Species-specific Substrate Interaction of Picornavirus 3C Proteinase Suballelic Exchange Mutants*

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The substrate recognition properties of the poliovirus type 1 and coxsackievirus B3 3C proteinases have been examined in vitro by allelic and suballelic exchange of 3C between the cloned virus genomes. The activity of the altered 3C proteinases was examined by translation of synthetic RNA in a rabbit reticulocyte lysate/HeLa cell extract translation system. Analysis of the subsequent processing of virus polyproteins by the altered 3C proteinases showed that all of the mutant proteinases maintained some catalytic activity. The disruption of polyprotein cleavages mediated by 3C followed a distinct pattern, suggesting a specific order of events in processing the polyprotein. Differences in cleavage activity of mutant proteinases when tested on coxsackievirus or poliovirus protein substrates suggest that, although structural elements throughout the proteinase play a role in efficient substrate utilization, the carboxyl-terminal region of the 3C proteinase contains elements most important in species-specific substrate recognition.

The modification of protein activity through specific intermolecular interaction with regulatory effectors is an important mechanism of controlling biochemical processes. The ability of a protease to recognize a specific target protein and to modify its activity by subsequent cleavage is an essential element in the post-transcriptional control of gene expression. Such a mechanism of regulation of gene expression is a prominent feature of the intracellular life cycle of members of the Picornaviridae. The picornaviruses are small RNA viruses which encode their genetic information in a single RNA molecule of message polarity (~7.5 kilobases in the case of the genus enterovirus). The viral genome is capable of serving as an mRNA upon uncoating and release into the host cell cytoplasm. The ~250,000-Da polyprotein which results from the cap-independent translation of the genome of poliovirus (PV)1 type 1, the prototype enterovirus, contains all the enzymatic activities and structural entities required to direct the replication of the viral RNA and the assembly of progeny virions.

To overcome the constraint of a monocistronic genome in the production of widely differing protein products, PV1 utilizes the proteolytic activity of two virus encoded cysteine proteinases, 2A (Toyoda et al., 1986) and 3C (Hancak et al., 1982), to cleave the polyprotein in a highly specific manner, resulting in the liberation of functional proteins (for reviews see Nicklin et al., 1986; Wellink and van Kammen, 1988; Dougherty and Carrington, 1989; Krauslich and Wimmer, 1988; Dewalt and Semler, 1989). A map of the PV1-encoded polyprotein is shown in Fig. 1 with the processing products normally seen in infected cells (Kitamura et al., 1981; Pal-lansch et al., 1984). The complex cascade of proteolytic events carried out by the viral proteinases serves to regulate the expression of other virus gene products. The 2A proteinase of poliovirus mediates the cleavage of the P1 precursor from the nascent polyprotein in an intramolecular (cis) fashion. The proper processing of the P1 precursor itself is dependent on this event, and larger, unprocessed P1 containing precursor proteins are not processed by the viral 3C activity (Ypma-Wong and Semler, 1987a). The 3C proteinase only inefficiently cleaves the capsid protein precursor VP1 from the capsid precursor P1 (Nicklin et al., 1988). However the 3CD protein, which additionally includes the 3D RNA polymerase sequences, has been shown to be the minimal protein capable of efficient in vitro processing of the P1 precursor to VP0, VP3, and VP1 (Jore et al., 1987; Ypma-Wong et al., 1988a). The cleavage of VP0 to VP4 and VP2 occurs only in mature viruses and has not yet been ascribed to a host or virus proteolytic activity.

A distinguishing feature of the picornavirus proteinases is their apparent specificity for virus substrates and their limited sequence specificity for amino acid pairs. Both the amino acid pair at the cleavage site and the surrounding amino acids have been shown to play a role in the determination of a cleavage site. The majority of cleavages in the PV1 polyprotein are mediated by the proteolytic activity of the 3C protein and occur exclusively between Q-G residues. However, not all Q-G sequences are cleaved. The proteolytic activities of other animal and plant RNA viruses are known, by amino acid sequence analysis of cleavage products or inferred from comparison of predicted amino acid sequences of cloned virus genomes, to occur at sequences other than Q-G (see above reviews). Genetic evidence suggests that the PV1 3C can also cleave at sites other than Q-G (Kirkegaard and Nelsen, 1990). Genetic manipulation of the 3C cleavage sites of the cardio-virus encephalomyocarditis virus has shown that the 3C proteinase of encephalomyocarditis virus can utilize dipeptide sequences not normally found in the virus polyprotein (Parks and Parnmenberg, 1987; Parks et al., 1989). A common characteristic found by examination of the amino acid sequences around the PV1 cleavage sites is the presence of an aliphatic amino acid residue in the P1 position of many cleavage sites.
specificity suggests that the proteinases recognize an extended amino acid sequence at the cleavage sites.

Proteinases are capable of processing the PV1 nonstructural proteinase may be involved in the recognition of cleavage sites with the ability of the Pl precursor protein to be processed to the substrates tested is important for efficient utilization of the substrates (Dewalt et al., 1989). The above observations indicate that some of the structural determinants of cleavage at the various sites in the polyprotein must be common among viruses bearing the mutations (Blair et al., 1990). Replacement of equivalent regions of the PV1 and CVB3 3C proteins to determine which regions of the 3C coding region and the 3'-nontranslated region, respectively. Poly(A) indicates the virus-encoded polyadenylate tract. The structure of the encoded polyprotein translation product is diagrammed with the map of the various products of proteolysis. Solid vertical lines indicate sites of cleavage in the polyprotein. The solid triangles (△) below the diagrammed polyprotein indicate sites cleaved by the proteolytic activity of the viral proteinase. Empty triangles (○) indicate sites cleaved by the proteolytic activity of the viral proteinase. The diamond (♦) indicates the site of cleavage in the capsid precursor VP0 which occurs only in mature virus particles to produce VP4 and VP2. Dashed vertical lines indicate sites of infrequent 3C- and 2A-mediated cleavage which have been described only in poliovirus type 1. Below the polyprotein is shown a processing cascade illustrating the proteins normally produced by virus encoded proteolytic activity.

EXPERIMENTAL PROCEDURES

Enzymes and Lysates—Restriction endonucleases were purchased from New England Biolabs or Boehringer Mannheim. Bacteriophage T4 DNA ligase, the Klenow fragment of E. coli DNA polymerase I, calf intestinal alkaline phosphatase, bovine pancreatic RNase A, creatine phosphate, creatine kinase, and aprotinin were purchased from Boehringer Mannheim. Rabbit reticulocyte lysate and RNAsin were purchased from Promega Biotech. Bacteriophage T7 DNA polymerase and polynucleotide kinase were purchased from Pharmacia LKB Biotechnology Inc.

