% agarose. **A:** ^{32}P-labeled poliovirus mRNAs in the gel were detected by autoradiography. **B:** The amounts of full-length ^{32}P-labeled wild-type poliovirus mRNA (■) and C24A mutant poliovirus mRNA (●) in the gel were quantitated using a phosphorimager and plotted versus time.

5′ exonuclease degradation of poliovirus mRNA

To determine if the C24A mutation rendered poliovirus mRNA sensitive to degradation by 5′ exonuclease, 5′ 7-methylguanosine caps were used on wild-type and mutant mRNAs (Fig. 8). 5′ 7-methylguanosine caps prevent the degradation of mRNA by 5′ exonuclease. ^{32}P-labeled viral mRNAs were synthesized by T7 polymerase in the presence (Fig. 8, lanes 9–16) and absence (Fig. 8, lanes 1–8) of 7-methylguanosine such that the resulting viral mRNAs were capped and uncapped, respectively. The radiolabeled viral mRNAs were then incubated in HeLa S10 translation-replication reactions and the integrity of the viral mRNA over time of incubation was determined by agarose gel electrophoresis, autoradiography, and phosphorimaging (Fig. 8). Wild-type poliovirus mRNA was stable over time both with and without a 5′ cap structure (Fig. 8A, lanes 1–4 and 9–12; Fig. 8B). As before, the C24A mutation rendered uncapped poliovirus mRNA unstable (Fig. 8A, lanes 5–8). A 5′ 7-methylguanosine cap restored stability to C24A mRNA (Fig. 8A, lanes 13–16 versus 5–8; Fig. 8B). These results support the conclusion that the C24A mutant mRNA was degraded by 5′ exonuclease activity.

To test whether the instability of wild-type poliovirus mRNA in the presence of ribohomopoly(C) competitor observed in Figure 1 was due to degradation by 5′ exonuclease, we determined whether a 5′ 7-methylguanosine cap restored stability to wild-type poliovirus mRNA in the presence of ribohomopoly(C) competitor (Fig. 8C,D). Uncapped wild-type poliovirus mRNA was stable in the absence of ribohomopoly(C) competitor and unstable in the presence of ribohomopoly(C) competitor (Fig. 8C, lanes 1–8 and Fig. 8D). The kinetics and magnitude of wild-type poliovirus mRNA degradation in the presence of ribohomopoly(C) competitor was virtually identical with the kinetics and magnitude of degradation of C24A mRNA (Fig. 8A, lanes 5–8 compared to Fig. 8C, lanes 5–8; Fig. 8B,D). In addition, a 5′ 7-methylguanosine cap restored stability to poliovirus mRNA in the presence of ribohomopoly(C) competitor (Fig. 8C, lanes 13–16; Fig. 8D). Although it is not possible to determine precisely the efficiency of 5′ capping in T7 transcription

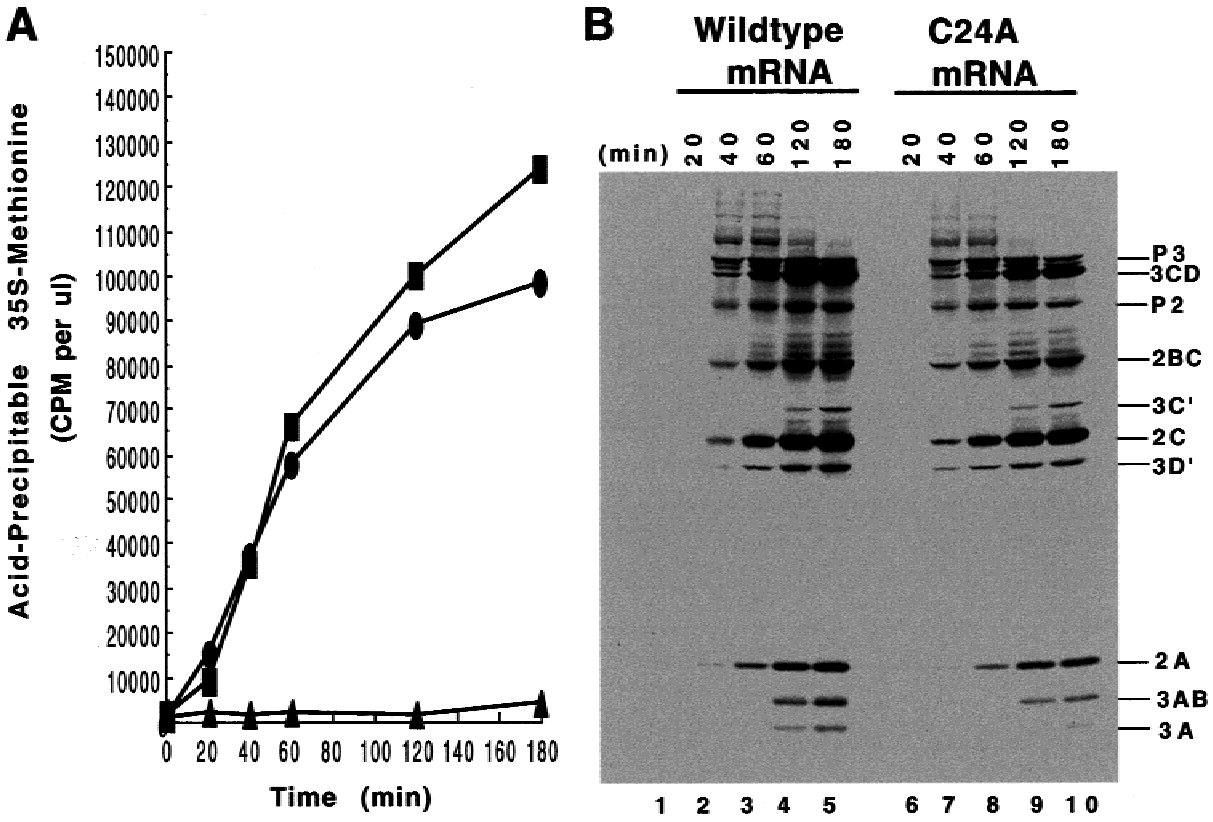

FIGURE 6. C24A mutation and poliovirus mRNA translation. HeLa S10 translation-replication reactions containing [^{35}S]methionine and the indicated poliovirus mRNAs at 75 μg/mL were incubated at 34 °C as described in Materials and Methods. Samples were removed after incubation for the indicated periods of time. **A:** Incorporation of [^{35}S]methionine into acid-precipitable material was plotted versus time. Wild-type mRNA (■), C24A mRNA (●), and no RNA (▲). **B:** Samples were separated by SDS-PAGE and ^{35}S-labeled proteins were detected by fluorography. No proteins were detected in SDS-PAGE from the mock (no viral mRNA) reaction (data not shown).

reactions, we can see from this data that approximately 80% of 5′-capped poliovirus mRNA was stable under conditions in which uncapped poliovirus mRNA was unstable (Fig. 8D). Thus, the phenotype of 5′ capped poliovirus mRNA relative to uncapped poliovirus mRNA suggests that ≥80% of the poliovirus mRNA synthesized in the presence of cap analog was actually capped. This is consistent with the capping efficiency predicted in bacteriophage T7 transcription reactions containing 3 mM cap analog and 0.75 mM GTP (Materials and Methods).

