Strand-Specific RNA Synthesis Determinants in the RNA-Dependent RNA Polymerase of Poliovirus

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The viral RNA-dependent RNA polymerase (3Dpol) is highly conserved between the closely related enteroviruses poliovirus type 1 (PV1) and coxsackievirus B3 (CVB3). In this study, we generated PV1/CVB3 chimeric polymerase sequences in the context of full-length poliovirus transcripts to determine the role of different subdomains within the RNA-dependent RNA polymerase of PV1 that are required for functions critical for RNA replication in vitro and in cell culture. The substitution of CVB3 sequences in the carboxy-terminal portion (thumb subdomain) of the polymerase resulted in transcripts incapable of RNA replication. In contrast, three of the seven chimeras were capable of synthesizing RNA, albeit to reduced levels compared to that of wild-type PV1 RNA. Interestingly, one of the replication-competent chimeras (CPP) displayed an inability to generate positive strands, indicating the presence of amino-terminal sequences within the 3D polymerase and/or the 3D domain of the 3CD precursor polyprotein that are necessary for the assembly of strand-specific RNA synthesis complexes. In some constructs, the partial reestablishment of PV1 amino acid sequences in this region was capable of rescuing RNA replication in vitro and in cell culture.

For an RNA virus to replicate successfully inside a host cell, it must carry out several processes that alter the intracellular milieu, making it a suitable environment for genomic RNA amplification and the synthesis of progeny virions. Members of the Picornaviridae family of RNA viruses are very efficient at carrying out these functions, since they utilize very few gene products generated from relatively small genomes. Part of this efficiency stems from the fact that many of the viral proteins, generated entirely from a single polypeptide that is processed by virally encoded proteinases (33), are multifunctional in nature (for a review, see reference 18). Combined with the observation that viral protein precursors often have functions distinct from those of the mature polyprotein, this allows picornaviruses to maximize their coding capacities and rapidly subvert the host cell, resulting in the production of thousands of new virus particles from just one RNA genome. Central to the process of genome amplification is the viral RNA-dependent RNA polymerase 3D. This enzyme participates in specific protein-protein and protein-RNA interactions with other viral proteins and their precursors to form replication complexes that allow the selective recognition and amplification of the viral genome among a vast excess of nonviral RNAs.

The structure and biochemical activities of several viral RNA polymerases have been described. In 1997, Schultz and coworkers determined the three-dimensional structure of the poliovirus type 1 (PV1) 3D RNA polymerase (9), which resembles a cupped right hand with “fingers,” “palm,” and “thumb” subdomains characteristic of all viral polymerases described thus far (for a review, see reference 24). Interestingly, the poliovirus 3D polymerase possesses the ability to form oligomers, mediated by two proposed interfaces of polymerase-polymerase contacts (interface I and interface II) that may allow higher ordered polymerase structures to form (9, 27). Previous studies have suggested that the ability of the PV1 3D polymerase to form these structures is necessary for full enzymatic activity (13, 28), possibly by producing a “lattice” network of polymerase molecules upon which membrane-associated RNA replication takes place (20). Interface I has been suggested to involve contacts between the back of the thumb of one polymerase molecule and the back of the palm of an adjacent polymerase molecule, forming head-to-tail interactions infinitely in both directions. It has been proposed that the interface I junction provides a binding space for double-stranded RNA, the equivalent of a primed template (13). An additional interface (interface II) has been proposed to form between the top of the thumb of one molecule and the base of the fingers of another molecule. These contacts could allow for extension of the polymerase oligomers in directions opposite those formed from interface I interactions, resulting in the formation of polymerase “sheets” (20). These specifically ordered structures may be a somewhat unique feature of picornavirus polymerases, although recently it has been shown that the NS5B polymerase of hepatitis C virus (a flavivirus) can also form oligomers involving two discrete polymerase interfaces (37).

It is possible that, in the context of certain replication complexes, one or more precursor forms of the polymerase assemble with other viral proteins and/or the viral RNA. An example of this is the 3CD polypeptide of poliovirus, which consists of amino acids from both the 3C viral proteinase and the 3D RNA-dependent RNA polymerase. In addition to acting as a viral proteinase, protein 3CD is capable of carrying out a number of functions critical to viral RNA replication. These include stimulating VPg (viral protein 3B) uridylylation (30),
the mechanism by which protein-primed RNA synthesis initiation is thought to occur, and forming complexes with host and viral proteins at the 5' and 3' ends of the genome, which play critical roles in RNA replication (1, 2, 6, 8, 11, 26, 40). Interestingly, the 3CD polypeptide does not possess RNA elongation activity (10, 36), nor does it have the ability to carry out VPg uridylylation (29). Therefore, 3CD may provide a source of 3D to these complexes.