Cells and Viruses—All plasmids were propagated in E. coli strain C600 or GM33 as noted. HeLa S3 cells grown in suspension were used for the preparation of all cell extracts. Poliovirus type 1 (Mahoney) and the Nancy strain coxsackievirus B3 were plaque-purified on HeLa cell monolayers, and virus stocks were prepared in HeLa S3 cells grown in suspension. Extracts of cells infected with PV1 or CVB3 were made 7 h post-infection or 8 h post-infection, respectively, and these new sites are maintained in viruses bearing the mutations (Blair et al., 1990). Replacement of regions of the PV1 cDNA that encode the 3C proteinase and neighboring sequences with the equivalent regions of coxsackievirus B3 (CVB3) and human rhinovirus 14 cDNA has shown in vitro that the CVB3 and human rhinovirus 14 proteinases are capable of processing the PV1 nonstructural P2 and P3 proteins, although the amino acid sequences at the cleavage sites differed from those found in their natural substrates (Dewalt et al., 1989). The above observations indicate that some of the structural determinants of cleavage at the various sites in the polyprotein must be common among the enteroviruses and rhinoviruses.

The experiments described in this paper were designed to investigate the substrate recognition properties of the PV1 and CVB3 3C proteins to determine which regions of the 3C proteinase may be involved in the recognition of cleavage sites in the polyprotein. The 3C proteinases of PV1 and CVB3 show ~60% amino acid identity (Lindberg et al., 1987). In the present study, we employ the technique of suballelic replacement of equivalent regions of the PV1 and CVB3 3C proteinases to examine the effects of domain replacement on the substrate recognition properties of both proteinases. We conclude that a carboxyl-terminal domain of 3C homologous to the substrates tested is important for efficient utilization of cleavage sites in the polypeptides of PV1 and CVB3. We also conclude from amino acid insertion mutagenesis of the CVB3 3C proteinase that the structural integrity of this region is important for the proper processing of the polyprotein.

An extensive study of the role of higher order structures in the processing of the P1 capsid precursor of PV1 has revealed that a disruption of amino acid sequences encoding secondary structural elements of the mature virus particle interferes with the ability of the P1 precursor protein to be processed (Ypma-Wong et al., 1988b). The natural cleavage sites in the P1 precursor occur in regions that are relatively unstructured. Insertion of new cleavage sites into these regions was tolerated by the proteinase, and these new sites are maintained in viruses bearing the mutations (Blair et al., 1990). Replacement of regions of the PV1 cDNA that encode the 3C proteinase and neighboring sequences with the equivalent regions of coxsackievirus B3 (CVB3) and human rhinovirus 14 cDNA has shown in vitro that the CVB3 and human rhinovirus 14 proteinases are capable of processing the PV1 nonstructural P2 and P3 proteins, although the amino acid sequences at the cleavage sites differed from those found in their natural substrates (Dewalt et al., 1989). The above observations indicate that some of the structural determinants of cleavage at the various sites in the polyprotein must be common among the enteroviruses and rhinoviruses.

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region of pCP-P3-NSE by C-G transversion and T-C transition of N5363 and N5366, respectively. The new plasmid, pCP-P3-NSE-S1, was then used to create a KpnI site at N5651 by C-T transition of that nucleotide. The plasmid resulting from the second round of mutation, pCP-P3-NSE-S1/1, was completely digested with Apal and PstI. The resulting 2.8-kbp DNA fragment was purified by gel electrophoresis, religated and ligated to the gel-purified 3.1-kbp Apal to PstI fragment of pMV3.9-Ls4/3. The new plasmid, pMV-7-PCBP, containing PstI coding sequences from N5660 to NT524 in which the PVI 3C coding sequence at the BclI site at N5511 between the codons for amino acid 51 and 52 of the wild-type 3C protein was isolated. The isolated DNA was further treated with the Klenow fragment of E. coli DNA polymerase I and calf intestinal phosphatase. The blunt-ended and dephosphorylated DNA was then ligated to the phosphorylated synthetic octameric XhoI linker (New England Biolabs). Excess linkers were removed by digestion with XhoI, and the resulting DNA was religated and transformed into E. coli C600. Individual clones were screened for the presence of a new XhoI site at N5857 and the presence of an additional XhoI site at that location. The mutant 3C proteinase was then cloned into the full-length transcription vector containing the complete PVI cDNA, p17-1/1 (Ypma Wong and Semler, 1987a), by replacement of the 2.4-kbp Xmal fragment from N4685 to NT107 of P1 with the equivalent fragment from the various recombinant subclones. The recombinant plasmids were designated by the restriction sites bordering the CVB3 insertion. For example, pT7-CP3C-S1/5, containing a 5-kbp fragment of pCP-P3C-S1 at the 3' end of the cDNA, was designated as pT7-CP3C-S1/5 containing a complete replacement of 3C with CVB3 (pT7-PC3C) and a PVI cDNA containing the new restriction endonuclease recognition sequences in 3C (pT7-PVI-NC) were also constructed. All the plasmid constructions are described in Fig. 2.

Construction of a CVB3 Transcription Vector—A T7 transcription vector containing a CVB3 cDNA clone from N1 to N5560, pT7-CP3C-5.5, was constructed by combining five fragments from previously described plasmids. The EcoRI to PstI fragment of pCP-S05 containing the 523-kbp fragment of pT7-CP3D (Damasahapatra et al., 1987) was digested with EcoRI and PstI to Sall and ligated to the Sall fragment of pBVII-1 containing N529-N746 (Tracy et al., 1985). The Sall to SpeI fragment of pC7B7, a derivative of pBD7, containing N746-N847 (Uasahapatra et al., 1987), the SpeI to Xhol fragment of pBVII-3 containing N8387-N9497 (Tracy et al., 1985), and the Xhol to EcoRV fragment of pCP-P3-NSE-S1/1, described above, containing N9497-N9590, were all ligated in a sequential manner into the transcription vector pGEM-1 (Promega Biotech) under the control of the transcriptional promoter for bacteriophage T7 RNA polymerase. The resulting 3C3 and 3D3 sequences from pT7-PVI-NC were recombined into pT7-CP3C-3.9 containing a 5.9-kbp fragment of pCP-305 between the codons for amino acid 52 and 53 of the wild-type 3C proteinase. A BglII linker insertion mutant encoding an additional four amino acids, D-L-L-A, between amino acids 103 and 104 of the CVB3 3C protein has been previously described (Dewalt et al., 1989). This insertion was engineered into the plasmids pT7-CP3C and pT7-PC3D and the mutant full-length clones were designated pT7-CP3CB and pT7-PC3DB, respectively. pT7-CBV3-5.9-Xc containing a four-amino-acid insertion (3C insertions: amino acid 167 and the 4.9-kbp fragment of pT7-CBV3-5.9 was inserted into an XhoI construct from N3660 through the 3' end of the cDNA) to the 5.3-kbp fragment was then dephosphorylated, ligated to the 3.8-kbp fragment, and transformed into E. coli C600. Individual clones were screened for the loss of the BglII site at N5857 and the presence of an additional XhoI site on the vector. The mutant 3C proteinase was then cloned into the full-length transcription vectors pT7-CP3C and pT7-PC3D containing a complete replacement of 3C with CVB3 (pT7-PC3C: Xc and pT7-PC3D: Xc). The plasmid constructions are described in Fig. 2.