DNVR2 mRNA stability was unaffected by ribohomopoly(C) competitor (Fig. 9). DNVR2 mRNA is 3′ coterminal with poliovirus mRNA but possesses the 5′ NTR of HCV as diagramed in Figure 4A. Thus, poliovirus mRNA instability in the presence of ribohomopoly(C) was specifically associated with the 5′ NTR of poliovirus mRNA as ribohomopoly(C) did not activate ribonuclease activity capable of degrading DNVR2 mRNA. The results of these experiments support the conclusion that both the C24A mutation and the ribohomopoly(C) competitor RNA rendered poliovirus mRNA susceptible to degradation by a 5′ exonuclease.

Poly(rC) binding protein–5′ cloverleaf RNA interactions

Previous studies established that poly(rC) binding proteins 1 and 2 bind to the 5′ terminal cloverleaf of poliovirus RNA (Gamarnik & Andino, 1997; Parsley et al., 1997) and that a C24A mutation within the cloverleaf RNA disrupts the interaction with poly(rC) binding proteins (previously known as p36 due to the apparent molecular weight of poly(rC) binding proteins; Andino et al., 1993). We used UV crosslinking to detect the interaction of poly(rC) binding proteins with the 5′ terminal cloverleaf of poliovirus mRNA (Fig. 10). ^{32}P-labeled 5′ cloverleaf RNA was crosslinked to a small number of cellular proteins in HeLa cell extract (Fig. 10A, lane 2). Two crosslinked proteins approximately 36 kDa in size immunoprecipitated specifically with antibodies to poly(rC) binding proteins 1 and 2 (Fig. 10A, lanes 3 and 4). Of the four ribohomopolymers, only ribohomopoly(C) prevented the crosslinking of poly(rC) binding proteins to the poliovirus 5′ cloverleaf RNA (Fig. 10B). When ribohomopoly(C) prevented binding of poly(rC) binding proteins with the 5′ cloverleaf RNA, the amount of cloverleaf RNA crosslinked to a 90-kDa

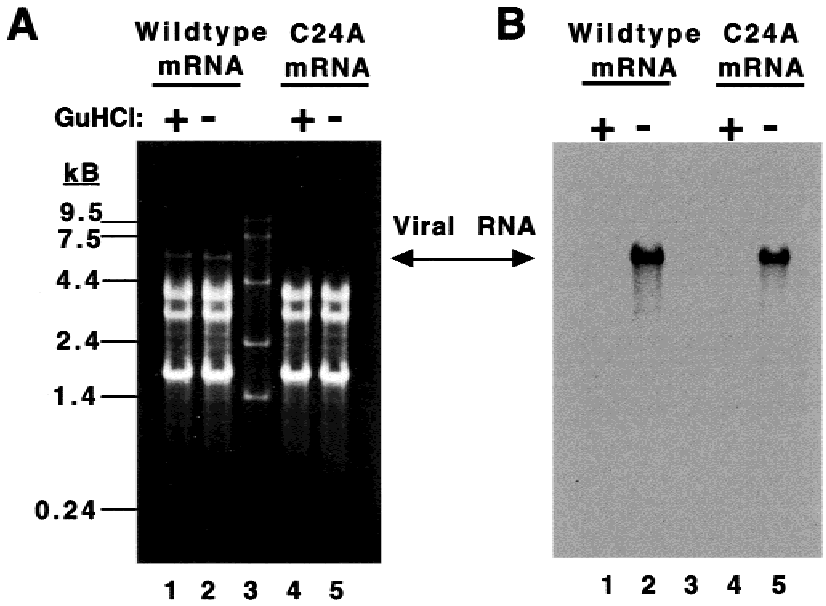

FIGURE 7. C24A mutation and poliovirus negative-strand RNA synthesis. Preinitiation RNA replication complexes were formed in HeLa S10 translation-replication reactions containing 75 μg/mL of wild-type poliovirus mRNA (lane 1 and 2) and reactions containing C24A mutant poliovirus mRNA (lanes 4 and 5) after incubation for 3 h at 34 °C. To assay negative-strand RNA synthesis within the preinitiation RNA replication complexes, they were isolated from the HeLa S10 translation-replication reactions by centrifugation and resuspended in labeling reactions containing [^{32}P]CTP in the presence (lanes 1 and 4) and absence (lanes 2 and 5) of 2 mM guanidine HCl and incubated at 37 °C for 30 min. The RNA products of each reaction were denatured with 50 mM methylmercury hydroxide and separated by electrophoresis in 1% agarose along with an RNA marker ladder (lane 3). **A:** RNAs in the gel were stained with ethidium bromide and visualized using UV light. **B:** ^{32}P-labeled poliovirus negative-strand RNA was detected by autoradiography and quantitated using a phosphorimager.

cellular protein increased (Fig. 10B, lane 5). The identity of this 90-kDa cellular protein is yet unknown. A C24A mutation in the 5′ cloverleaf RNA also prevented crosslinking with poly(rC) binding proteins (Fig. 10C, lane 4). The C24A mutation did not affect the interaction of the cloverleaf RNA with the 90-kDa protein (Fig. 10C, lane 4). Indeed, the amount of 90-kDa protein crosslinked to C24A 5′ cloverleaf was similar to the increased amount crosslinked to wild-type cloverleaf in the presence of ribohomopoly(C) (Fig. 10C, lanes 3 and 4). Thus, both ribohomopoly(C) and a C24A mutation disrupted the interaction of poly(rC) binding proteins with the 5′ terminal cloverleaf of poliovirus RNA.