We have previously generated recombinant 3CD polypeptides with chimeric 3D polymerase subdomains representing suballelic exchanges between PV1 and the closely related enterovirus, coxsackievirus B3 (CVB3). We initially carried out biochemical assays in vitro to examine the effects of these mutations on the ability of 3CD to bind viral RNA and process viral polyprotein (7). Furthermore, we have generated cDNA constructs harboring each of these chimeric polymerase sequences that reside in different structural regions of the 3D polymerase based on the published three-dimensional structure of the molecule. T7-derived transcripts from these cDNAs do not yield infectious virus in cell culture (C. T. Cornell, K. M. Bedard, and B. L. Semler, unpublished observations). In the present study, we utilized an in vitro translation-RNA replication assay (3, 4, 21, 34, 35) to determine the abilities of each PV1/CVB3 chimeric transcript to carry out RNA replication. Chimeric substitutions within the thumb subdomain of 3D that would be predicted to disrupt interface I result in a polymerase incapable of RNA synthesis in vitro. Genetic modification of the amino-terminal one-third of the polymerase sequence revealed a positive-strand RNA synthesis defect. This defect could be rescued, in some instances, by partial restoration of HindIII vector fragment, the BglII-Bsal PV1 fragment, and the necessary combinations of PV1 and PCR-derived CVB3 fragments in the presence of T4 DNA ligase (see Fig. 3B). All pPrb (+) JLRucM plasmids were confirmed by restriction enzyme digests and nucleotide sequencing (Biotech Diagnostic, Laguna Niguel, Calif.). pPrb (+) JLRucM(CPP) harbors CVB3 sequence in regions 1 to 3 in a PV1 background (Cornell and Semler, unpublished).

In vitro synthesis of T7-derived transcripts. To generate templates for in vitro transcriptions, full-length PV1/CVB3 chimeric pPrb (+) JLRucM replicase construct (Fig. 1) were linearized with MluI. Each linearized template was phenol-chloroform extracted, ethanol precipitated, washed with 70% ethanol, and resuspended in diethyl pyrocarbonate-treated water. In vitro transcription reactions were carried out with T7 RNA polymerase at 37°C in a total volume of 20 μl. Transcripts to be utilized in HeLa monolayer transfections were stored at −70°C without further purification, whereas transcripts to be utilized for in vitro translation and RNA replication experiments were treated with 2 U of RNase-free DNase and then incubated for an additional 15 min at 37°C. These reactions were quenched with the addition of 375 μl of sodium dodecyl sulfate (SDS) stop buffer (0.5% SDS, 100 mM NaCl, 10 mM Tris-HCl [pH 7.5], 1 mM EDTA) and 100 μg of predigested proteinase K. The resulting mixture was incubated at 37°C for an additional 30 min, phenol-chloroform extracted, and ethanol precipitated. RNA pellets were washed with 70% ethanol, dried, and resuspended in diethyl pyrocarbonate-treated water. All transcripts were quantitated on an ethidium bromide-stained agarose gel by using RNA of the same length and known quantity as a standard.

In vitro translation-RNA replication reactions. For translation-replication experiments, 50-μl reactions were prepared containing 65% (vol/vol) HeLa S10 cytoplasmic extract, 1.3 μl of wild-type or chimeric transcript RNA, 10% (vol/vol) of 10°C replication mix (10 mM ATP, 2.5 mM GTP, 2.5 mM UTP, 600 mM potassium acetate, 300 mM creatine phosphate [Boehringer Mannheim], 4 mg of creatine kinase [Boehringer Mannheim]/ml, 155 mM HEPES-KOH [pH 7.4], and 2 mM guanidine hydrochloride. For translation analysis, 10 μl of this reaction was added to 10 μCi of [35S]methionine (≈1,000 Ci/mmol; Amersham Pharmacia Biotech). The remaining 40 μl was used for RNA replication analysis. Both reactions were incubated at 30°C for 5 h, at which time 10 μl of 2× Laemmli sample buffer (17) was added to each translation reaction. In vitro translation reactions were boiled and resolved on a 12.5% polyacrylamide gel containing SDS. The gel was fluorographed and subjected to autoradiography on X-MR film (Kodak). The 40-μl RNA replication reactions were subjected to centrifugation for 20 min at 15,000 × g at 4°C, and the supernatants were removed. Pellets containing replication complexes were resuspended in 9 μl of fresh HeLa S10 cytoplasmic extract, 1.3 μl of 10°C replication mix, and 2.5 μl (25 μCi) of [α-32P]CTP (3,000 Ci/mmol; Amersham Pharmacia Biotech) and then incubated for 2 h at 34°C. After incubation, total RNA in each reaction was isolated by RNeasy spin column purification (Qiagen), subjected to a final ammonium acetate precipitation, washed with 70% ethanol, resuspended in 10 μl of diethyl pyrocarbonate-treated water–RNA loading buffer (Ambion), and subjected to gel electrophoresis on a native 1% agarose Tris-borate–EDTA gel containing ethidium bromide. The amount of 18S and 28S rRNA present in each lane was used to confirm equal loading of samples before the gel was dried and subjected to phosphorimagery analysis on a Personal Molecular Imager FX (Bio-Rad).

