Chimeric Picornavirus Polyproteins Demonstrate a Common 3C Proteinase Substrate Specificity

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Cross-species proteolytic processing was demonstrated by the 3C proteinases of human rhinovirus 14 and coxsackievirus B3 on poliovirus-specific polypeptide precursors. Chimeric picornavirus cDNA genomes were constructed in a T7 transcription vector in which the poliovirus 3C coding region was substituted with the corresponding allele from human rhinovirus 14 or coxsackievirus B3. In vitro translation and processing of the polypeptides encoded by the chimeric genomes demonstrated that the proteolytic processing of poliovirus P2 region (nonstructural) proteins could be functionally substituted by the heterologous proteinases. In contrast, the 3C proteinase activities expressed from the chimeric genomes were incapable of recognizing the poliovirus-specific processing sites within the capsid precursor. Since the amino acid sequences flanking and inclusive of the P2 region cleavage sites of the three viruses are not stringently conserved, these results provide evidence for the existence of common conformational determinants necessary for 3C-mediated processing.

The coordinate gene expression and replication of the picornaviruses is controlled by a highly complex cascade of proteolytic processing events. All of the members of this clinically and economically important group of viruses, which includes the enteroviruses (poliovirus, echovirus, coxsackievirus, and hepatitis A virus), rhinoviruses (with 100 serotypes of human common cold viruses), aphthoviruses (foot-and-mouth disease virus of ungulates), and cardioviruses (encephalomyocarditis virus and mengovirus), express their genetic information as a large precursor polypeptide which is processed by at least two virally-encoded proteinases (9, 30, 43) to generate the mature structural and nonstructural viral proteins. (For recent reviews of picornavirus proteolytic processing, see references 19, 27, and 29).

In the case of poliovirus, one of the best-studied members of the *Picornaviridae*, the majority of the posttranslational processing events occur between Gln-Gly (Q-G) pairs (28) and are mediated by the proteinase activity of viral protein 3C (9). This enzyme is a member of the cysteine class of proteinases (1, 8, 18, 32, 42). It is unique in its autocatalytic activity (10, 13, 31) and specificity for picornaviral substrates. Comparison of direct end-terminal amino acid sequence data derived from viral polypeptides (28, 36, 37) with the complete nucleotide sequence of the poliovirus RNA genome (16, 34) indicates that only 9 of a predicted 13 Q-G pairs are ever utilized as proteolytic processing sites during the course of infection. Determinants other than the presence of a particular linear amino acid sequence must play a role in the specific primary site recognition of a viral polypeptide substrate by the 3C enzyme. Proteinase 3C-mediated cleavage of the viral polyprotein in other picornaviruses generates viral proteins homologous to those produced by poliovirus. Although Q-G pairs are the most commonly utilized processing sites among the picornaviruses, the various 3C enzymatic activities expressed from these viruses also cleave between Gln-Ala (Q-A), Gln-Ser (Q-S), Gln-Leu (Q-L), and Glu-Gly (E-G) amino acid pairs (27, 29). The lack of stringent conservation of the amino acid pairs cleaved by

the different picornavirus 3C proteinases suggests that the enzyme recognizes a three-dimensional conformation of the substrate in addition to a particular dipeptide sequence. Examination of the 3C-coding regions of a number of picornaviruses has revealed that whereas the carboxy-terminal one-third of the protein is highly conserved and contains the putative active site of the enzyme, the amino-terminal domains of the various 3C enzymes are not as highly conserved (1, 46). Consequently, it is likely that the determinants which confer substrate specificity are located in the amino-terminal portion of 3C.

In order to determine whether the poliovirus 3C activity can be functionally substituted with a 3C enzyme from another picornavirus, we generated chimeric cDNA genomes in which the 3C- and 3CD-coding regions of human rhinovirus 14 (HRV14) or the 3C-coding region of coxsackievirus B3 (CB3) were substituted for the corresponding poliovirus sequences within the context of a full-length poliovirus type 1 (PV1-Mahoney) cDNA. Although the HRV14 and CB3 cleavage sites have not been determined by protein sequencing, the amino acid sequence predicted from the nucleotide sequence data (4, 41) suggests that the proteolytic cleavage sites of HRV14 and poliovirus are conserved, with the exception of an E-G pair at the VP3-VP1 junction and a Q-A pair at the 2B-2C junction in the HRV14 polyprotein. In the predicted amino acid sequence of the CB3 and CB1 polyproteins, a Q-N pair is the most probable site of cleavage at the 2B-2C junction. The remainder of the 3C cleavage sites for both CB3 and CB1 are predicted to be Q-G pairs (12, 22). We report that the 3C proteinase activities expressed from each of the chimeric picornavirus genomes recognized a subset of the poliovirus Q-G cleavage sites in an in vitro translation and processing system. The HRV14 3C proteinase was shown to be capable of correctly processing poliovirus nonstructural precursor polypeptides (including the Q-G pair between 2B and 2C) but was unable to process the poliovirus capsid proteins. A similar pattern of selective, cross-species processing was obtained when the poliovirus 3C allele was replaced with the corresponding sequences from CB3.