DISCUSSION

Poly(rC) binding proteins 1 and 2 interact with two distinct RNA structures within the poliovirus 5′ NTR (Fig. 11A). Poly(rC) binding proteins 1 and 2 bind to the cloverleaf RNA structure at the extreme 5′ terminus of poliovirus RNA (Gamarnik & Andino, 1997; Parsley et al., 1997). In addition, poly(rC) binding protein 2 binds to stem-loop IV of the poliovirus IRES (Blyn et al., 1996). In this study, ribohomopoly(C) competitor RNA (Fig. 11B) and a C24A mutation (Fig. 11C) were used to block the binding of poly(rC) binding proteins to poliovirus RNA in HeLa S10 translation-replication reactions. Quantitative assays were used to measure poliovirus mRNA stability, poliovirus mRNA translation, and poliovirus negative-strand RNA synthesis in the context of these reactions. In the presence of ribohomopoly(C) competitor RNA, poliovirus mRNA stability was greatly diminished and poliovirus mRNA was not translated (Fig. 11B); yet when poliovirus replication proteins were provided *in trans* from a helper mRNA impervious to translation inhibition by ribohomopoly(C), poliovirus mRNA (DJB14 mRNA, Fig. 4) functioned as a template for poliovirus negative-strand RNA synthesis. Therefore, we conclude that poliovirus negative-strand RNA synthesis does not require poly(rC) binding proteins (Fig. 11B). A C24A mutation, which specifically diminishes the interaction of poly(rC) binding proteins with the 5′-terminal cloverleaf RNA (Fig. 10C; Andino et al., 1993), rendered poliovirus mRNA susceptible to degradation by 5′ exonuclease (Fig. 11C). The C24A mutation did not affect the efficiency of translation initiation nor affect the ability of the viral mRNA to serve as a template for viral negative-strand RNA synthesis (Fig. 11C). These results support the conclusion that poly(rC) binding proteins mediate poliovirus mRNA stability by binding to the 5′-terminal cloverleaf of poliovirus mRNA.

Poly(rC) binding proteins and poliovirus mRNA stability

Poliovirus mRNA is stable despite the absence of a 5′ cap structure. In theory, poliovirus mRNA stability could