Transfections and lucerase assays. Chimeric lucerase replicon constructs were transfected into HeLa monolayers as previously described (5). Parallel transfections and subsequent incubations were carried out in the presence of 2 mM guanidine hydrochloride to prevent RNA replication and to allow for the measurement of lucerase levels at each time point that resulted solely from the translation of input replicon RNA. When necessary, lysisates were diluted to remain within the linear range of the luminometer instrument (Monolight 2010). Each transfection was carried out in triplicate to obtain standard deviations.

RESULTS

Design and rationale of PV1/CVB3 chimeric cDNAs. The 3D RNA-dependent RNA polymerases of PV1 and CVB3 share ca. 75% amino acid identity. To examine the functional role of different regions within the 3D polymerase domain of 3CD that are critical for 3C-mediated biochemical activities, we previously generated seven chimeric 3CD polypeptides representing suballelic exchanges between the polymerase sequences of PV1 and CVB3 (7). The junctions of the chimeric...
sequences were placed in regions of sequence identity to allow (i) proper folding of the resulting polypeptides and (ii) an analysis of the effects of amino acid mutations involving residues likely to be surface exposed on the 3CD protein. Furthermore, in the absence of a three-dimensional structure for the 3CD precursor polypeptide, we utilized the crystal structure of the PV1 3D polymerase (9) to predict which region(s) of the polymerase subdomain of the 3CD polypeptide were altered by our chimeric substitutions. The results from these initial studies indicated the presence of separate determinants within the 3D polymerase subdomain of the 3CD polypeptide that were critical for capsid (P1) protein processing versus RNA binding [indicated by the formation of a complex consisting of the viral protein 3CD, host protein poly r(C)-binding protein 2 (PCBP2), and the viral 5’ cloverleaf RNA] (7).

To expand our analysis of the effects of these chimeric substitutions to other functions known to be mediated by the polymerase (or the polymerase subdomain of 3CD), we cloned PV1 cDNAs containing each of the seven PV1/CVB3 suballelic exchanges from which RNA transcripts could be generated (Fig. 1A). The 3D polymerase gene is divided into thirds, with the location of each region of the protein (based on the published three-dimensional structure for the poliovirus 3D polymerase [9]) indicated in Fig. 1B. The columns show the secondary structures found within the three segments of the 3D polymerase that have been replaced with CVB3 sequence in
the chimeras (9). Transcripts generated from these constructs, each with a precise 5' end resulting from cis cleavage by the 5' hammerhead ribozyme (12) (see Fig. 1A), were used to provide not only a functional readout of alterations of 3CD precursor functions extending beyond protein processing and RNA binding but also an indication of the ability of each chimeric 3D polymerase to mediate critical life cycle functions.

Initially, a transfection analysis carried out in HeLa cell monolayers with transcripts generated from each chimeric cDNA revealed that none of the chimeric substitutions allow production of infectious virus at 33 or 37°C (data not shown). Although previously published biochemical data from our laboratory indicate wild-type or near wild-type RNA binding and protein processing by some of the chimeric 3CD polypeptides (7), the transfection results indicated that one or more additional 3CD and/or 3D polymerase functions have been altered, abolishing the production of infectious virus in cell culture.