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The results of these experiments have important implications for the mechanism of primary site recognition by 3C. The ability of proteinase 3C activity from one picornavirus to effectively cross-process a precursor polypeptide from another picornavirus at an amino acid pair distinct from its normal processing site provides experimental evidence for the existence of specific conformational requirements for correct substrate selection by 3C. In addition, the data indicate that there are differences between the picornavirus Q-G cleavage activities that process structural polypeptides and those that generate nonstructural proteins.

MATERIALS AND METHODS

Construction of chimeric picornavirus cDNAs. The source of HRV14 cDNA was a subgenomic clone, pHRVΔ55, which has been previously described (4). A 0.8-kilobase (kb) fragment of the HRV14 genome (nucleotides 5240 to 6017) was prepared by complete digestion with *Ava*II and *Acc*I and treatment with calf intestine alkaline phosphatase (Boehringer Mannheim Biochemicals). The subgenomic poliovirus cDNA clone, pMV3.9, served as the source of the following three DNA fragments (pMV3.9 contains a 3.9-kb segment of the poliovirus genome between nucleotides 3660 and 7524 that has been inserted via *Eco*RI linkers into the *Eco*RI site of a pBR322 derivative): a 0.3-kb *Ava*II fragment extending from nucleotides 5111 to 5438, a 1.2-kb fragment extending from the *Bst*EII site at 3925 to the *Ava*II site at 5111 which was subsequently treated with calf intestine alkaline phosphatase, and a 3.6-kb vector fragment prepared by digestion with *Bst*EII and *Acc*I. The four fragments were ligated and transformed into *Escherichia coli* C600 to obtain a subgenomic plasmid, pRP111, in which the entire 3C-coding region and first 234 nucleotides of poliovirus 3D had been substituted with the homologous HRV14-coding sequences.

pRP111 was digested to completion with *Bst*EII and *Acc*I to generate a 2.3-kb insert that could be subcloned into a full-length infectious cDNA plasmid, pSVP37-5, which has been previously described (5). In order to complete the upstream poliovirus cDNA sequences, a 0.7-kb *Bst*EII fragment was prepared from pSVP37-5. A 7.5-kb vector fragment was generated by the complete digestion of pSVP37-5 with *Bst*EII and *Acc*I. The three fragments were ligated to yield the full-length chimeric cDNA clone, pRP212.

The subgenomic clone that provided the CB3 (Nancy) cDNA, pCBIII-3'A (obtained from S. Tracy and N. Chapman), was constructed from two previously described smaller subgenomic clones, pCBIII-211 and pCBIII-35 (44). pCBIII-3'A was digested with *Nco*I and *Sac*I to yield a 1.3-kb fragment extending from nucleotide 4647 through 5934 of the CB3 genome and encoding the carboxy-terminal half of 2C, 3ABC, and the amino-terminal 8 amino acids of 3D. The CB3 fragment was substituted for the corresponding sequences in a previously described (39) subgenomic PV1 cDNA clone, pMV7-2.9, which had been digested with *Nco*I and *Eco*RV at PV1 nucleotides 4729 and 6026, respectively. A synthetic double-stranded oligonucleotide corresponding to amino acid residues 9 to 13 of CB3 3D was used to maintain the translational reading frame and to bridge the two cDNA fragments between the *Sac*I site of CB3 and the *Eco*RV site of PV1. The resulting chimeric plasmid, pCP-P3-NSE, was subsequently subcloned into the background of pSVP37-5 to yield the full-length chimeric cDNA plasmid pCP-P3-FL.

Transfections. Subconfluent monolayers of COS-1 cells were transfected with 0.1 to 5.0 μg of CsCl-purified pRP212,

FIG. 1. Genomic structure and chimeric polyprotein encoded by the T7 transcription vectors pT7-RP3C (A) and pT7-PCP (B). The construction of the chimeric picornavirus cDNAs is detailed in Materials and Methods. ■, Region substituted into the poliovirus cDNA background; ▨, T7 vector sequences. MCS, Multiple cloning site. The polyproteins that could be translated from the bacteriophage T7 transcripts are represented below the cDNA constructs. Major cleavage sites utilized in the processing of the poliovirus polyprotein are indicated as follows: ▲, Gln-Gly sites mediated by 3C activity; △, Tyr-Gly sites processed by proteinase 2A; ◆, Asn-Ser maturation cleavage of capsid precursor VP0.

pCP-P3-FL, or pSVP37-5 DNA as previously described (5). Initial transfections were incubated for up to 7 days at 33 or 37°C.