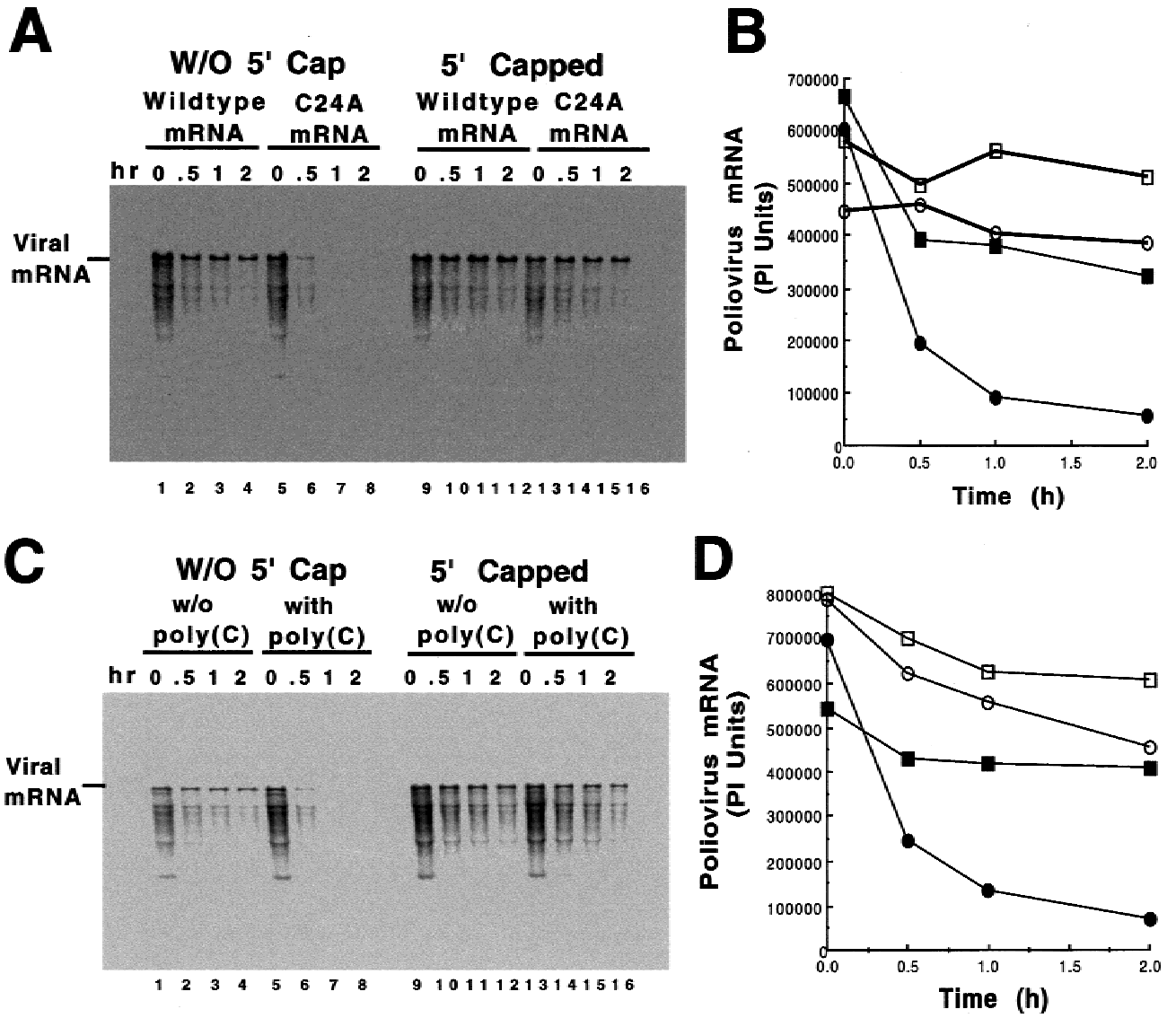

FIGURE 8. 5′ 7-methylguanosine caps and poliovirus mRNA stability. To assay poliovirus mRNA stability, ^{32}P-labeled poliovirus mRNAs (125,000 CPM/μg; 25 μg/mL) were incubated in HeLa S10 translation-replication reactions at 34 °C as described in Materials and Methods. Samples of each reaction were solubilized in 0.5% SDS buffer after incubation for 0 (lanes 1, 5, 9, 13), 0.5 (lanes 2, 6, 10, 14), 1 (lanes 3, 7, 11, 15), and 2 h (lanes 4, 8, 12, 16) of incubation. RNA from the samples was denatured with 50 mM methylmercury hydroxide and separated by electrophoresis in 1% agarose. RNAs in the gel were stained with ethidium bromide and visualized using UV light. Observation of ribosomal RNA in the gel indicated that each lane was loaded with equal portions of each reaction (data not shown). **A:** ^{32}P-labeled poliovirus mRNAs in the gel were detected by autoradiography. Reactions contained wild-type poliovirus mRNA (lanes 1–4), C24A mutant poliovirus mRNA (lanes 5–8), 5′ capped wild-type poliovirus mRNA (lanes 9–12), and 5′ capped C24A mutant poliovirus mRNA (lanes 13–16) at 25 μg/mL of reaction. **B:** The amounts of full-length ^{32}P-labeled poliovirus mRNAs in each lane of the gel in **A** were quantitated using a phosphorimager and plotted versus time. Wild-type mRNA (■), C24A mRNA (●), 5′ capped wild-type mRNA (□), and 5′ capped C24A mRNA (○). **C:** ^{32}P-labeled wild-type poliovirus mRNA, without or with 5′ 7-methylguanosine caps, was incubated in reactions in the absence and presence of 25 μg/mL ribohomopoly(C), fractionated by electrophoresis in 1% agarose, and detected by autoradiography. Reactions contained wild-type poliovirus mRNA (lanes 1–4), wild-type poliovirus mRNA and 25 μg/mL ribohomopoly(C) (lanes 5–8), 5′ capped wild-type poliovirus mRNA (lanes 9–12) and 5′ capped wild-type poliovirus mRNA and 25 μg/mL ribohomopoly(C) (lanes 13–16). **D:** The amounts of full-length ^{32}P-labeled poliovirus mRNAs in each lane of the gel in **C** were quantitated using a phosphorimager and plotted versus time. Wild-type poliovirus mRNA (■), wild-type poliovirus mRNA in reactions containing 25 μg/mL ribohomopoly(C) (●), 5′ capped wild-type poliovirus mRNA (□), and 5′ capped wild-type poliovirus mRNA in reactions containing 25 μg/mL ribohomopoly(C) (○).

be mediated by an RNA structure alone, or by a ribonucleoprotein complex. A well-characterized cloverleaf RNA structure exists at the 5′-terminus of poliovirus mRNA (Andino et al., 1990), and this cloverleaf RNA structure forms various ribonucleoprotein complexes (Andino et al., 1990, 1993; Harris et al., 1994; Gamarnik & Andino, 1997; Parsley et al., 1997). As shown in this article, ribohomopoly(C) competed with the stabil-

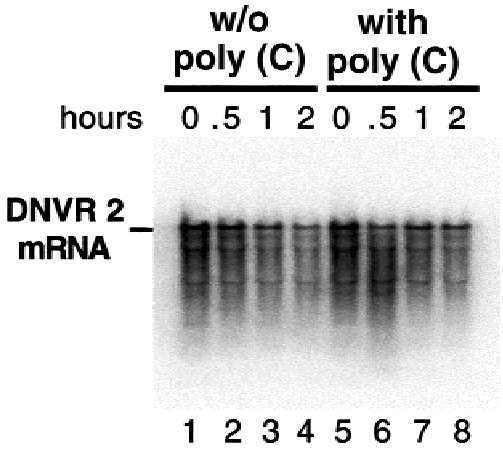

FIGURE 9. No effect of ribohomopoly(C) on DNVR2 mRNA stability. ^{32}P-labeled DNVR2 mRNA stability was assayed in the presence and absence of 25 μg/mL ribohomopoly(C) as described in Figure 8. DNVR2 mRNA possesses the 5′ NTR of HCV in place of the 5′ NTR of poliovirus mRNA (as illustrated in Fig. 4).

ity of wild-type poliovirus mRNA (Figs. 1 and 8). Ribohomopoly(C) was used previously to disrupt both α-globin and erythropoietin messenger ribonucleoprotein complexes containing poly(rC) binding proteins (Wang et al., 1995; Czyzyk-Krzeska & Bendixen, 1999). We demonstrated that ribohomopoly(C) disrupted the interaction of poly(rC) binding proteins with the 5′ terminal cloverleaf of poliovirus RNA (Fig. 10B). The ability of ribohomopoly(C) to compete with the stability of poliovirus mRNA suggested that ribohomopoly(C) binding protein(s) mediated, in part, poliovirus mRNA stability and that RNA structure alone was not sufficient to mediate mRNA stability. Poly(rC) binding proteins bind to the 5′-terminal cloverleaf structure of poliovirus mRNA (Gamarnik & Andino, 1997; Parsley et al., 1997). UV-crosslinking studies indicate that poly(rC) binding proteins 1 and 2 specifically interacted with nt 24 of the cloverleaf RNA structure (Andino et al., 1993). Indeed, a C24A mutation prevented interaction of the poliovirus