Transcription of Full-length cDNA Clones in Vitro—Prior to transcription, all plasmid DNAs were linearized by complete digestion with Sall, which cleaves in the multiple cloning site 3' to the inserted viral cDNA. Plasmid cDNAs were transcribed using bacteriophage T7 RNA polymerase as described (Ypma Wong and Semler, 1987a), except that 50-100 µCi ml-1 of [α-32P]GTP (3000 Ci mmol-1) was included to facilitate quantitation. Transcription reactions were performed using 40 µg ml-1 of plasmid DNA. After transcription, synthesized RNA was purified by phenol/chloroform extraction. The organic phase was re-extracted using an equal volume of 10 mm TrisCl, pH 9, 1 mM EDTA, and 0.1% SDS. The aqueous phases from extraction were combined, made 2.5 M NH4OAc, and then precipitated with 2.5 volumes of ethanol. Precipitates were pelleted, washed twice with 70% ethanol, and resuspended in 1/10th transcription volume of H2O. The RNA was quantitated by spotting an aliquot onto DE81 filter paper (Whatman), washing extensively in 5% NaOH/0.5 M Na2HPO4, and counting by scintillation according to a described method (Sambrook et al., 1989).

Translation of RNA in Vitro—The conditions of translation have been previously described, with the exception that creatine phosphate was replaced with auxin (20 µm Dorrin et al., 1984). Translation reactions were carried out at 30 °C in a rabbit reticulocyte lysate supplemented with a HeLa cell cytoplasmic extract (Ypma Wong and Semler, 1987a). The amount of extract used, typically 20-25% of the reaction volume, was optimized for the inhibition of internal initiation events. In vitro transcribed RNA were used as substrates for wild-type CVB3 and CIII 3C activities, to be tested as substrates of wild-type CVB3 or CIII 3C activity, reactions were carried out in the presence of 30 µg ml-1 of proteinase A to a final concentration of 5 µg ml-1. Reactions were terminated after 3 h by the addition of bovine pancreatic RNAase A to a final concentration of 200 µg ml-1 and incubated at 30 °C for an additional 20 min. If the synthesized proteins were to be tested as substrates of wild-type CVB3 or CIII 3C activity, reactions were carried out in the presence of cycloheximide at a final concentration of 5 µg ml-1 in addition to the RNAase A treatment. Post-translational processing was then carried out for 1 h at 30 °C by incubation of 30% vol/vol of CII- or CIII-infected HeLa cells. All reactions were diluted 10-fold in Laemmli sample buffer and 10-20 µl of the [35S]methionine-labeled proteins were analyzed by electrophoresis on 15% SDS-polyacrylamide gels (Laemmli, 1970). Gels were fluorographed with 2.5-diphenyloxazole and exposed to Kodak XAR film at -70 °C.

Identification of Proteins by Immunoprecipitation—Proteins produced in vitro were analyzed by immunoprecipitation using either wild-type and mutant 3C activity were immunoprecipitated from translation reactions with rabbit antiserum directed against the poliovirus 2C or 3D proteins by a previously described protocol (Semler et al., 1982). Antiserum against poliovirus 2C has been reported to be cross-reactive with the 2C protein of CVB3 (Emini et al., 1985). Cross-reactivity of the PV1 3D antisera with the 3D protein of CIV from CIV-infected cells was detected by immunoprecipitation of proteins analyzed by SDS-polyacrylamide gel electrophoresis and visualized by fluorography as described above.
RESULTS

To define regions of the 3C proteinase of enteroviruses which may confer the substrate recognition properties of the enzyme, new restriction endonuclease recognition sequences were engineered into the respective cDNA clones of PV1 and CVB3 at codons encoding amino acids 1, 67, and 126 of the 3C proteinase to allow suballelic exchange of regions encompassing approximately one-third of the encoded 3C proteinases. The effects of such substitutions on the processing of the PV1 and CVB3 polyprotein were examined by in vitro translation of RNA synthesized from the altered cDNA clones. The structures of the various recombinant proteinases are shown in Fig. 2. All plasmids encoding replacements into the PV1 3C region were termed pT7-CP3C with the further extension AK, KA, AR, AA, KA', or KR to designate the restriction sites bordering the insertion of CVB3 sequences into the PV1 cDNA. Plasmids bearing replacements of CVB3 cDNA sequences with the chimeric PV1/CVB3 cDNA were termed pT7-PC3CD to reflect the presence of PV1 3D in all cDNA clones. The extensions were maintained in the nomenclature of the PV1-based plasmids to designate identical chimeric proteinases in the CVB3 or PV1 cDNA background. Additionally, amino acid insertions into the CVB3 3C sequences after amino acids 51, 103, and 167 were separately constructed into the full-length PV1 and CVB3 cDNA clones. The effect on processing of the insertions into 3C was analyzed as above.

Processing of the PV1 Polyprotein by the pT7-CP3C-encoded 3C Activity—The modified cDNA clone pT7-PV1-NC, which contains the newly constructed restriction endonuclease sites engineered in the wild-type PV1 cDNA of pT7-1, was found to have an identical processing phenotype as that of pT7-1 when RNA synthesized in vitro from the plasmid was translated in the rabbit reticulocyte/HeLa cell extract translation system (data not shown). Translation in vitro of RNA synthesized from the plasmid pT7-CP3C, which replaces the PV1 3C coding region and 13 amino acids of the 3D coding region with CVB3 sequences, exhibited a pattern of processing shown in lane 4 of Fig. 3A. By comparison with the translational and subsequent processing of the wild-type PV1 proteinases encoded by pT7-1 shown in lane 3 of Fig. 3A, it can be readily seen that the PV1 structural proteinases VP0, VP1, and VP3 (normally produced by the processing activity of the encoded wild-type 3C activity of pT7-1) are not produced by the CVB3 3C activity encoded by pT7-CP3C. The nonstructural P2 and P3 proteins are efficiently processed to 2BC, 2C, 2A, and 3CD by the CVB3 3C activity as provided in the context of a single polyprotein, consistent with observations reported previously (Dewalt et al., 1989). The remaining P3 proteins 3D, 3C, and 3AB are not efficiently produced in vitro translation reactions (Ynpa-Wong and Semler, 1987a).

The altered mobility of the P3 and 3CD proteins is most likely a consequence of the presence of CVB3 sequences in the P0 region (compare lanes 3 and 4 of Fig. 3A).