Construction of chimeric PV1-HRV14 and PV1-CB3 transcription vectors. The chimeric viral cDNAs from pRP212 and pCP-P3-NSE were substituted for the wild-type poliovirus sequences in derivatives of the bacteriophage T7 transcription vectors, pT7-1 or pT7PV1-5, which have been previously described (45, 49). The resulting plasmids, pT7-RP3C and pT7-PCP, are diagrammed in Fig. 1. An additional transcription vector, pT7-RP3CD, was generated in which the entire HRV14 3CD-coding region was substituted for the corresponding sequences of wild-type poliovirus cDNA. A 6.3-kb fragment from pT7-RP3C was generated by complete digestion with *Pvu*II, which cuts within the vector, and *Acc*I, which cuts within the chimeric 3D-coding region. The extreme 3'-terminal HRV14 cDNA, containing the carboxy-terminal portion of 3D and the 3'-nontranslated region, was supplied by digestion of pHRVΔ55 with *Acc*I and *Pst*I to yield a 1.2-kb fragment. The two viral cDNA fragments were ligated into a 2.8-kb T7 transcription vector which was prepared by complete digestion of pGEM-1 (Pharmacia, Inc.) with *Pvu*II and *Pst*I. The 3C deletion mutant, pT7-RP3C:X, was generated by the complete digestion of pT7-RP3C with *Bgl*II (HRV14 nucleotides 5515 and 5703). The resulting 10.3-kb fragment was treated with the Klenow fragment of DNA polymerase I and rejoined via ligation with

a phosphorylated *Xho*I linker, d(CCCTCGAGGG) (New England BioLabs, Inc.). Insertion mutagenesis of the CB3 3C-coding region was accomplished by the introduction of a *Bgl*II linker d(CAGATCTG) into the *Nhe*I site (CB3 nucleotide 5663) of pCP-P3-NSE after the *Nhe*I-digested DNA had been treated with the Klenow fragment to produce blunt ends. A 2.4-kb *Xmn*I-*Xmn*I fragment containing the additional sequences was substituted back into the full-length transcription vector, pT7-PCP:B, which encodes the chimeric PV1-CB3 polyprotein containing the four additional amino acids inserted in the amino portion of CB3 3C.

In vitro transcription and translation. Prior to transcription, the templates were linearized at the 3' end of the poliovirus-specific sequences with *Eco*RI or *Sal*I. Transcription reactions were carried out with bacteriophage T7 RNA polymerase (Pharmacia) as previously described (49, 50), with the modification that the transcription reactions were not treated with DNase after completion.

In vitro translation of transcripts derived from chimeric cDNAs. Translation reactions were carried out in a rabbit reticulocyte lysate (Promega Biotec) supplemented with a postmitochondrial extract from uninfected HeLa cells (prepared as described in reference 2). Reaction conditions were essentially as previously described (50) and included 0.75 mCi of [³⁵S]methionine (Amersham Corp.) per ml. The translation reactions were diluted in Laemmli sample buffer and analyzed on 10 or 12.5% sodium dodecyl sulfate-polyacrylamide gels (20).

Immunoprecipitation of [³⁵S]methionine-labeled translation reactions. Aliquots of translation reactions in Laemmli sample buffer were boiled, diluted 10-fold with ice-cold extraction buffer, and immunoprecipitated as described elsewhere (38). Rabbit antisera had been previously prepared against poliovirus polypeptides 2C and 3D (9, 38) and a tryptic digest of VP1 (B. L. Semler, S. A. Lynch, and E. Wimmer, unpublished results).

RESULTS

Generation of PV1-HRV14 and PV1-CB3 chimeric genomes. In order to determine whether the 3C proteinase activity of poliovirus could be functionally substituted with the corresponding enzymatic activity from another picornavirus, chimeric viral cDNAs were generated in which the coding region for poliovirus was replaced by the homologous sequences from either HRV14 or CB3. A PV1-HRV14 chimera was generated in which the coding region for poliovirus 3C and the first 78 amino acids of 3D were replaced by the equivalent sequences from HRV14. The PV1-CB3 chimera resulted from a more extensive substitution of CB3 sequences into the PV1 genome, which included the carboxy-terminal 127 amino acids of CB3 2C, the entire 3ABC coding region, and the amino-terminal 13 residues of 3D.