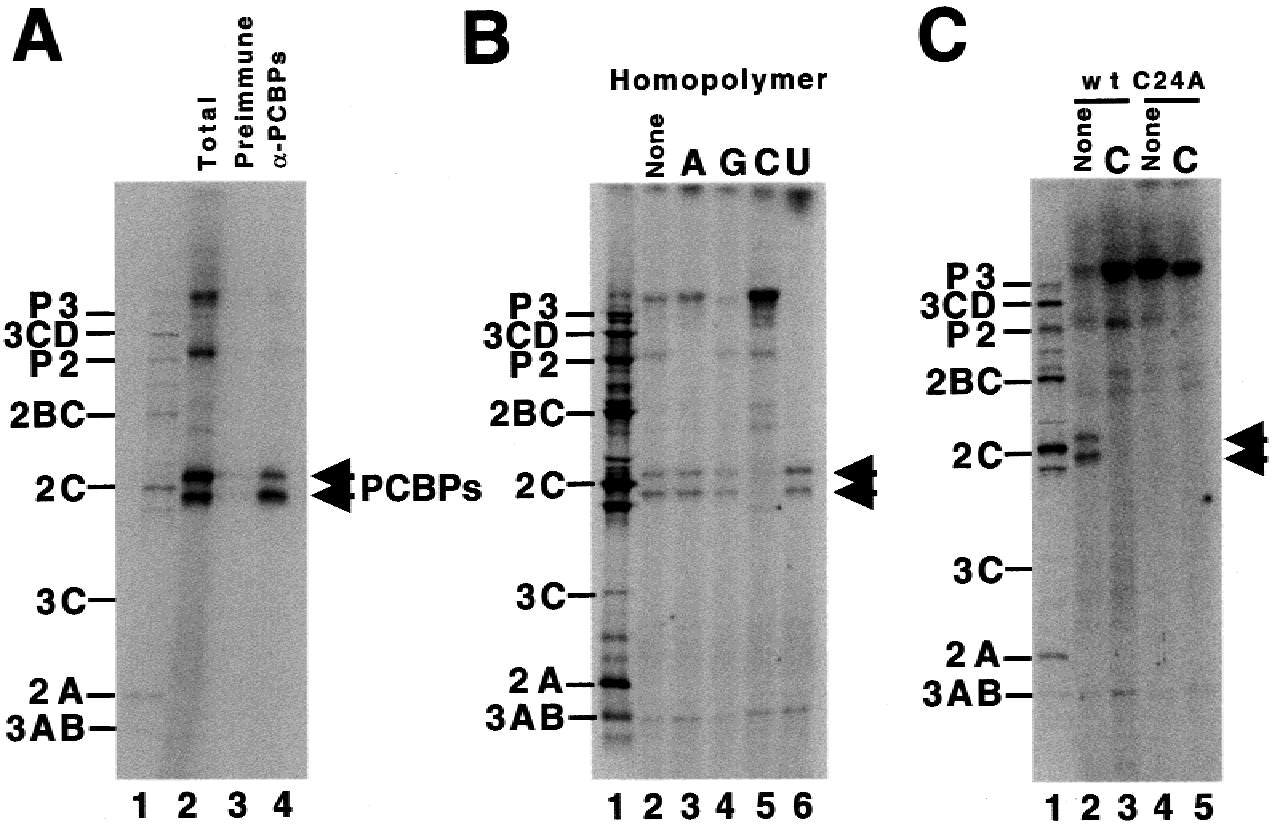

FIGURE 10. Ribohomopoly(C) and C24A mutation disrupt poly(rC) binding protein–5′ cloverleaf RNA interactions. ^{32}P-labeled 5′ cloverleaf RNA was crosslinked to HeLa cell proteins as described in Materials and Methods. UV-crosslinked proteins were fractionated by SDS-PAGE and detected by phosphorimaging. Poliovirus proteins expressed from RNA2 were used as molecular weight markers (lane 1 in **A**, **B**, and **C**); P3, 84 kDa; 3CD, 72 kDa; P2, 65 kDa; 2BC, 48 kDa; 2C, 37 kDa; 3C, 20 kDa; 2A, 17 kDa; 3AB, 12 kDa. **A:** Immunoprecipitation of poly(rC) binding proteins crosslinked to cloverleaf RNA. ^{32}P-labeled 5′ cloverleaf RNA was crosslinked to HeLa cell IFs. A portion of the crosslinked proteins were fractionated by SDS-PAGE before (lane 2) and after immunoprecipitation with preimmune rabbit serum (lane 3) or α-poly(rC) binding protein 1 and 2 immune serum (lane 4). **B:** Ribohomopolymer competition of UV-crosslinking. ^{32}P-labeled 5′ cloverleaf RNA was crosslinked to HeLa cell IFs in the presence of 25 μg/mL ribohomopolymers as indicated (lanes 2–6). **C:** Wild-type and C24A 5′ cloverleaf RNAs. Wild-type 5′ cloverleaf and C24A 5′ cloverleaf RNAs were crosslinked with HeLa cell IFs in the absence (lanes 2 and 4) and presence (lanes 3 and 5) of 25 μg/mL ribohomopoly(C).

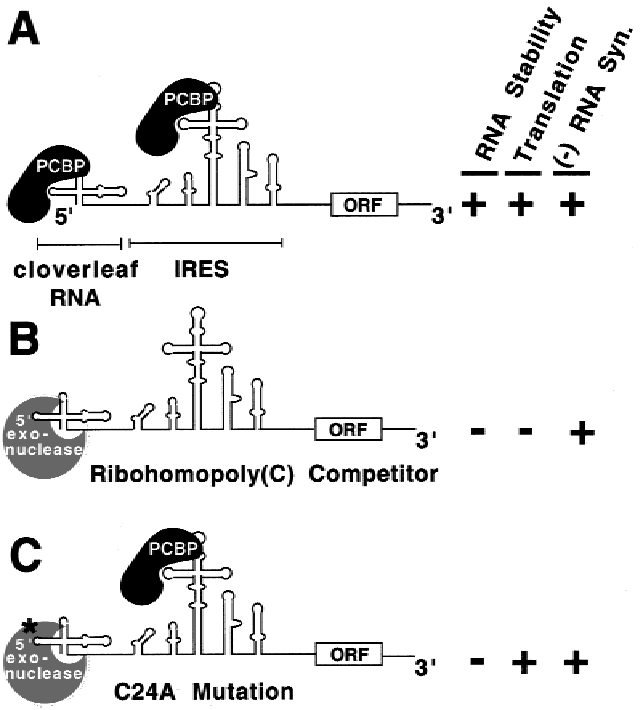

FIGURE 11. Poly(rC) binding proteins bind distinct *cis*-active RNA structures to separately mediate poliovirus mRNA stability and poliovirus mRNA translation. **A:** The 5′-terminal cloverleaf RNA structure of wild-type poliovirus mRNA binds poly(rC) binding proteins 1 and 2 (Gamarnik & Andino, 1997; Parsley et al., 1997). In addition, poly(rC) binding protein 2 binds to stem-loop IV of the poliovirus IRES (Blyn et al., 1996). **B:** Ribohomopoly(C) competitor RNA prevented the binding of poly(rC) binding proteins to the 5′ NTR of poliovirus. In the presence of ribohomopoly(C), poliovirus mRNA was degraded and translation of the mRNA was prevented. Ribohomopoly(C) did not prevent poliovirus RNA (DJB14 RNA) from functioning as a template for negative-strand RNA synthesis. **C:** A C24A mutation blocked poly(rC) binding proteins from binding to the 5′-terminal cloverleaf without affecting the interaction of poly(rC) binding protein 2 with the IRES. The C24A mutation rendered poliovirus mRNA susceptible to degradation by 5′ exonuclease. The C24A mutation did not diminish the efficiency of poliovirus mRNA translation initiation. The C24A mutation did not diminish the ability of the viral RNA to serve as a template for negative-strand RNA synthesis.

cloverleaf structure with poly(rC) binding proteins (Fig. 10C; Andino et al., 1993). In vivo, a C24A mutation leads to a 20% to 40% reduction in plaque size (Andino et al., 1993), but the step(s) of poliovirus replication affected by this mutation are not known. In this article, we demonstrate that the C24A mutation rendered poliovirus mRNA unstable in HeLa S10 translation-replication reactions (Figs. 5 and 8). These results strongly support the conclusion that poly(rC) binding proteins mediate poliovirus mRNA stability by binding to the 5′-terminal cloverleaf of poliovirus mRNA. 5′ 7-methylguanosine caps restored stability to C24A mutant mRNA (Fig. 8). 5′ 7-methylguanosine caps also restored stability to wild-type poliovirus mRNA in reactions containing ribohomopoly(C) competitor (Fig. 8). Because 5′ cap structures block 5′ exonuclease, these results suggest that cellular 5′ exonuclease is responsible for the degradation of poliovirus mRNA. Poly(rC) binding protein bound to the cloverleaf RNA may simply block the ability of 5′ exonuclease to access the 5′-terminus of poliovirus mRNA. The modest reduction in plaque size associated with a C24A mutation could be due to a defect in poliovirus mRNA stability; although the magnitude of mRNA instability in HeLa S10 translation-replication reactions may be larger than that in vivo.