Processing of the PV1 Polyprotein by the pT7-CP3C-AK, pT7-CP3C-AR, and pT7-CP3C-AR-encoded 3C Activity—The processing of PV1 proteinases encoded by RNA synthesized from cDNA clones in which the first, second, or last one-third of the PV1 3C coding region has been replaced by CVB3 sequences is markedly different (Fig. 3A, lanes 5, 6, and 8) compared with the processing of PV1 proteinases by the wild-type pT7-1 and allelic replacement pT7-CP3C cDNAs shown in lanes 3 and 4 of Fig. 3A. The processing of the P2-derived proteins is differentially affected by the replacement of PV1 sequences with those of CVB3. Replacement of the first one-third (67 amino acids) of the PV1 3C with CVB3 3C sequences (pT7-CP3C-AK, lane 5 of Fig. 3A) created an enzyme which cannot mediate the production of 2C. The proteolytic activity of the PV1 3C proteinase containing a replacement of the central one-third (amino acids 68–126) of the PV1 3C protein (pT7-CP3C-AR, lane 6 of Fig. 3A) with CVB3 sequences is unable to mediate the production of 2C or 2BC (by inference 2A). The replacement of the PV1 sequences from amino acid 127 to the carboxyl terminus of 3C with CVB3 sequences (pT7-CP3C-AR, lane 8 of Fig. 3A) produces a proteinase incapable of processing the PV1 polyprotein into any authentic P2 region protein.

The processing of the P3 region proteins was also differentially affected among the amino-terminal, central, and carboxyl-terminal one-third 3C replacement mutants. The amino-terminal replacement mutant (pT7-CP3C-AK) produces only the P3 protein and none of its authentic cleavage products in detectable quantities. The central-one-third replacement (pT7-CP3C-AR) does not produce any detectable P3 protein. The level of 3CD produced by the carboxyl-terminal replacement mutant (pT7-CP3C-AR) were very low. The appearance of aberrant processing products in lanes 5, 6, and 8 of Fig. 3A and the presence of proteins of greater size than the P1 protein (97,000 Da) indicate that the loss of nonstructural protein processing capability is accompanied by slow or inappropriate cleavage, which may represent both altered cleavage pathways by which individual proteins are produced and altered rates of cleavage at the various C-G cleavage sites.

To test the ability of the proteinases produced by translation
Further incubated in the presence of a crude extract of PV1-infected HeLa cells for 1 h. The P1 precursors encoded by all mutant cDNAs were processed by the wild-type PV1 3C activity (Fig. 3A, lanes 11, 12, and 14). Further processing of nonstructural proteins by the wild-type PV1 3C activity was also detected, with the exception of the proteins produced by pT7-CP3C-AR, from which 2C was produced but not 2BC and 2A (Fig. 3A, lane 14).

**Processing of the PV1 Polyprotein by the pT7-CP3C-AA, pT7-CP3C-KA’, and pT7-CP3C-KR-encoded 3C Activities**—The cleavage of PV1 proteins by the activity of chimeric 3C proteinases in which two-thirds of the PV1 sequences have been replaced with equivalent CVB3 sequences is shown in Fig. 3A, lanes 7 and 13 and B, lanes 5, 6, 9, and 10. The chimeric 3C activity encoded by pT7-CP3C-AA-derived RNA, in which the amino-terminal two-thirds of the protein are derived from CVB3, can mediate the production of authentic P2, 2BC, 2C, and 2A proteins as well as P3 and 3CD (Fig. 3B, lane 5). The appearance of additional proteins that are not normally observed in translation of pT7-1 RNA (compare with B, lane 3) indicates the inappropriate use of cleavage sites in the polyprotein. The 3C activity encoded by pT7-CP3C-KA’ RNA (A, lane 7), in which the first and last one-third of the 3C proteinase are derived from CVB3, also shows efficient production of P2 and its processed products as well as P3 and 3CD. Additional proteins of high apparent molecular weight and proteins resulting from the alternate cleavage of P3-derived proteins, possibly at the proteinase 2A Y-G cleavage site in the PV1 3D sequence, are also seen. The proteins produced by the cleavage activity of the pT7-CP3C-KR-encoded 3C proteinase, the last two-thirds of which is CVB3-derived, are shown in lane 6 of Fig. 3B. Although the P2 protein was produced by the mutant 3C activity, the production of 2BC and 2C is limited relative to the 3C activity encoded by pT7-CP3C-AA. Normal P3 proteins are detected and additional aberrant processing products are seen.

**Identification of Proteins Produced by Altered 3C Cleavage Activity**—To identify P2-derived proteins produced by *in vitro* translation and subsequent processing by the altered 3C activities, all translation reactions were immunoprecipitated with antiserum directed against the poliovirus proteins 2C (Hanecak et al., 1982). Immunoprecipitated proteins were subjected to electrophoresis on 12.5% SDS-polyacrylamide gels with the products of the original translation reaction to facilitate identification of proteins. To identify proteins derived from the P3 region of the polyprotein, translation reactions were also immunoprecipitated with antiserum directed against the poliovirus protein 3D (Semler et al., 1983) and analyzed as above.

The normal P2, 2BC, and 2C proteins produced by wild-type 3C-mediated cleavage of the PV1 polyprotein are shown in the immunoprecipitate displayed in lane 3 of Fig. 4A next to the original translation reaction of pT7-1-derived RNA in lane 2. The immunoprecipitation of 2C containing proteins produced by the CVB3 3C activity from translation of pT7-CP3C-derived RNA is shown in lane 5 of Fig. 4A. The immunoprecipitation shows that the processing of nonstructural P2 proteins is similar to that of the wild-type 3C activity. The normal processing of P3 derived proteins was confirmed by a similar analysis (data not shown).

**Identification of Proteins Produced by pT7-CP3C-AK, pT7-CP3C-KA, and pT7-CP3C-AR-encoded 3C Activities**—The immunoprecipitation of the P2 proteins produced by translation of pT7-CP3C-AK-derived RNA and subsequent processing by the chimeric 3C activity confirms the production of P2 and 2BC proteins and the inability to produce the 2C...
of an apparent molecular mass slightly greater than P2 (65,000 Da, Fig. 4A, lanes 12 and 13). The above translation reactions were also immunoprecipitated with antisera directed against the 3D protein. The proteins with electrophoretic mobilities equal to or less than that of the P1 protein that were reactive with the 2C antisera were found to also be reactive with the 3D antisera (data not shown). The estimated molecular masses of the proteins are consistent with proteins that would be produced by alternate cleavage of the nonstructural precursor containing both P2 and P3 (see Pallerinsh et al., 1984) resulting in proteins containing 2C and 3D sequences, such as 2ABC3ABC' (with a predicted molecular mass 113,400 Da) 2C3AABC (predicted molecular mass 122,000 Da), and 2B2C3ABCD (predicted molecular mass 132,000 Da). The smaller proteins which also appear in the translations of pT7-CP3C-KA-, pT7-CP3C-AK-, and pT7-CP3C-AR-derived RNA are most likely the cleavage counterparts of the large proteins produced by such alternative processing pathways.