Recombinant plasmids containing the full-length chimeric cDNAs were assayed for infectivity by transfection of COS-1 cell monolayers. No plaque formation or other evidence of virus-induced cytopathic effects was observed. Since the chimeric cDNAs did not yield infectious virus upon transfection, we wanted to determine the nature of any possible proteolytic processing defects by using a cell-free translation system. To that end, the full-length chimeric cDNAs were introduced into a bacteriophage T7 transcription vector which had been previously modified to optimize the in vitro translational efficiency of poliovirus-derived transcripts (49). A schematic diagram of the PV1-HRV14 and PV1-CB3

transcription vectors (pT7-RP3C and pT7-PCP, respectively) and the virus-specific polypeptides that could potentially be produced by translation of the transcribed RNA are shown in Fig. 1. In vitro translation analysis of the polypeptides expressed from the hybrid viral genomes provided a novel opportunity to assess which Q-G cleavage sites within the chimeric polyprotein might be poliovirus specific and which might be susceptible to heterologous processing.

In vitro translation of pT7-RP3C RNA and processing of poliovirus polypeptides by HRV14 3C activity. Both wild-type poliovirus and the PV1-HRV14 recombinant transcripts were used to program the synthesis of virus-specific polypeptides in an in vitro translation system. The rabbit reticulocyte lysate used in these experiments was supplemented with a postmitochondrial extract from uninfected HeLa cells which has been demonstrated to minimize the internal initiation of protein synthesis on the poliovirus RNA genome (6, 33). The virus-specific polypeptides produced by the in vitro translation of the viral transcripts and concomitant processing of the primary translation products are shown in lanes 3 (pT7-1) and 4 (pT7-RP3C) of Fig. 2. Figure 2, lanes 6 and 7 represent samples to which poliovirus-infected HeLa cell extract was added after translation was stopped by the addition of cycloheximide and RNase. As previously demonstrated in the wild-type poliovirus translation system (26, 49), this experiment was designed to test the ability of exogenous 3C proteinase activity (present in the infected extract) to carry out the Q-G cleavages *in trans*.

It can be clearly seen in Fig. 2, lane 3 that via the activity of newly synthesized 3C proteinase, the wild-type poliovirus P1 precursor was processed into the capsid proteins VP0, VP1, and VP3 (VP2 and VP4 are not generated in the cell-free translation system since the requisite virion assembly does not occur). The processing of P1 was more efficient when exogenous 3C activity was provided by the infected-cell extract, as evidenced by the reduction in the amount of P1 (Fig. 2, lane 6). The P2 precursor and its cleavage products, 2BC and 2C, were also detected in the wild-type poliovirus translation. Extensive processing of the P3-derived proteins is not readily detected in in vitro translations of wild-type poliovirus genomic RNA or in vitro transcripts. However, as evidenced by the production of mature virus-specific polypeptides derived from the P1 and P2 precursors, 3C proteinase (or 3CD proteinase [see below]) must be generated in sufficient quantities to effect the Q-G cleavages that give rise to these proteins.

In contrast to the results obtained from the wild-type poliovirus translations, it appears that the 3C proteinase activity expressed from the pT7-RP3C chimeric transcript was capable of processing the P2 and possibly the P3 region polypeptides but was incapable of processing its P1 precursor (Fig. 2, lane 4). The mature viral capsid proteins resulting from Q-G-processing activity were generated after post-translational incubation of the chimeric translation reaction with the poliovirus-infected cell extract, confirming that the P1 polypeptide expressed from the chimera was indeed a substrate for authentic poliovirus 3C (or 3CD) processing (Fig. 2, lane 7). The data shown in Fig. 2, lane 4 demonstrate that polypeptides which comigrated with polio proteins P2, 2BC, and 2C were produced following translation of the RP3C-derived transcript. The identities of these P2-region polypeptides were later confirmed by immunoprecipitation of the translation samples (see below).

In vitro translation and processing of a chimeric PV1/CB3 polyprotein by CB3 3C proteinase activity. In a fashion similar to that described above, the PV1-CB3 chimeric