In vitro translation and RNA replication properties of PV1/CVB3 chimeras. We next wanted to analyze RNA replication directly in the absence of presumably more stringent requirements for virus production in cell culture. To do this, we carried out translation and RNA replication studies in vitro with the chimeric PV1/CVB3 transcripts shown in Fig. 1. Figure 2A shows two exposures of the RNA replication results with purified virion RNA (lane 1), wild-type PV1 (lane 2) and each of the seven chimeric PV1/CVB3 transcripts (lanes 4 to 10). As expected, both virion RNA and wild-type transcript were capable of synthesizing high levels of radiolabeled RNA (Fig. 2A, lanes 1 and 2). The presence of the species labeled RI/RF is an indication, in part, of negative-strand RNA synthesis, whereas the species labeled ssRNA indicates positive-strand RNA synthesis. Interestingly, three of the seven chimeras tested were capable of RNA synthesis (Fig. 2A, lanes 4, 5, and 8). Two of the three replication-competent chimeras (PCP and CCP) were capable of synthesizing detectable levels of both negative- and positive-strand RNA (Fig. 2A, lanes 5 and 8), with PCP RNA replication products accumulating to considerably higher levels than those of CCP. However, chimera CPP was only capable of generating negative-strand RNA in this assay (Fig. 2A, lane 4), as indicated by the presence of RI/RF species and the absence of any detectable ssRNA signal, even after a long exposure of the agarose gel. Interestingly, this is not a by-product of an overall RNA synthesis defect, since CPP is more effective in making negative strands compared to CCP (Fig. 2A, compare lane 4 to lane 8 on the long exposure autoradiogram), a chimera which is capable of generating low levels of positive-strand RNAs. Therefore, the CPP chimeric substitution, mapping to amino acids within the fingers and the top of the thumb subdomain of the 3D polymerase (Fig. 1), results in a strand-specific RNA synthesis defect. Furthermore, other chimeric substitutions (PPC, CPC, PCC, and CCC) could mediate one or more functional defects in the 3D polymerase or polymerase precursor (3CD) that inhibit RNA synthesis in vitro. Given the lack of correlation between in vitro
RNA replication (shown in Fig. 2A) and virus production in cell culture (data not shown) for the PCP and CCP chimeras, there are likely other macromolecular interactions extending beyond those required for RNA synthesis that involve 3D polymerase sequences that have been disrupted in these chimeras, thereby precluding virion production.

Shown in Fig. 2B are the results of the in vitro translation reactions, carried out in the presence of [35S]methionine, from the corresponding RNA replication reactions. Overall, the translation levels observed for all RNAs tested were approximately equal, as indicated by the comparable amounts of radiolabeled viral proteins present in each lane. Furthermore, the presence of 3D mutations in each of the chimeras is evident from the altered electrophoretic mobilities of the 3D-containing polyepitides (P3 and 3CD) and mature 3D protein products in each lane. Importantly, chimeras whose 3CD proteins were previously described by our laboratory as being deficient in processing the capsid (P1) precursor protein at the VP0-VP3 junction (CPP, PPC, CPC, PCC, and CCC) also showed reduced proteolysis in this assay (Fig. 2B, lanes 4, 6, 7, 9, and 10). No other processing defects are apparent that could result in the generation of suboptimal amounts of viral proteins necessary for RNA replication in vitro.

Generation of PV1/CVB3 amino-terminal chimeric luciferase replicons. To determine which amino acids in the amino-terminal one-third of the poliovirus polymerase were most critical for polymerase contacts necessary for positive-strand RNA synthesis, we constructed six additional chimeric constructs. Each was cloned into a poliovirus-luciferase replicon cDNA from which RNA could be transcribed in vitro and transfected into HeLa cells (Fig. 3B). The kinetics and levels of positive-strand RNA synthesis were then assessed by determining the levels of luciferase activity. The chimeric luciferase replicon constructs utilized for these RNA transfections harbored CVB3 sequences in different regions of the amino-terminal one-third of their polymerase sequences, and all contain PV1 sequence in the carboxy-terminal two-thirds of the 3D polymerase gene. Each “region” (designated 1, 2, and 3; see Fig. 3B) represents a subdivision of the amino-terminal one-third of the 3D polymerase (amino acids [a.a.] 1 to 176), the junctions of which are based on PV1/CVB3 sequence identity. Region 1 contains sequences residing at the top of the thumb subdomain of the polymerase (a.a. 12 to 37), whereas region 2 contains amino acids found at the base of the fingers (see Fig. 3A). The sequence designated region 3 consists of amino acids that form the amino-terminal portion of the palm subdomain. The sequence alignment of the amino-terminal one-third of the polymerases from PV1 and CVB3 is given in Fig. 3A. It should be noted that the first nine amino acids within the polymerase gene of all luciferase replicons tested are derived from poliovirus.