Identification of Proteins Produced by the pT7-CP3C-AA, pT7-CP3C-KA', and pT7-CP3C-KR-encoded 3C Activities—Proteins produced by **in vitro** translation and processing by the chimeric 3C activities of pT7-CP3C-AA, pT7-CP3C-KA', and pT7-CP3C-KR were also analyzed by immunoprecipitation with the 2C and 3D antisera. Electrophoretic analysis of pT7-CP3C-AA (Fig. 4B, lanes 6 and 7), pT7-CP3C-KA' (A, lanes 10 and 11), and pT7-CP3C-KR (B, lanes 8 and 9) immunoprecipitates revealed that all chimeric activities were capable of producing authentic P2, 2BC, and 2C proteins, although to varying degrees. As above, the proteins identified by immunoprecipitation with both antisera and which do not comigrate with protein species normally seen in translation of PV1 wild-type pT7-1-derived RNA can be accounted for by the use of alternative pathways of cleavage at Q-G and Y-G amino acid sequences known to be utilized in the wild-type PV1 polyprotein. The variety of 2C containing proteins that are visualized by immunoprecipitation of translation reactions programmed with RNA encoding the chimeric 3C proteinases, particularly those involving the first two-thirds of the 3C proteinase, indicates that the normal processing of the polypeptide is inhibited by the mutations, allowing aberrant cleavage events to occur that are never or rarely seen in normal 3C-mediated processing of the wild-type polyprotein.

**Processing of the PV1 Polyprotein by the pT7-CP3C,X, pT7-CP3C,B, and pT7-CP3C:X2-encoded 3C Activities**—The PV1 cDNA clone in which the 3C proteinase coding region after the codons for amino acids 51, 103, and 167 as described under Experimental Procedures was replaced with an insertion mutation has been constructed into the chimeric cDNA in which the 3C coding region of CVB3 has replaced that of the wild-type PV1 sequences is capable of normal processing of the PV1 nonstructural proteins but not the structural precursor polypeptides. Three different insertion mutations were constructed into the chimeric cDNA in each of the CVB3 3C proteinase encoding region after the codons for amino acids 51, 103, and 167 as described under Experimental Procedures. Analysis of the processing capabilities of the insertion mutations on the PV1 polyprotein substrates showed similarities to the suballelic replacement mutations described above. The proteins produced in translation reactions programmed with RNA synthesized from pT7-CP3C,X, encoding a four-amino acid (A-S-R-D) insertion at amino acid 51 of the CVB3 3C proteinase, were analyzed on 12.5% polyacrylamide gels. The processing of the nonstructural proteins derived from the P2 and P3 regions was unaffected (Fig. 5, lane 5). The presence of authentic P2 and P3 proteins was further supported by immunoprecipitation of translation reactions with 2C and 3D antisera (data not shown).

The four-amino acid (A-D-L-L) insertion at amino acid 103 of the CVB3 3C protein encoded in the chimeric cDNA pT7-
program translation react&s as described under "Experimental Pro-
cedures." The protein processing profiles of the encoded wild-type
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compositions of the cDNA tem-
an infected HeLa cell extract (+ inf HeLa) as described under
described in Fig. 2. Lane 1 is marker PVl proteins prepared as
aliquots of the reactions in lanes 3-7 which have been post-transla-
and mutant 3C activities are displayed in lanes 3-7. Lanes 8-12 are
stitution mutants of the CVB3 3C proteinase tested on the
PC3C:B exhibits pronounced defects in P3 processing (Fig. 5,
lane 6). The proteinase activity produced by in uitro transla-
tion and processing of pT7-CP3C:CB does not mediate the
production of detectable amounts of 2C, but does produce
detectable levels of P2 and 2BC (Fig. 5, lane 6).
The four-amino acid (R-S-S-G) insertion at amino acid 167
and the Q's" deletion in the CVB3 3C proteinase encoded in the
structural precursor P2 was produced, but no further P2
processing was detected by anti-2C immunoprecipitation. Sig-
ificant aberrant processing was also observed (Fig. 5, lane 7).
Immunoprecipitation of the proteins produced by the
processing activity of pT7-CP3C:X2 with 3D antiserum de-
lected low levels of normal P3 protein, although the majority
of P3-derived proteins were aberrantly processed by 2A activ-
ity. Collectively, the aberrant P2 and P3-derived proteins
produced by the altered 3C activities of pT7-CP3C:CB, pT7-
CP3C:B, and pT7-CP3C:X2 are similar to those seen in the
translations of the suballelic replacement mutations in 3C
which involve the same regions of the proteinase.

The processing activities of the suballelic replacement mu-
tants and the processing activities of the amino acid insertion
mutants of the CVB3 proteinase on the PV1 polyprotein are summarized in Fig. 7. Several conclusions can be made from
the summary, and these are presented under "Discussion"
(see below). If conclusions drawn from the observations made
of the processing activity of the chimeric PV1/CVB3 3C
proteinases on the PV1 polyprotein are valid, similar effects
of the suballelic replacements on the processing of the CVB3
polyprotein should also be observed. To test this, the chimeric
3C proteinases and the amino acid insertion mutants were cloned into the CVB3 cDNA plasmid pT7-CB3-5.9.

Processing of the CVB3 Polyprotein by the pT7-PC3CD and
pT7-PC3D-encoded 3C Activities—The cDNA clone pT7-
PC3CD contains the CVB3 sequences from nucleotides 1
through 5363, encoding the entire PV1 polyprotein up
through the carboxyl terminus of 3B. The 3C and 3D proteins
are those of PV1 joined in-frame with the newly engineered
ApaI site described under "Experimental Procedures." The
cDNA clone pT7-PC3D is identical to that of pT7-PC3CD
except that the PV1 sequences begin at amino acid 14 of 3D
and the plasmid contains the CVB3 3C coding region. All full-
length cDNA clones of CVB3 contain 3D sequences derived
from PV1. The presence of poliovirus 3D sequences allowed
identification of P3 proteins with the antisera directed
against the PV1 3D protein. Transcription in vitro of these
two cDNA clones and subsequent translation of the RNA
revealed similar processing phenotypes (Fig. 6A, lanes 3 and 4).
Proteins labeled in CVB3-infected HeLa cells at 5–7 h
post-infection are shown in Fig. 6A, lane 1. The P2 and P3
derived nonstructural proteins seen in the pT7-PC3CD and
pT7-PC3D translations were confirmed by immunoprecipita-
with 2C or 3D antiserum (data not shown). A low level
of processing of the CVB3 capsid precursor P1 was detected
by the PV1 3C proteinase, as shown by the presence of VP1
in Fig. 6A, lane 3. A marked increase in the efficiency of
cleavage of P1 to VP1 and 1ABC (~63,000 Da, above P2 and
in lane 4) was also observed. As noted above, the altered
electrophoretic mobilities of the P3 region proteins can be
attributed to differences in the 3C sequences between CVB3
and PV1.