FIG. 2. In vitro translation of PV1-HRV14 chimeric T7 transcripts. Full-length transcripts from wild-type poliovirus cDNA (lanes 3 and 6), a chimeric PV1-HRV14 (pT7-RP3C) cDNA encoding a HRV14 3C proteinase (lanes 4 and 7), or a chimeric PV1-HRV14 cDNA bearing a deletion within the HRV14 3C-coding region (lanes 5 and 8) were used to direct the synthesis of viral proteins in a rabbit reticulocyte translation system supplemented with uninfected HeLa cell extract as described in Materials and Methods. Translation reactions were programmed with 5 μ g of RNA per ml. Lanes 6 to 8 contain samples which were incubated posttranslationally with poliovirus-infected HeLa cell extract after the arrest of translation by the addition of 5 μ g of cycloheximide and 10 μ g of RNase A per ml. Lanes 6 to 8 have been underexposed to facilitate the resolution of the processed capsid proteins. Lane 1 is a marker lane containing [35 S]methionine-labeled poliovirus proteins prepared from HeLa cells 6 h postinfection. Samples were diluted in Laemmli sample buffer and subjected to electrophoresis on a 12.5% sodium dodecyl sulfate-polyacrylamide gel. Shown is an autoradiogram of the fluorographed gel. Positions of viral proteins are indicated to the left of lanes 1 and 6.

transcripts from pT7-PCP were translated in the rabbit reticulocyte system to investigate the ability of the CB3 3C proteinase to process both poliovirus precursor polypeptides and CB3 cleavage sites within the context of a chimeric polypeptide. The pattern of proteolytic processing observed for the polypeptides expressed from the pT7-PCP RNA was similar to that obtained with the PV1-HRV14 chimera. Note that the P3-derived PV1-CB3 chimeric polypeptides do not precisely comigrate with their wild-type poliovirus counter-

FIG. 3. In vitro translation of PV1-CB3 chimeric T7 transcripts. (A) Full-length transcripts derived from wild-type poliovirus cDNA (pT7-1 [lanes 2 and 3]) or a chimeric PV1-CB3 cDNA (pT7-PCP [lanes 4 and 5]) were used to direct the synthesis of viral proteins in a rabbit reticulocyte translation system as described in the legend to Fig. 2. The translation reactions were programmed with 10 (lanes 2 and 4) or 5 (lanes 3 and 5) μ g of RNA per ml. Numbers above the lanes indicate nanograms of RNA per 10- μ l reaction. (B) Translation reactions programmed with 5 μ g of pT7-1 (lane 2), pT7-PCP (lane 3), or the insertion mutant pT7-PCP:B (lane 4) RNA per ml were incubated with a poliovirus-infected HeLa cell extract after translation was stopped by the addition of 5 μ g of cycloheximide and 10 μ g of RNase A per ml. Lane 1 in both panels is a marker lane containing [35 S]methionine-labeled PV1 proteins prepared from HeLa cells 6 h postinfection. Positions of viral proteins are indicated to the left of the lanes.

parts. In contrast to the translation and processing of the wild-type poliovirus genome (Fig. 3A, lanes 2 and 3), the most striking aspect of the PV1-CB3 translation was the production of nonstructural polypeptides derived from the P2 and P3 regions coupled with the absence of capsid proteins from the poliovirus P1 precursor. Posttranslational addition of 3C activity (in the form of poliovirus-infected HeLa extract) to the translation of pT7-PCP RNA resulted in the apparently wild-type processing of P1 into VP0, VP1, and VP3 (Fig. 3B, lane 3), confirming the integrity of P1

derived from the chimeric polyprotein to serve as a substrate for poliovirus 3C (or 3CD).

In vitro processing of the PV1-CB3 polyprotein by CB3 3C yielded polypeptides which comigrated with the P2 cleavage products, P2, 2BC, and 2C. However, the decreased levels of 2BC and 2C in Fig. 3A, lanes 4 and 5 indicate that the P2 processing effected by the chimera was somewhat less efficient than wild-type PV1 processing.

Deletion and insertion mutagenesis of chimeric 3C-coding regions. We confirmed that the proteolytic processing of the P2 region polypeptides produced by the PV1-HRV14 and the PV1-CB3 recombinants was indeed carried out by the 3C activities expressed from the chimeras through the introduction of mutations in the 3C-coding regions which would abrogate proteolytic activity. The sequences corresponding to the putative catalytic site of 3C were deleted from pT7-RP3C by the excision of 188 nucleotides between the *Bgl*II sites at positions 5515 and 5703 of the HRV14-derived cDNA. The sites were rejoined via the ligation of a 10-nucleotide *Xho*I linker, which maintained the translational reading frame of the polyprotein while inserting 4 new amino acids (Pro-Leu-Glu-Gly) into the 3C-coding region.

The translation products derived from the transcripts of the deletion construct pT7-RP3C:X are shown in Fig. 2, lanes 5 and 8. It can be clearly seen in lane 5 that the proteolytic processing of the poliovirus-specific P2 precursor was abolished by the deletion in the HRV14 3C-coding region. Processing of the pT7-RP3C:X polyprotein was restored by the addition of exogenous poliovirus 3C to the translation reaction (Fig. 2, lane 8).