RNA replication kinetics with amino-terminal chimeric luciferase replicons. In addition to the original CPP polymerase chimeric substitution utilized in the context of a full-length PV1 transcript (see Fig. 2), we analyzed the ability of a luciferase replicon harboring this same chimeric polymerase to replicate in HeLa cells. We also analyzed the kinetics of RNA replication in transfection experiments with the six amino-terminal substitution mutant transcripts described in Fig. 3. The use of luciferase replicons has previously been shown to afford a high degree of sensitivity in detecting positive-strand RNA synthesis for picornaviruses (1, 15), allowing us to determine slight increases or decreases in replicative fitness brought about by our amino-terminal chimeric polymerase substitutions. Figure 4 shows the results from a 14-h time course experiment, indicating the levels of luciferase produced from the translation of each replicon RNA in HeLa monolayers at different times posttransfection. As controls for this approach, in the presence of 2 mM guanidine-HCl (Fig. 4, closed circles) all transcripts yielded approximately equal levels of luciferase at each time point. Since guanidine-HCl inhibits negative-strand RNA synthesis, these data give a quantitative measure of luciferase produced from the translation of input RNA in the absence of genome amplification. Without guanidine-HCl, wild-type luciferase replicon RNA was capable of replicating its RNA to a level that resulted in a 2- to 3-log increase in luciferase units over that of the guanidine-HCl control (Fig. 4, closed squares). The CPP replicon, with a complete CVB3 substitution in the amino-terminal one-third of its polymerase, was incapable of synthesizing levels of luciferase greater than ~0.5 log units above those observed in the presence of guanidine-HCl, providing additional evidence that this construct is severely deficient in positive-strand RNA synthesis. The ~0.5-log difference in luciferase activity detected at late times after transfection of the CPP replicon may reflect the production of a small amount of positive-strand RNA, accumulating to a level that is not detectable in the in vitro translation-RNA replication assay shown in Fig. 2. Nonetheless, these data are consistent in suggesting that the CPP construct displays a strand-specific RNA synthesis defect.

Constructs harboring CVB3 sequence in only one region within the amino-terminal one-third of the polymerase (chimeras C1,1PP, C2,3PP, and C3,3PP; refer to Fig. 3B) were capable of near-wild-type levels of positive-strand RNA synthesis, indicated by the levels of luciferase that accumulated to ca. 2- to 3-log units above the guanidine-HCl control (Fig. 4, closed squares). The CPP replicon, with a complete CVB3 substitution in the amino-terminal one-third of its polymerase, was incapable of synthesizing levels of luciferase greater than ~0.5 log units above those observed in the presence of guanidine-HCl, providing additional evidence that this construct is severely deficient in positive-strand RNA synthesis. The ~0.5-log difference in luciferase activity detected at late times after transfection of the CPP replicon may reflect the production of a small amount of positive-strand RNA, accumulating to a level that is not detectable in the in vitro translation-RNA replication assay shown in Fig. 2. Nonetheless, these data are consistent in suggesting that the CPP construct displays a strand-specific RNA synthesis defect.

In vitro translation-RNA replication with amino-terminal chimeras. Although the luciferase replicon approach should afford a higher degree of sensitivity in detecting RNA replication in cell culture, it is sometimes subject to signal-to-noise problems that do not allow the detection of subtle differences in the levels of RNA replication. Furthermore, the use of luciferase replicons does not provide an indication as to the ability of each RNA to synthesize negative strands. To confirm our results shown in Fig. 4 and to determine whether any
Amino-terminal chimeras display a strand-specific RNA synthesis defect, we used in vitro translation and replication reactions with the same luciferase replicon transcripts. Figure 5A shows two exposures of in vitro RNA replication reactions with wild-type full-length PV1 RNA (RzPV1; lane 1), wild-type PV1 luciferase replicon transcript (Rzluc; lane 2), and each of the chimeric luciferase replicon RNAs (lanes 4 to 10). Substituting either the top of the thumb or the base of the fingers of 3D with CVB3 sequences resulted in a replicon RNA that was capable of synthesizing both negative- and positive-strand RNA (C1PP, C2PP, C3PP, and C2,3PP; Fig. 5A, lanes 4, 5, 6, and 9). Our original analysis of the RNA replication phenotypes of these constructs in cell culture (Fig. 4) did not reveal any major differences in the overall ability of each to synthesize positive-strand (and presumably negative-strand) RNA. In contrast, Fig. 5A indicates that chimeric substitutions made on the 3D polymerase of PV1 resulted in a replicon RNA that was capable of synthesizing both negative- and positive-strand RNA. These results suggest that the structural changes in the fingers and top of the thumb of 3D are important for strand specificity in RNA synthesis.