Processing of the CVB3 Polyprotein by the pT7-PC3CD-AK,
pT7-PC3D-KA, and pT7-PC3D-AR-encoded 3C Activi-
ties—The processing activity of the PV1 3C proteinases in
which one-third of the protein has been replaced with CVB3
sequences was tested on the CVB3 polyprotein by in vitro
transcription and translation of the chimeric CVB3 cDNAs
pT7-PC3CD-AK, pT7-PC3D-KA, and pT7-PC3D-AR.
The proteins produced by translation of the RNA and subse-
quently processing by the encoded mutant 3C proteinases are
shown in Fig. 6A, lanes 5, 6, and 8. The 3C proteinase encoded
by pT7-PC3CD-AK was capable of producing only the P2 and
P3 nonstructural proteins and not their cleavage products
(Fig. 6A, lane 5). A protein of greater mass than P1 is seen.
This protein is detected by both the 2C and 3D antiserum
(data not shown). The 3D antiserum additionally detects two
proteins with apparent masses of ~60,000 Da. Although sim-
lar in size to the 4a protein described in PV1 (Pallansch et
al., 1984), the exact origins of these proteins are not known.
The 2C antiserum also detects a protein which comigrates
with the P3 protein, consistent with a 2B3C3ABC fusion
protein. The 2A protein predicted by the presence of such a
large P2-P3 fusion protein was not seen, since only low levels
of the fusion protein were seen in the translation and proc-
cessing assays.

The processing of nonstructural proteins by the pT7-
PC3CD-KA-encoded 3C proteinase is shown in lane 6 of Fig.
6A. The inability of the 3C proteinase to mediate authentic
cleavage of the CVB3 polyprotein is evident. A large protein
of an apparent mass identical to that of the largest polypeptide
seen in lane 5 of Fig. 6A is observed. This protein is immu-

Fig. 5. In vitro translation of T7 transcripts of amino acid
insertion mutants of the CVB3 3C proteinase tested on the
PV1 polyprotein. Transcribed RNAs derived from the insertion
mutants of pT7-CP3C:CB (lanes 3 and 11), pT7-CP3C:B (lanes 6 and 12),
and pT7-CP3C:X (lanes 7 and 12) as well as RNAs derived from
the wild-type PV1 cDNA pT7-1 (lanes 3 and 8) and the allelic
replacement of PV1 3C, pT7-CP3C:C (lanes 4 and 9) were used to
program translation reactions as described under "Experimental Pro-
cedures." The protein processing profiles of the encoded wild-type
and mutant 3C activities are displayed in lanes 3-7. Lanes 8–12 are
aliquots of the reactions in lanes 3-7 which have been post-transla-
tionally incubated in the presence of wild-type PV1 3C activity from
an infected HeLa cell extract (+ inf HeLa) as described under
"Experimental Procedures." The compositions of the cDNA tem-
plates used to synthesize the RNA designated above the lanes are
described in Fig. 2. Lane 1 is marker PV1 proteins prepared as
described in the legend to Fig. 3. Lane 2 is a no RNA control
translation.

CP3C:B exhibits pronounced defects in P3 processing (Fig. 5,
lane 6). The proteinase activity produced by in uitro transla-
tion and processing of pT7-CP3C:CB does not mediate the
production of detectable amounts of 2C, but does produce
detectable levels of P2 and 2BC (Fig. 5, lane 6).

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noprecipitated from both translation reactions with both 2C and 3D antisera. A protein detectable only by the 2C antiserum which migrates between P1 and P3 is observed. We also detect anti-3D reactive proteins of apparent masses of ~60,000 Da. The combined masses of the 2C- and 3D-containing proteins are consistent with alternative cleavage of the large P2-P3 fusion protein.

A dramatic difference in the processing of the CVB3 nonstructural proteins (when compared with that of the pT7-PC3CD-AK and pT7-PC3CD-KA 3C proteinases) occurs in the replacement of the carboxyl-terminal one-third of the PV1 proteinase with equivalent CVB3 sequences (pT7-PC3CD-AR), shown in lane 8 of Fig. 6A. Authentic P2-derived proteins are observed at low levels upon translation and subsequent processing by the mutant 3C proteinase. Normal P3 region proteins are observed, although in different relative amounts than observed in the processing by the wild-type CVB3 and PV1 3C activities. A low level of VP1 production is also observed. The activity of the pT7-PC3CD-AR-encoded 3C proteinase in a CVB3 polyprotein background is in contrast to the 3C activity of identical composition encoded by pT7-CP3C-AR, which was incapable of authentic proteolytic processing of the PV1 polyprotein (Fig. 3A, lane 8).

Processing of the CVB3 Polyprotein by the pT7-PC3CD-AA, pT7-PC3CD-KA’, and pT7-PC3CD-KR-encoded 3C Activities—The replacement of two-thirds of the PV1 3C proteinase with CVB3 sequences had effects on the processing of the CVB3 polyprotein similar in nature to the effects seen when tested on the PV1 polyprotein. The CVB3 replacement of the amino-terminal two-thirds of the proteinase of pT7-PC3CD-AA results in the loss of P2 and P3 processing ability, although the P2 and P3 proteins themselves are produced normally (Fig. 6B, lane 5). In addition to the P3 and P2 proteins, low levels of a 2BC protein and a P3-derived 4a-like protein are seen.

The first and last one-third replacement of the PV1 3C proteinase with CVB3 sequences encoded by pT7-PC3CD-KA’ affected the processing of the CVB3 polyprotein as shown in lane 7 of Fig. 6A. Normal processing of the P2 protein is seen but the processing of P3 is limited. Overexposure of the gel shown in Fig. 6A also reveals a low level of VP1, indicating some P1 processing capability.

The CVB3 replacement of the carboxyl-terminal two-thirds of the PV1 3C proteinase in pT7-PC3CD-KR also limited the processing of the CVB3 polyprotein, as seen in Fig. 6B, lane 6. The polypeptides P2, 2BC (and by inference, 2A), and P3 were produced by the chimeric 3C activity of pT7-PC3CD-KR. Overexposure of the gel shown in Fig. 6B also shows a limited degree of VP1 production. Similar to the P2 processing characteristics of the other chimeric proteinases with a replacement of the central one-third of the 3C protein, a protein containing 2C but not 3D sequences which comigrates with P3 is seen, consistent with a 2BC3ABC protein.

Processing of the CVB3 Polyprotein by the pT7-PC3D-X, pT7-PC3D-B, and pT7-PC3D-X,-encoded 3C Activities—The three 4-amino acid insertion mutations in the CVB3 3C proteinase described above were also tested on the CVB3 polyprotein. The processing of nonstructural proteins by pT7-PC3D-X, as in the identical mutation in pT7-CP3C-X (containing a PV1 polyprotein), was unaffected (Fig. 6B, lane 7). The 3C activity of pT7-PC3D-X also maintained the ability to mediate the cleavage of the structural protein VP1 from the P1 precursor.

The processing activity of the pT7-PC3D-B-encoded proteinase was markedly affected by the insertion of four amino acids at amino acid 103, as shown by the pattern of proteins seen in lane 8 of Fig. 6B. Authentic P2 protein was detected, as confirmed by immunoprecipitation with 2C antiserum and a low level of 2BC was seen (data not shown). A 4a-like P3-derived protein which comigrates with P2 is detected by
immunoprecipitation with 3D antiserum. As in the chimeric 3C proteinase mutants, the larger 2C containing protein and the 3D-derived 4A-like protein can be accounted for by an alternative cleavage of the P2-P3 nonstructural precursor.