Translation of a mutagenized PV1-CB3 cDNA transcript showed the effect of the insertion of 4 amino acids (Ala-Asp-Leu-Leu) into the 3C region of pT7-PCP (Fig. 4B, lane 4). The absence of P2 cleavage products in the pT7-PCP:B translation reaction demonstrated that these polypeptides were indeed the result of 3C proteolytic activity. Processing of pT7-PCP:B polyprotein was demonstrated by addition of exogenous poliovirus 3C activity to the translation reaction (Fig. 3B, lane 4).

Identification of virus-specific polypeptides from cell-free translations. Translation reactions programmed with transcripts from pT7-1 or the chimeric picornavirus RNAs were immunoprecipitated with antisera prepared against poliovirus-specific polypeptides in order to verify the identities of the products of 3C-mediated proteolytic processing. Immunoprecipitation with anti-2C serum clearly demonstrated that the HRV14 3C expressed from the PV1-HRV14 chimera correctly processed the poliovirus P2 precursor into its immunologically related cleavage products, 2BC and 2C (Fig. 4A, lane 3). These results confirm that the HRV14 3C proteinase activity is capable of recognizing and processing the Q-G sites within the poliovirus P2 region. The relatively higher amount of P2 that persisted in the pT7-RP3C translation (Fig. 4A, lane 3) versus in the wild-type poliovirus pT7-1 sample (Fig. 4A, lane 4) supports the results from the translation experiments which suggest that the rhinovirus 3C is not as efficient as authentic poliovirus 3C in the execution of the poliovirus P2 cleavages.

Immunoprecipitation of the pT7-PCP translation reactions with poliovirus anti-2C serum also verified the presence of P2, 2BC, and 2C, the end products of 3C-processing activity (Fig. 4B, lane 9). The P2 region polypeptides were not detected after immunoprecipitation of the insertion mutant pT7-PCP:B translation products.

P3 region polypeptides P3, 3BCD, and 3CD, were immunoprecipitated with poliovirus anti-3D serum from the pT7-1

FIG. 4. Immunoprecipitation of polypeptides produced from the in vitro translation of T7 transcripts derived from wild-type poliovirus or chimeric virus genomes. Translation reactions were diluted with Laemmli sample buffer and immunoprecipitated with poliovirus-specific anti-2C or anti-3D sera as described in references 9 and 38. (A) Immunoprecipitation of a [35 S]methionine-labeled poliovirus-infected HeLa cell extract (lane 2), pT7-RP3C translation (lane 3), or pT7-1 translation (lane 4) with antiserum prepared against poliovirus 2C. Lane 1 is an immunoprecipitation of a [35 S]methionine-labeled HeLa cell extract with preimmune serum. M, Marker lane as described in the legend to Fig. 1. (B) In vitro translation of pT7-1 (lane 2), pT7-PCP (lane 3), and pT7-PCP:B (lane 4) transcripts. Translation reactions immunoprecipitated with anti-3D (lanes 5 through 7) and anti-2C (lanes 8 through 10) were as follows: pT7-1 (lanes 5 and 8), pT7-PCP (lanes 6 and 9), and pT7-PCP:B (lanes 7 and 10). Lane 1, marker lane prepared as described in the legend to Fig. 3. Positions of viral proteins are indicated to the left of the lanes.

translation (Fig. 4B, lane 5). The lower-molecular-weight proteins 3D and 3D', which immunologically react with anti-3D, are not normally produced in detectable quantities when pT7-1 RNAs are translated in cell-free extracts. Immunoprecipitation of pT7-PCP translation products with anti-3D serum detected the presence of P3 region polypeptides P3 and 3BCD (Fig. 4B, lane 6), which did not precisely comigrate with wild-type P3 or 3CD during electrophoresis in sodium dodecyl sulfate-polyacrylamide gels (compare with Fig. 4B, lane 5). We attribute this lack of comigration to

the chimeric nature of the P3 region polypeptides derived from the coxsackievirus and poliovirus sequences and possibly to differential cleavage (either in *trans* or via autocatalysis) within these chimeric polypeptides. No detectable 3CD was observed after immunoprecipitation of the pT7-PCP translation reaction with poliovirus anti-3D serum. Though the Q-G pair at the amino terminus of 3C represents an authentic CB3 cleavage site, it appears that it may not be cleaved by CB3 3C expressed from the chimeric P3 region. We observed similar results upon immunoprecipitation of the pT7-RP3C translation reaction with anti-3D serum (data not shown).