**FIG. 3.** (A) Sequence alignment of the amino-terminal one-third of PV1 and CVB3 3D polymerases, which share ca. 75% amino acid identity. Shaded white letters show regions of sequence divergence, and the amino acids in the top of the thumb and base of the fingers are boxed (aa 12 to 37 and 67 to 97). In the alignment, circled letters A to D indicate junctions that are the boundaries for regions 1, 2, and 3 (i.e., region 1 consists of CVB3 amino acids from junction A to B, region 2 consists of amino acids from junction B to C, etc.). (For the PV1 sequence, see reference 16; for the CVB3 sequence, see reference 19). (B) Schematic of amino-terminal PV1/CVB3 chimeric luciferase replicons. The plasmid construct (top of panel) pRib(+)/RLucM contains a cis-acting 5' hammerhead ribozyme and the firefly luciferase gene in place of the viral capsid sequence. In vitro transcriptions with T7 RNA polymerase yield the RNAs appearing below, with different regions (shown by hatched boxes) of the amino-terminal one-third of the PV1 polymerase changed to CVB3 sequence. Also shown are region number designations used in the nomenclature for these constructs. The right portion of the panel indicates the location of the chimeric substitutions based on the published three-dimensional structure of the PV1 3D RNA polymerase, although most substitutions (other than αA within the base of the fingers) are in an unresolved region of the structure. †, based on previously published data (9).
closer to the central portion of the polymerase (i.e., C3PP) have a more significant effect on both negative- and positive-strand RNA synthesis than those made closer to the amino terminus (i.e., C1PP). Consistent with the data shown in Fig. 4, the in vitro RNA synthesis assay reveals an inability of the C1,3PP and C1,2PP (Fig. 5A, lanes 7 and 8) chimeras to synthesize both negative- and positive-strand RNA, indicating that one or more functions mediated by 3D polymerase sequences have been disrupted. Finally, the CPP luciferase replicon construct yielded somewhat lower levels of negative-strand RNA synthesis than its full-length counterpart (compare Fig. 5A, lane 10, to Fig. 2A, lane 4) but nonetheless shows a phenotype consistent with the strand-specific defect previously described for this chimera.

The [35S]methionine-labeled translation products synthesized from the corresponding RNA replication reactions are displayed in Fig. 5B. The asterisk to the right of the figure indicates the mobility of firefly luciferase, which is synthesized in roughly equal amounts from each luciferase replicon RNA (Fig. 5B, lanes 2 to 10). The wild-type luciferase replicon (Rzluc) displayed a slightly lower efficiency in generating viral replication proteins (Fig. 5B, lanes 2 and 3) compared to full-length PV1 RNA (lane 1). The reason for this is unclear, but the reduced levels of nonstructural proteins could be responsible for the lower level of RNA replication seen in the wild-type luciferase replicon compared to that of the full-length construct (Fig. 5A, compare lanes 1 and 2). As previously observed, chimeric substitutions (Fig. 5B, lanes 4 to 10) within polymerase sequences resulted in an altered mobility of the P3 and 3CD precursor proteins, as well as the mature 3D RNA polymerase. The overall translation levels between the chimeric constructs were approximately equal, confirming that the differences in RNA replication shown in Fig. 5A are not due to protein synthesis or proteolytic processing defects.

**DISCUSSION**

Picornavirus RNA replication involves the assembly of complexes containing viral and host proteins that specifically recognize viral RNA in the presence of a vast excess of cellular transcripts. The first step in the process of genome amplification involves the synthesis of a negative-strand RNA that is complementary to the positive-strand genome, presumably requiring ribonucleoprotein complexes that are strand specific in function. Once the positive-strand RNA is copied, multiple copies of progeny plus strands must be synthesized from a negative-strand or double-stranded intermediate with a high degree of efficiency. Complexes that assemble for positive-strand synthesis likely differ from those that assemble for the initial rounds of negative-strand synthesis, both with respect to the host and viral proteins involved and the surfaces in contact between them and the viral RNA. A key player in this process is the 3D RNA-dependent RNA polymerase. The poxivirus 3D polymerase has been shown to interact with other replication proteins such as 3AB (31, 32, 38, 39), in addition to associating with other polymerase molecules (9, 13, 20, 27, 38). As mentioned above, it is possible that the 3D polymerase is delivered to strand-specific replication complexes in one or
more precursor forms (i.e., 3CD). 3CD has been shown to strongly interact with 3AB (and 3B) (38) and weakly with itself and 3D (Cornell and Semler, unpublished).