The amino acid insertion at aa167 and the Q115 deletion encoded by pT7-PC3D.X2 had a greater effect on processing of the polyprotein (data not shown). The only authentic viral proteins produced in translation of pT7-PC3D:Xr-derived RNA were P1 (produced by 2A activity), P2, 2BC, and P3. The only proteolytic event mediated by the mutant proteinase is the cleavage of the P2-P3 and 2A-2B junctions. The processing capabilities of the chimeric PV1-CVB3 3C proteinases and amino acid insertion mutants as tested on the CVB3 polyprotein substrate are summarized in Fig. 7.

**DISCUSSION**

The majority of polyprotein cleavage events in the processing of the picornavirus polyprotein are mediated by the activity of the 3C protein. The cascade of proteolytic events is most likely highly specific, as several examples of mutations in the 3C protein which affect polyprotein processing result in wide ranging alterations in the virus life cycle (Dewalt and Semler, 1987; Dewalt and Semler, 1989; Kean et al., 1988; Dewalt et al., 1990). Mutations in other regions of the polyprotein have also been reported to influence protein processing (Kuhn et al., 1988a; Kuhn et al., 1988b; Ypma-Wong et al., 198813; Dewalt et al., 1989). The need for a specific pathway of cleavage illustrates that cleavage of the polyprotein in itself is not sufficient to regulate picornavirus gene expression, but that the order in which cleavage occurs is paramount. The pleiotropic effects of mutations which primarily disrupt processing attest to the intimate role of processing in assuring a successful infectious cycle.

A key to understanding how a specific proteolytic cascade serves to regulate gene expression lies in determining the nature of the protein-protein interactions that the proteolytic enzymes use to recognize specific features of a substrate. Recent reports have made it clear that higher order structures in substrate proteins rather than primary structure have a significant role in directing the 3C proteinases of picornaviruses to correct cleavage sites (Ypma-Wong et al., 1988b). The need for a specific pathway of cleavage illustrates that cleavage of the polyprotein in itself is not sufficient to regulate picornavirus gene expression, but that the order in which cleavage occurs is paramount. The pleiotropic effects of mutations which primarily disrupt processing attest to the intimate role of processing in assuring a successful infectious cycle.

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**Effect of Suballelic Replacement on P1 Processing** — The effects on PV1 polyprotein processing of CVB3 replacement of, or insertion into, the PV1 3C proteinase are summarized in Fig. 7. It is clear that processing of the PV1 P1 capsid precursor is abrogated in all mutants. The lack of P1 processing is consistent with earlier observations which indicate that the interaction between the capsid protein and the processing enzyme 3CD is specific, and most likely dependent on structural features not shared between CVB3 and PV1, or on specific amino acid sequences which are not common between the two viruses. The mutant proteinases were also tested on the CVB3 polyprotein (also summarized in Fig. 7) and some were found to maintain some P1 cleavage capability. There was considerable difference between the ability of the PV1 3C activity of pT7-PC3CD to cleave the CVB3 P1 and that of the CVB3 3C activity of pT7-PC3D to carry out the same cleavages (Fig. 6a, lanes 3 and 4). Since the 3D sequences of both clones were derived from PV1, we must assume that the increased efficiency of cleavage is due to the presence of the CVB3 3C in pT7-PC3D. Both chimeric plasmids were more efficient at VP3/P1 cleavage than the CVB3 3C alone (data not shown). The chimeric 3C proteinases which show CVB3 P1 cleavage activity are those which contain CVB3 sequences from a aa127 of 3C to the carboxyl terminus. These observations suggest that the efficient recognition of the VP3/P1 cleavage site in the CVB3 P1 protein is partially dependent on interaction between the amino acids at the cleavage site and structural elements in the carboxyl-terminal region of the CVB3 proteinases. It should be noted that the VP3/P1 cleavage site of CVB3 and PV1 share the least degree of similarity with other cleavage sites within the two polyproteins. Neither the 3CD protein encoded by pT7-PC3D nor pT7-PC3CD was capable of complete P1 processing, suggesting that the overall

![Fig. 7. Summary of wild-type and mutant 3C processing activity produced by in vitro translation of RNA synthesized from cDNA templates. The composition of the 3C coding regions of the various PV1/CVB3 wild-type and mutant 3C proteinases is shown above each column and is described in Fig. 2. The polyprotein background in which the proteinases were tested is listed below the 3C description. The left column shows viral proteins normally observed by translation of wild-type viral or in vitro transcribed RNA and subsequent polyprotein processing. The production of a protein by translation of full-length RNA and processing of the resultant polyprotein by the encoded wild-type or mutant 3C activity is indicated by a + in the appropriate column. Proteins produced by aberrant cleavage events are not included.](http://www.jbc.org/)

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**FIG. 7. Summary of wild-type and mutant 3C processing activity produced by in vitro translation of RNA synthesized from cDNA templates.** The composition of the 3C coding regions of the various PV1/CVB3 wild-type and mutant 3C proteinases is shown above each column and is described in Fig. 2. The polyprotein background in which the proteinases were tested is listed below the 3C description. The left column shows viral proteins normally observed by translation of wild-type viral or in vitro transcribed RNA and subsequent polyprotein processing. The production of a protein by translation of full-length RNA and processing of the resultant polyprotein by the encoded wild-type or mutant 3C activity is indicated by a + in the appropriate column. Proteins produced by aberrant cleavage events are not included.
integrity of the 3CD enzyme is necessary for efficient P1 substrate utilization. The CVB3 VP3/VP1 cleavage site does share greater similarity with VP1 nonstructural cleavage sites, which may explain, in part, the ability of the VP1 3C to cleave that site.

It has been demonstrated in studies of the cleavage activity of bacterially expressed poliovirus 3C and 3CD (Nicklin et al., 1988; Burns et al., 1989) as well as in in vitro translation studies (Jore et al., 1988) that the poliovirus 3C protein alone is capable of mediating the VP3/VP1 cleavage, albeit at very low efficiency. Our observation that VP1 production in pVT7-PC3D-directed translation reactions was very efficient but that P1 processing did not progress to completion indicates that the role of 3D in P1 processing is more specific than that of a mediator of general protein-protein contact between 3CD and P1.