We were unable to identify any authentic 3C-mediated cleavage products after immunoprecipitation of the pT7-PCP:B translation reactions with poliovirus anti-3D or anti-2C sera (Fig. 4B, lanes 7 and 10). Both antisera brought down a polypeptide with an apparent molecular weight of ~150 kilodaltons as well as other high-molecular-weight polypeptides. Presumably, this product represents a P2-P3 fusion protein that was not processed as a result of the inactivation of CB3 3C by the linker insertion. It should be noted that other chimeric polyproteins carrying the same 4-amino-acid insertion in the CB3 3C sequence as that encoded in pT71-PCP:B are capable of producing a polypeptide that comigrates with poliovirus P2 and immunoprecipitates with anti-2C serum (M. A. Lawson and B. L. Semler, unpublished observations).

Translation and processing of polypeptides expressed from pT7-RP3CD. It has been demonstrated that processing of the poliovirus P1 capsid precursor in vitro requires 3C proteinase activity in the form of polypeptide 3CD (15, 47). Cleavage within the P2 and P3 regions which generates nonstructural viral proteins can be executed by either 3C or 3CD with similar degrees of efficiency. The requirement of 3CD for capsid protein processing suggests the existence of a domain contained in the additional 3D sequences that is involved in stabilizing the interaction of the P1 precursor and the proteinase. Therefore, the possibility remained that the inability of the 3C activity expressed from pT7-RP3C to process the poliovirus P1 precursor was due to the chimeric nature of 3CD rather than simply an incompatibility between the heterologous substrate and enzyme. In order to answer this question, a recombinant picornavirus cDNA was constructed in which the intact HRV14 3CD-coding region was precisely substituted for the corresponding poliovirus sequences in the T7 transcription vector.

The resulting chimeric cDNA, pT7-RP3CD, was transcribed and translated in vitro as described for pT7-RP3C. The translation products of pT7-1, pT7-RP3C, and pT7-RP3CD are shown in lanes 2, 5, and 8, respectively, of Fig. 5. Immunoprecipitation of the translation reactions with poliovirus anti-2C serum demonstrated that the 3C proteinase activity expressed by pT7-RP3CD (Fig. 5, lane 10) is similar to that of pT7-RP3C in its ability to process the P2 region proteins. Relatively low amounts of the P1 capsid precursor remained in the translation of the wild-type poliovirus transcript, pT7-1. As evidenced by immunoprecipitation of VP1 with poliovirus anti-VP1 serum, most of the P1 was processed during translation (Fig. 5, lane 3). Parallel immunoprecipitation of the pT7-RP3C and pT7-RP3CD translation reactions with anti-VP1 showed no production of VP1 but rather an accumulation of unprocessed P1 (Fig. 5, lanes 6 and 9).

FIG. 5. In vitro translation and immunoprecipitation of polypeptides encoded in wild-type PV1 or chimeric PV1-HRV14 genomes. In vitro translation of pT7-1 (lane 2), pT7-RP3C (lane 5), and pT7-RP3CD (lane 8). Translation reactions immunoprecipitated with anti-VP1 (lanes 3, 6, and 9), and anti-2C (lanes 4, 7, and 10) sera were as follows: pT7-1 (lanes 3 and 4), pT7-RP3C (lanes 6 and 7), and pT7-RP3CD (lanes 9 and 10). Lane 1, marker lane prepared as described in the legend to Fig. 3.

DISCUSSION

We have generated defined chimeric picornavirus genomes to provide the first experimental evidence for cross-processing of a picornavirus polyprotein by 3C proteinases derived from different viruses. Previously unsuccessful attempts to detect cross-processing between poliovirus and encephalomyocarditis virus or between foot-and-mouth disease virus and encephalomyocarditis virus involved mixing experiments in which exogenously expressed 3C was added to substrates that had been previously synthesized in vivo or in cell extracts (29). The approach we have undertaken involved the direct allelic replacement of the 3C-coding region of one picornavirus with that of another and addresses the question of whether the 3C enzyme can be functionally substituted with an enzyme of similar but not identical substrate specificity.

Statistical analysis of the predicted amino acid sequences of poliovirus and HRV14 predicts that the location of specific secondary structural motifs of the respective 3C poly-

peptides are quite similar (46). However, the two proteins share only 45% amino acid homology (4, 46) and are not immunologically cross-reactive (7). Although most of the Q-G cleavage sites appear to be shared between poliovirus and HRV14, the 3C proteinase of HRV14 may recognize an E-G pair between VP3 and VP1 and a Q-A pair between 2B and 2C in the HRV14 genome (based on analogy of the predicted amino acid sequence of HRV14 and the apparent molecular weight of HRV14 polypeptides compared with that of poliovirus). The degree of amino acid conservation between the 3C proteins of PV1 and CB3 is 60%, which reflects the closer relatedness that exists between these two enteroviruses (22). On the basis of the amino acid sequence deduced from the CB3 nucleotide sequence, the Q-G cleavage sites are conserved between PV1 and CB3 with the exception of a Q-N site at the 2B-2C junction. It was of interest to determine which, if any, of the Q-G cleavage sites present in the poliovirus polypeptide could be recognized and processed by heterologous 3C proteinases.