Previous studies (Simoes & Sarnow, 1991; Andino et al., 1993) examined the effects of 5′-terminal cloverleaf RNA mutations on poliovirus mRNA stability; however, in contrast to the results of this study, no defects in poliovirus mRNA stability were observed. In these studies, poliovirus mRNAs were transfected into cells and the stability of the poliovirus mRNAs was assayed by RNase protection (Simoes & Sarnow, 1991) or by reporter protein (luciferase) accumulation (Andino et al., 1993). Neither of these assays were validated using unstable mRNA controls to prove their reliability in detecting unstable mRNAs (Simoes & Sarnow, 1991; Andino et al., 1993). In contrast, exogenous mRNAs incubated in cytoplasmic extracts of HeLa cells appear to exhibit regulated mRNA stability under appropriate conditions, as shown in this article with HeLa S10 extracts and with HeLa S100 extracts previously (Ford & Wilusz, 1999). Nonetheless, we must emphasize that in vivo experiments confirming the results presented here have not been done. The stability of poliovirus mRNA in vivo requires examination following the validation of the experimental system to be used.

Poly(rC) binding proteins and poliovirus mRNA translation

Poly(rC) binding protein 2 binds two distinct *cis*-active RNA structures within the 5′ NTR of poliovirus, the 5′-terminal cloverleaf structure as discussed above and stem-loop IV of the IRES (Fig. 11; Blyn et al., 1996). Binding of poly(rC) binding protein 2 to stem-loop IV in the IRES is required for poliovirus mRNA translation (Blyn et al., 1997; Walter et al., 1999). Ribohomopoly(C) competitor completely prevented poliovirus mRNA translation in HeLa S10 translation-replication reactions (Fig. 2). This inhibition could have been due to the degradation of the viral mRNA induced by ribohomopoly(C) competitor RNA (Fig. 1) or to a requirement for poly(rC) binding protein(s) in the mechanism of translation initiation itself. A C24A mutation, which rendered poliovirus mRNA unstable (Fig. 5), did not inhibit poliovirus mRNA translation (Fig. 6). This result suggests that under the conditions of saturating concentrations of viral mRNA used in HeLa S10 translation-replication reactions, the instability of viral mRNA does not by itself prevent observable translation. Unstable mRNA can be translated before being degraded if the mRNA contains a functional IRES (Fig. 6). Therefore,

ribohomopoly(C) most likely prevented poliovirus mRNA translation by competing with the available poly(rC) binding protein required by the IRES for the mechanism of translation initiation. Thus, the results of this study support previous conclusions (Blyn et al., 1997; Walter et al., 1999) that poly(rC) binding protein 2 is required for poliovirus IRES-mediated translation initiation.

Binding of poly(rC) binding protein to the 5′-terminal cloverleaf structure of poliovirus mRNA was not required for efficient translation initiation (Fig. 6). The initial rate of viral mRNA translation was the same in reactions containing wild-type poliovirus mRNA and C24A mRNA (Fig. 6, 20- and 40-min time points). Diminished translation of viral mRNA with a C24A mutation was only evident at times corresponding to those in which significant amounts of the input C24A mRNA would have been degraded (Fig. 5, after 60 min of incubation). Degradation of significant amounts of the input C24A mRNA would lead to nonsaturating concentrations of C24A mRNA in the HeLa S10 translation-replication reaction and diminished translation relative to reactions containing higher concentrations of wild-type mRNA. Parsley et al. (1997) previously reported that 5′-terminal cloverleaf RNA mutations diminished poliovirus mRNA translation and prevented RNA replication. These authors used less subtle mutations to diminish poly(rC) binding protein interactions with the cloverleaf RNA than the C24A mutation described in this article. Furthermore, these authors did not examine the stability of mutant viral mRNA within the in vitro translation reactions (Parsley et al., 1997). The results of this study suggest that the primary role of poly(rC) binding protein interactions with the 5′-terminal cloverleaf RNA is to mediate poliovirus mRNA stability.

Poly(rC) binding proteins and poliovirus negative-strand RNA synthesis

The 5′-terminal cloverleaf RNA and associated ribonucleoprotein structures have been proposed to function as *cis*-active structures necessary for viral negative-strand RNA synthesis (Gamarnik & Andino, 1998; Barton et al., 2001). Clearly, degradation of poliovirus mRNA would prevent its ability to become the template RNA within a replication complex. Therefore, we predicted that a C24A mutation would diminish viral negative-strand RNA synthesis. Nonetheless, a C24A mutation did not diminish the ability of poliovirus mRNA to form preinitiation RNA replication complexes in HeLa S10 translation-replication reactions nor did the mutation diminish the ability of these complexes to catalyze the initiation and synthesis of viral negative-strand RNA (Fig. 7). This result suggests that only a small portion of the viral mRNA in HeLa S10 translation-replication reactions is converted into template RNA for viral negative-strand RNA synthesis within preinitiation RNA replication complexes. More importantly, this result suggests that poly(rC) binding protein may not need to bind the 5′-terminal cloverleaf RNA to mediate the initiation and synthesis of negative-strand RNA. Alternatively, the C24A mutation may not completely block the binding of poly(rC) binding proteins with the 5′-terminal cloverleaf RNA in the context of preinitiation RNA replication complexes.