Considerable published data support 3D polymerase oligomerization (via crystallographically observed interfaces I and II) as important for polymerase function. Specifically, interface I has been suggested to be involved in RNA binding, and interface II may be necessary for efficient elongation and catalytic activity (9, 13, 27, 28). Consistent with these observations, changing amino acids in the thumb portion of the polymerase (as would occur in our chimeras PPC, CPC, PCC, and CCC) could severely affect interface I formation. This is supported by the lack of detectable RNA replication seen in each of these four chimeras (Fig. 2A). A previously published study indicated that mutations in the thumb region of interface I dramatically inhibit polymerase function, whereas mutations within the palm are tolerated (28). Our results with chimera PCP, which is capable of both negative- and positive-strand RNA synthesis and harbors primarily palm subdomain mutations (see Fig. 1), are in agreement with these data.

Analysis of the CVB3 substitutions in the context of the three-dimensional structure of the polymerase molecule and its proposed oligomerization interfaces further supports the possibility that structural perturbations might contribute to the defective enzymatic functions of the PV1/CVB3 chimeric 3D polymerases. The atomic coordinates of the three-dimensional structure of the PV1 polymerase (9) were used in molecular modeling studies to substitute CVB3 amino acids in place of PV1 amino acids. Figure 6A to C each show four polymerase molecules interacting via the proposed interfaces I and II (boxes X and Y, respectively). In each, the locations of the amino acids (shown in red) changed in CPP (Fig. 6A), PCP (6B), and PPC (Fig. 6C) are indicated. Figure 6E and D show the amino acids changed at proposed interface I (in the PPC chimera) and interface II (in the CPP chimera) with the identity of the CVB3 amino acids labeled. Due to the number of mutations observed in these chimeras, it is plausible that interface I and its neighboring interactions may be affected by the amino acid substitutions. For instance, wild-type D349 in helix I may hydrogen bond with R455 in helix N (of the opposite molecule), but in the four chimeras noted above this residue is changed to G349, which could disrupt such a hydrogen bond. This residue has also been studied by Pathak et al. (28), who described a D349A/S341A/D339A triple mutant that has a lower rate of RNA synthesis resulting in a small plaque phenotype. However, Hobson et al. (13) described a D349R mutant that was wild-type in phenotype when engineered into a poliovirus cDNA. Therefore, the D349G mutation alone may not be sufficient to disrupt interface I but in the context of the other changes (e.g., Y454R) may have an adverse effect on the stability of interface I and enzymatic functions of the polymerase. Y454 in helix N, although facing internally, is proximal to several aromatic amino acids, as well as R455 and R456, two amino acids that have been shown to be important for two-dimensional lattice formation (20) and RNA binding (13). Therefore, the change of D349G and Y454R together may be disruptive. Similarly, L446, which is in the loop immediately preceding helix N, has been changed to T446 in the chimeras. Hobson et al. have shown that poliovirus transcripts harboring a L446A mutation did not yield viable virus, and the purified 3D polymerase containing this mutation had a reduced ability to bind RNA (13). Therefore, it is possible that L446T may

FIG. 5. In vitro translation and RNA replication of wild-type (full-length and luciferase replicon) and amino-terminal chimeric PV1/CVB3 luciferase replicon transcripts. (A) RNA replication carried out as described in the legend for Fig. 2A, utilizing full-length PV1 transcript (lane 1) and wild-type (lane 2) or amino-terminal chimeric luciferase replicon RNA (lanes 4 to 10). The autoradiogram shown below is a longer exposure of the same agarose gel. (B) [35S]methionine-labeled translations from the corresponding reactions shown in panel A, carried out as in Fig. 2B. The mobility of firefly luciferase (observed in lanes 2 to 10) is indicated by an asterisk to the right of the panel.
also affect 3D polymerase functions. Furthermore, the E449A mutation present in the chimeras may affect the local environment in this region by replacing a larger glutamic acid side chain with the smaller one of alanine.

Another important feature of the chimeric substitutions described above is that they change several surface-exposed amino acids that could participate in additional protein-protein interactions necessary for polymerase activity. These
amino acids are primarily clustered in helix L, a helix that does not participate in interface I formation. In the PPC, CPC, PCC, and CCC chimeras, four mutations (K431E, A434R, E427H, and N430E) change the charge characteristics on this surface of the polymerase and could disrupt interactions between the polymerase and other viral or host factors. Although these changes could result in structural perturbations that impact interface I, it is possible that this region of the polymerase contains additional determinants proximal to interface I that participate in protein-protein interactions involving the polymerase that are critical to RNA replication.