The most striking result of these experiments was the inability of the chimeric proteinase to efficiently process the poliovirus-specific P1 precursor into mature capsid proteins. Although X-ray crystallographic data obtained for poliovirus (11) and HRV14 (35) indicate that the virion particles and their constituent capsid proteins share a basic common core structure, fundamental differences in the three-dimensional conformations of the three viruses are suggested by the lack of immunological cross-reactivity among their individual capsid proteins and by the fact that they bind to distinct cell surface receptors (23). Recent data from *in vitro* translation and processing of genetically altered poliovirus P1 polypeptides suggest a highly specific interaction between the capsid precursor and the poliovirus Q-G cleavage activity (48). Consequently, it is likely that the putative site recognition domain(s) of either heterologous 3C proteinase is unable to efficiently direct the catalytic region of the enzyme to the Q-G sites when they are presented in the context of the poliovirus P1 precursor. Alternatively, in light of experimental evidence indicating that 3CD rather than 3C actually carries out the proteolytic processing of the P1 Q-G sites (15, 25, 47, 49), the inability of the hybrid P3 precursor polypeptides to process the poliovirus capsid proteins may be a reflection of the heterologous nature of 3CD. Our experimental evidence rules out the latter possibility for HRV14 proteolytic activity. A chimeric genome containing an exact replacement of HRV 3CD into the poliovirus full-length cDNA was unable to direct the synthesis of proteinase activity that will cleave the poliovirus P1 precursor polypeptides (Fig. 5). In light of this data, it is not surprising that the chimeric cDNAs initially proved to be noninfectious. It should be noted that we have recently generated a chimeric genome containing poliovirus 3CD-coding sequences cloned into a cDNA encoding the CB3 polypeptide. Our preliminary experiments suggest that poliovirus polypeptide 3CD expressed from this genome is capable of generating low levels of CB3 capsid polypeptides from the heterologous P1 (CB3) precursor polypeptides (M. A. Lawson and B. L. Semler, manuscript in preparation).

It was particularly interesting that the HRV14 and CB3 proteinases were capable of correctly processing the poliovirus P2 precursor, since the primary sequence of the 2B-2C-processing site is not identical among the three viruses employed in this study. Mutational inactivation in the coding regions of the HRV14 and CB3 3C proteinases correlated with the inhibition of P2 processing, thus confirming that the foreign 3C enzymatic activity was directly

responsible for mediating the proteolytic processing of the poliovirus P2 precursor expressed from the recombinant. The identity of the proteolytic cleavage products was confirmed by immunoprecipitation with antiserum directed against poliovirus 2C. These results provide experimental evidence that structural determinants, in addition to the presence of a particular amino acid pair, are required for the recognition of a cleavage site by the 3C proteinase activity. The ability of the chimeras to generate 2C supports earlier conclusions, based on amino acid sequence comparisons (1) and antigenic cross-reactivity (7), that the 2C polypeptides are structurally conserved among the picornaviruses.

It is apparent from our experiments that processing of the P2 precursor may be functionally substituted with 3C from another picornavirus, whereas processing of the P1 capsid precursor is more virus specific and dependent on the presence of specific substrate recognition domains located in 3CD. The picornaviruses have evolved from a primordial ancestor into a group of viruses that exhibit a highly diverse host range and a myriad of different physiological effects. Although these viruses share a common genome organization and basic virion structure, fine structural differences in the architecture of the virion surface are likely to play a role in host cell tropism. The stringent enzyme-substrate requirements for structural protein processing can be therefore presumed to be the result of the coevolution of the capsid protein genes and the substrate recognition determinants of their requisite processing enzymes.

The isolation of chimeric picornavirus cDNAs has proved to be a valuable tool for probing the functions of the 5'-noncoding region of poliovirus (14, 21, 40) and provides a potential alternative for the development of new picornavirus vaccines (3, 17, 24). The generation of interspecies recombinants within the nonstructural regions of picornavirus genomes provides the opportunity to functionally map the domains that are important for conferring substrate specificity to the 3C proteinase activity. This information is a key to our complete understanding of the regulation of picornavirus protein processing. From a practical standpoint, this line of experimentation will also determine whether the picornaviral proteinases are sufficiently similar to serve as a common target for antiviral chemotherapy.

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