In a *trans*-replication experiment, we found that poliovirus negative-strand RNA synthesis was not inhibited by the constant presence of 25 μg/mL ribohomopoly(C) (Fig. 4). This amount of ribohomopoly(C) competitor was sufficient to render poliovirus mRNA unstable, completely inhibit PCBP-dependent translation, and block UV crosslinking of PCBPs to 5′ cloverleaf RNA. To assay poliovirus negative-strand RNA synthesis in the presence of ribohomopoly(C), we took advantage of the ability of DNVR2 mRNA to translate efficiently in the presence of ribohomopoly(C). In contrast with the IRES of poliovirus, the hepatitis C virus IRES does not require PCBPs for translation (Fig. 4B). In addition, we used 5′ 7-methylguanosine caps to prevent the degradation of DJB14 mRNA templates in the presence of ribohomopoly(C). Thus, these conditions stringently assayed the requirement for PCBPs in poliovirus negative-strand RNA synthesis and demonstrated that PCBPs are not directly required for poliovirus negative-strand RNA synthesis.

The results of this study suggest that the interaction of poly(rC) binding protein(s) with the 5′-terminal cloverleaf of poliovirus RNA is necessary for the stability of poliovirus mRNA. Because the poliovirus 5′-terminal cloverleaf RNA sequence and structure is highly conserved among enteroviruses and rhinoviruses (Zell & Stelzner, 1997), poly(rC) binding proteins likely mediate, in part, mRNA stability for all of these viruses. The poly(rC) binding protein–RNA cloverleaf interaction is not directly required for efficient translation initiation. Furthermore, the poly(rC) binding protein–cloverleaf RNA interaction may not function directly in the initiation and synthesis of negative-strand RNA within preinitiation RNA replication complexes. It will be important to determine in future studies whether the quantitative distribution of poly(rC) binding proteins 1 and 2 in different tissues contributes to the pathogenesis of enteroviruses. Skeletal muscle, where poly(rC) binding protein 1 is expressed at relatively high levels (Aasheim et al., 1994; Leffers et al., 1995), is an important site of poliovirus replication in the pathogenesis of poliomyelitis (Gromeier & Wimmer, 1998).

MATERIALS AND METHODS

cDNA and cloning

A plasmid containing a cDNA clone of a subgenomic RNA replicon of poliovirus RNA (Mahoney, Type 1) was kindly provided by James B. Flanegan, University of Florida College of

Medicine, Gainesville, Florida. This plasmid encoded RNA2, a subgenomic poliovirus RNA replicon containing an in-frame deletion of poliovirus nt 1175–2956 within the capsid genes (Collis et al., 1992). A C24A mutation was engineered into a subclone of this plasmid using two complementary 5′ phosphorylated DNA oligonucleotide primers (5′-pGCTCTGGGG TTGTACACACCCCAGAGGCCCACG-3′;5′-pCGTGGGCCT CTGGGGTGTGTACAACCCCAGAGC-3′) and Strategene's "QuickChange" mutagenesis kit (Strategene, La Jolla, California). An *Mlu*I to *Blp*I DNA fragment containing the C24A mutation in the subclone was then exchanged with the corresponding fragment of the plasmid encoding RNA2. The presence of the C24A mutation was confirmed by DNA sequencing.

A plasmid, pNCR-C(AUG), encoding the hepatitis C virus IRES, was kindly provided by Aleem Siddiqui, University of Colorado Health Science Center, Department of Microbiology, Denver, Colorado. Plasmid pT7-PV1(A)$_{80}$, encoding an infectious cDNA clone of poliovirus RNA, and plasmid pDJB14, a derivative of pT7-PV1(A)$_{80}$ containing deletions of poliovirus nt 629–6011 and nucleotides 6057 to 6516, were kindly provided by James B. Flanegan.

pDNVR1

PCR-based cloning was used to removed nonviral nucleotides between the T7 promoter of pNCR-C(AUG) and the 5′ nontranslated region of HCV in pNCR-C(AUG), creating pDNVR1 plasmid. PCR primers (5′-CTGTAATACGACTCA CTATAGGCCAGCCCCCTGAG-3′ and 5′-CTGGCCATTGA GGTTTAGGATTCGTGCTCATGG-3′) were used to amplify the HCV 5′ NTR from pNCR-C(AUG). pNCR-C(AUG) was cut with *Pvu*II and *Eco*RI to remove the unwanted T7 promoter and HCV 5′ NTR. The 395-bp PCR product encoding the T7 promoter immediately upstream of the HCV 5′ NTR was blunt ligated into the *Pvu*II and *Eco*RI-cut pNCR-C(AUG).

pDNVR2

pT7-PV1(A)$_{80}$ and pDNVR1 were used as parental plasmids to create pDNVR2. The *Msc*I to *Pvu*I fragment of pDNVR1 was ligated to the *Sna*BI to *Pvu*I fragment of pT7-PV1(A)$_{80}$ to make pDNVR2.

Viral mRNA

Poliovirus mRNA was generated by T7 transcription of *Mlu*I linearized plasmids using a commercial T7 transcription kit (Epicentre, Madison, Wisconsin). *Mlu*I linearized plasmids immediately downstream of the poly(A) tail of the viral cDNA. The T7-transcribed RNA2 ("wild-type poliovirus mRNA") was 5,741 bases in length. This subgenomic wild-type poliovirus mRNA possessed two 5′-terminal nonviral guanosine residues, poliovirus RNA with an internal deletion of nt 1175–2956, and a 3′-terminal poly(A) tail 83 bases in length. The viral mRNA was purified by phenol-chloroform extraction, G-50 desalting, and ethanol precipitation. Viral mRNA was quantitated by OD_{260}. For RNA stability assays, radiolabeled viral mRNA was synthesized by including [α-^{32}P]CTP in the T7 transcription reactions. The specific radioactivity of the viral mRNAs was determined by acid-precipitation and scintillation counting along with an OD_{260}. To synthesize viral mRNA with a 5′ 7-methylguanosine cap, 3 mM cap analog (m^7G5′ppp5′G) was included in the T7 transcription reactions and the concentration of GTP was reduced to 0.75 mM as recommended by the manufacturer (Epicentre). Under these T7 transcription conditions (a 4:1 ratio of cap analog to GTP), one could predict that up to 80% of the transcripts would be capped.