Although the chimeric substitutions within the amino-terminal one-third of the molecule (see Fig. 1) are not predicted to be as severe as those that affect interface I, the substitutions alter the overall hydrophobicity of this region (data not shown). Structural perturbations caused by these changes could be reflected in the reduced RNA synthesis phenotypes observed for the CPP and CCP chimeras (see Fig. 2). Interestingly, the CPP chimera was capable of only low levels of negative-strand RNA synthesis with no detectable positive-strand RNA synthesis, whereas CCP (which further substituted CVB3 sequences into the central one-third [or palm subdomain] of the polymerase) was capable of positive-strand RNA synthesis. Therefore, aa 67 to 97 and selected palm subdomain sequences of the polymerase present in the absence of changes to the amino-terminal one-third (i.e., chimera PCP; see Fig. 1) could maintain this surface.

The amino acid changes in each of the chimeric substitutions involving the amino-terminal one-third of the polymerase (i.e., CPP and CCP) fall within proposed Interface II. The CPP chimera was only capable of synthesizing negative strands in vitro, with almost undetectable levels of positive-strand RNA synthesis in luciferase assays in cell culture transfections. Interestingly, the CCP chimera was capable of very low levels of positive-strand RNA synthesis, suggesting that making additional chimeric substitutions in the central one-third portion of the polymerase with CVB3 sequence could at least partially reestablish protein-protein contacts. This is true if it is assumed that the PV1/CVB3 3D polymerase can form functional protein-protein contacts similar to those formed by PV1 due to its ∼75% amino acid identity with the PV1 polymerase.

Our data provide insights into strand-specific RNA synthesis complexes and protein-protein interactions involving the polymerase. For negative-strand synthesis initiation, it is possible that higher-ordered structures necessary for template recognition by the polymerase involve such proposed interface I contacts, which are sufficient for the copying of the positive-strand genome. Interface I contacts may only require the very low stoichiometric amount of polymerase initially present early in an infection. During negative-strand RNA synthesis, the protolytic processing of replication proteins continues, resulting in an increase in mature 3D polymerase available to participate in interface II contacts that could then form a polymerase lattice (as proposed by Lyle et al. [20]) or other protein-protein contacts utilized for positive-strand synthesis.

There are other possible explanations for the positive-strand synthesis defect observed in the CPP chimera. The amino-terminal one-third (the fingers subdomain) of the polymerase may contain determinants necessary for associations with heterologous protein or RNA-binding partners, and due to one or more deficiencies in these contacts, the CPP polymerase is incapable of gaining access to complexes that form for positive-strand RNA synthesis. Recently, it has been shown that cre-dependent VPg uridylylation is necessary for positive-strand RNA synthesis (22, 23), suggesting that the 3’ poly(A) tract could act as the biologically relevant template for 3D-catalyzed VPg uridylylation that generates the primer for negative-strand RNA synthesis. It is possible that the CPP 3D RNA polymerase is capable of participating in protein-protein and protein-RNA interactions necessary for 3’ poly(A) tract-dependent VPg uridylylation but unable to form complexes that mediate the analogous cre-dependent reaction required for positive-strand RNA synthesis. This argument can be extended to the role of the 3CD polypeptide, which has been shown to stimulate both cre- and poly(A)-dependent VPg uridylylation. Although we have previously shown that 3CD (CPP) is active in ribonucleoprotein complex formation, it is deficient in capsid protein processing (7), which could suggest other deficiencies in critical protein-protein interactions with other host or viral polypeptides necessary for RNA replication. It is not clear why the CPP construct (with CVB3 sequence present in regions 1 to 3 of its amino-terminal one-third) is capable of generating negative-strand RNA, whereas the C13-PP construct, harboring CVB3 sequence only in regions 1 and 2, is incapable of RNA synthesis altogether. In the context of region 1 and 2 substitutions, the presence of CVB3 sequence in region 3 could allow the 3D polymerase to somehow regain RNA synthesis activity, possibly by facilitating proper folding of the molecule. In the form of its precursor polypeptide, 3CD, specific subdomains within 3D modulate the 3C-mediated functions of protein-processing and RNA binding (7, 25). Since the mature 3D polymerase is itself multifunctional, carrying out both the uridylylation of VPg and mediating the synthesis of RNA molecules, the presence of functionally specific subdomains is not surprising. Finally, we have not carried out a direct analysis of PV1/CVB3 chimeric polymerase biochemical functions in the present study but rather have used in vitro RNA synthesis as an indicator for polymerase activity. Efforts are currently under way to purify each chimeric 3D RNA polymerase and examine their activities in VPg uridylylation, nucleic acid binding, and RNA chain elongation.

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