HeLa S10 translation-replication reactions

HeLa cell S10 extract (S10) and HeLa cell translation initiation factors (IFs) were prepared as previously described (Barton et al., 1996). HeLa S10 translation-replication reactions contained 50% by volume S10, 20% by volume IFs, 10% by volume 10× nucleotide reaction mix (10 mM ATP, 2.5 mM GTP, 2.5 mM CTP, 2.5 mM UTP, 600 mM KCH_3CO_2, 300 mM creatine phosphate, 4 mg/mL creatine kinase, 155 mM HEPES-KOH, pH 7.4), 2 mM guanidine HCl, and viral mRNA at the indicated concentrations (25 to 75 μg/mL). Reactions were incubated at 34 °C.

mRNA stability

Poliovirus mRNA stability was assayed by incubating ^{32}P-labeled poliovirus mRNA in HeLa S10 translation-replication reactions. Portions of the reactions were solubilized in 0.5% SDS buffer [0.5% sodium dodecyl sulfate (Sigma Chemical Company, St. Louis, Missouri), 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl] after incubation at 34 °C for the indicated times, extracted with phenol-chloroform, and the RNA from the reactions was ethanol precipitated. The RNA from the reactions was then denatured in 50 mM methylmercury hydroxide and separated by electrophoresis in 1% agarose. The RNAs in the gels were stained with ethidium bromide and visualized by UV light. The gels were then dried and radiolabeled poliovirus mRNA was detected by autoradiography and quantitated using a phosphorimager (Molecular Dynamics, Sunnyvale, California or Biorad, Hercules, California).

mRNA translation

Poliovirus mRNA translation was assayed by including [^{35}S]methionine (1.2 mCi/mL, Amersham) in HeLa S10 translation-replication reactions. Incorporation of [^{35}S]methionine into acid-precipitable material was assayed by collecting 1 μL samples of the HeLa S10 translation-replication reactions in 100 μL of 0.1% KOH and 1% casaminoacids at the indicated times. Samples were then precipitated with 5% trichloroacetic acid (5 mL) on ice for 10 min. Precipitated proteins were collected by filtration on 2.5 cm diameter nitrocellulose filters (Millipore) and the radiolabel retained on the filters was quantitated by scintillation counting. CPM of acid-precipitable[^{35}S]methionine was plotted versus time of incubation.

[^{35}S]methionine-labeled proteins synthesized in HeLa S10 translation-replication reactions were also analyzed by SDS-PAGE. Samples (4 μL) of the HeLa S10 translation-replication reactions containing [^{35}S]methionine were solubilized in Laemmli sample buffer after incubation for the indicated times. The samples were heated at 100 °C for 5 min

and separated by gel electrophoresis in 9–18% SDS-polyacrylamide gels as previously described (Barton et al., 1996). The gels were fixed by soaking in 50% trichloroacetic acid. The gels were then fluorographed using DMSO/PPO, dried, and [^{35}S]methionine-labeled proteins were detected on film (Kodak XL1-Blue).

Negative-strand RNA synthesis

Poliovirus negative-strand RNA synthesis was assayed using preinitiation RNA replication complexes formed in HeLa S10 translation-replication reactions as previously described (Barton & Flanegan, 1997). Following 3 h of incubation at 34 °C, preinitiation RNA replication complexes were isolated from HeLa S10 translation-replication reactions containing wild-type or C24A mutant poliovirus mRNA by centrifugation at 13,000 × *g*. Pellets containing preinitiation RNA replication complexes were then resuspended in 50 μL labeling reactions containing [^{32}P]CTP and incubated at 37 °C for 30 min as previously described (Method 4, Barton & Flanegan, 1997). Under these conditions, radiolabel is incorporated into nascent negative-strand RNA as it is synthesized by the viral RNA replication complexes (Barton & Flanegan, 1997). Two nonviral G residues at the 5′ end of T7 RNA transcripts prevent positive-strand RNA synthesis within preinitiation RNA replication complexes (Barton et al., 1999; Herold & Andino, 2000). The reactions were terminated by the addition of 0.5% SDS buffer. The products of the reaction were phenol-chloroform extracted, ethanol precipitated, denatured with 50 mM methylmercury hydroxide, and separated by electrophoresis in 1% agarose. RNA in the gels was stained with ethidium bromide and visualized by UV light. ^{32}P-labeled poliovirus negative-strand RNA was detected by autoradiography and quantitated by phosphorimaging (Molecular Dynamics or BioRad).

UV crosslinking

^{32}P-labeled 5′ cloverleaf RNAs (~250,000 CPM/ng) were synthesized using *Dde*I linearized DJB14 or C24A DJB14 plasmid templates and bacteriophage T7 RNA polymerase (Riboscribe Kit, Epicentre). Reactions (20 μL) contained 2 μL HeLa cell IFs, 25 ng ^{32}P-labeled 5′ cloverleaf RNA, 20 mM HEPES-KOH, pH 7.4, 60 mM KCH_3CO_2, 2.75 mM $Mg(CH_3CO_2)_2$, 5 mM KCl, and 3 mM DTT. Reactions were incubated at 34 °C for 15 min before being exposed to UV light (Stratalinker, Stratagene) for 30 min at 4 °C. RNase A (1.5 μL of 8.6 mg/mL; USB, Cleveland, Ohio) was added and the reactions were incubated at 37 °C for 60 min to digest the 5′ cloverleaf RNA. Reactions were diluted 1:2 with 2× Laemmli sample buffer, heated at 100 °C for 5 min, and separated by electrophoresis in 9–18% SDS polyacrylamide gels. Gels were fixed in 50% trichloroacetic acid, dried, and radiolabeled proteins were detected by phosphorimaging.

Immunoprecipitation

UV-crosslinked proteins (20 μL) were mixed with 20 μL of immunoprecipitation buffer (1% Triton X-100, 5 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.05 mM EDTA, 1 mM DTT, 5% glycerol). Samples were cleared by centrifugation; 20 s, 4 °C, 12,000 × *g*. Rabbit serum (4 μL of preimmune or α-PCBPs 1 and 2, as indicated), kindly provided by Raul Andino, University of California, San Francisco, was added and the reactions were incubated at room temperature for 35 min. Protein A-agarose beads (15 μL equilibrated in immunoprecipitation buffer; Calbiochem) were added and the reactions were incubated for 45 min at 0 °C. Reactions were centrifuged 12,000 × *g* for 20 s at 4 °C to pellet immunoprecipitates. Immunoprecipitates were washed with 300 μL immunoprecipitation buffer, repelleted, solubilized in 40 μL of Laemmli sample buffer, heated 3 min at 100 °C, cleared by centrifugation, and fractionated by electrophoresis in a 9–18% SDS-polyacrylamide gel. Gels were fixed in 50% trichloroacetic acid, dried, and radiolabeled proteins were detected by phosphorimaging.

Ribohomopolymer competitor RNAs

Ribohomopolymer competitor RNAs (poly(A), poly(G), poly(U), and poly(C); Sigma; 25 μg/mL) were added to HeLa S10 translation-replication reactions and UV-crosslinking reactions as indicated.

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