Effect of Suballelic Replacement on P2 Processing—The VP1 3C proteinases which contain substitutions of CVB3 into the first or second one-third of the VP1 3C protein exhibit similar deficiencies in processing when tested on both CVB3 and VP1 P2 proteins (compare pT7'-CP3C-AK and pT7'-CP3C-KA with pT7'-PC3CD-AK and pT7'-PC3CD-KA in Fig. 7). We see that the loss of P2 processing ability progresses as the replacement involves the region between amino acids 68-126 of 3C. The amino acid insertion mutations into the CVB3 3C at aa51 and aa103 show similar disturbances in processing of both VP1 and CVB3 polyproteins. That such different types of mutational analyses yield similar effects on proteolytic activity provides evidence that similar structural elements are disturbed or changed. We interpret the results to mean that the elements disturbed by the mutations are important in recognition of nonstructural proteins in trans. The orderly pattern in which P2 processing is disrupted by the insertions encoded by pT7'-CP3C-AK, pT7'-CP3C-KA and by pT7'-CP3C:X and pT7'-CP3C:B, as well as their counterparts in the CVB3 background predicts a specific order of processing the P2 protein, P2→2BC+2A→2B+2C. Although the precursor-product relationship of P2 to 2BC and 2C has been well established (Butterworth and Korant, 1974; Shih et al., 1978; Pallansch et al., 1984; Dewsalt and Semler, 1987; Kean et al., 1988), whether 2BC is a direct precursor to 2C or an end product itself is not clear due to the rapid in vivo processing of P2 to its smaller constituents. That 2BC is a precursor to 2C has been brought into question by recent data comparing the rates of cleavage of synthetic peptides identical to natural PV2 cleavage sites by purified PV2 3C proteinase expressed in E. coli (Pallai et al., 1989). In the analysis of the mutant proteinases described here, we have detected the 2C protein independent of the appearance of 2BC in only one instance (pT7'-CP3C-AR, Fig. 3A, lane 14) in which it was produced from an aberrant 3C containing precursor (Fig. 4A, lane 14). In addition, only very low levels of 2AR are ever detected in PV1-infected cells (Pallansch et al., 1984).

It should be noted that the ability of a mutant proteinase to produce 2C corresponds with the ability to efficiently process the P3 precursor, suggesting further that large proteins containing 3C are not equally capable of processing P2. It has been proposed that only 3C is capable of efficient P2 processing (Kean et al., 1988). Our data show that some 3C containing proteins other than P3 may be necessary for P2 processing, since the presence of P3 alone does not insure P2 processing (see Fig. 7). Alternatively, such a correlation between P2 and P3 processing may reflect a disruption in a common mechanism of substrate recognition in trans. It has been shown for encephalomyocarditis virus that large P3-derived proteins are fully capable of P2 processing (Parks et al., 1989) and that P2 proteins are produced before detectable levels of 3C are produced (Jackson, 1986; Jackson, 1989). Every altered 3C proteinase is capable of at least one normal cleavage event in the PV1 or CVB3 polyprotein, suggesting that cleavage in trans is most greatly affected. These observations alone do not clearly distinguish between the two interpretations.

In consideration of how the location of the CVB3 insertion into the VP1 3C affects P2 processing ability, those proteinases which contain a carboxyl-terminal region homologous in origin to that of the protein substrates being tested are the least affected in P2 processing. In the case of the linker insertion mutants, the insertion after aa167 and Q168 deletion in the CVB3 proteinases is most detrimental. The carboxyl-terminal portion of the proteinase is important for efficient P2 substrate utilization, as is most evident in the carboxy-terminal one-third insertion of CVB3 sequences into the VP1 3C encoded by pT7'-CP3C-AR and pT7'-PC3CD-AR. The encoded proteinase is incapable of authentic cleavage of P1-derived proteins but is capable of efficient cleavage of CVB3 polyprotein. Since the proteolytic enzymes are identical, we must conclude that elements in the carboxyl-terminal region of the proteinase play an important role in recognition and cleavage of substrate proteins.

Effect of Suballelic Replacement on P3 Processing—The effects of the suballelic replacement on P3 processing are difficult to assess for the following reasons: 1) the replacements engineered into 3C can be considered mutations of both enzyme and substrate, and 2) normally P3 proteins are processed inefficiently in the system used, even in the presence of exogenously added wild-type 3C activity (Ympa-Wong and Semler, 1987a). The effects of the replacements on P3 processing can be interpreted as a direct result of the disruption of the 3C sequences, making the P3 protein an unsuitable substrate for its own 3C activity, rather than significantly altering the catalytic activity of 3C itself. However, the processing of PV1 and CVB3 proteins by several of the suballelic replacement mutants is qualitatively different depending on the substrates analyzed. The processing phenotypes indicate that the most affected function is the ability of 3C to recognize a substrate. Other mutational analyses of 3C have shown the sensitivity of P3 processing to alterations in 3C, further supporting the interpretation that substrate recognition rather than loss of catalytic ability is primarily affected (Dewsalt and Semler, 1987, Dewsalt et al., 1990). The activity of pT7-PC3CD-AR versus that of pT7-CP3C-AR agrees with the interpretation that the carboxyl terminus contains determinants of species-specific substrate recognition. However, in light of the above considerations and the chimeric nature of P3, no definite conclusions can be made based on P3 processing alone.

Effect of Replacements and Insertions on 3C Structure and Catalytic Ability—It is surprising that all of the mutant 3C proteinases maintained some degree of catalytic ability. That replacement of one-third of a 183-amino acid protein with sequences of ~60% amino acid identity does not abrogate catalytic activity argues strongly for a highly conserved structure among the enteroviral 3C proteinases. Manipulation of transcarbamoylase enzymes by exact domain replacement has proven successful (Houghton et al., 1989). In our studies, the replacement of domains of the putative bi-lobar structure of the 3C proteinase is much less precise (Werner et al., 1986; Bazan and Pletterick, 1988).

Several studies have demonstrated the possible relationship of picornavirus cysteine proteinases to cellular serine proteinases of the trypsin family (Gorbalenya et al., 1986; Gorb-
lenya et al., 1988; Bazan and Fletterick, 1988; Gorbalenya et al., 1989; Bazan and Fletterick, 1989). Based on the comparison of picornavirus, comovirus, and potyvirus proteinases with cellular trypsin-like serine proteinases, amino acids predicted to be important in forming the S1 substrate-binding pocket of CVB3 and PV1 3C are absolutely conserved between the two viruses. All of these residues are present in the carboxy-terminal one-third of the proteinase. The marked difference in the activity on CVB3 substrates compared with PV1 substrates of the PV1 3C containing the carboxy-terminal one third from CVB3 indicates that residues other than those forming the putative S1-binding pocket are important for substrate utilization. The serine protease elastase has been shown to be dependent on more extended contact with a peptide substrate for cleavage than trypsin (for review see Kraut, 1977). The potyvirus tobacco etch virus 49-kDa proteinase shows activity on a 25-amino acid cleavage site cassette, and cleavage sites in the polyprotein exhibit extended amino acid sequence similarity (Carrington and Dougherty, 1988). The PV2 3C proteinase expressed in E. coli shows increased activity on a peptide containing Pp through P's peptide (Pallai et al., 1989). These experiments show the importance of extended substrate binding in substrate utilization by viral proteinases. As both the CVB3 and PV1 proteinases cleave at Q-G amino acid pairs, the specific activity of the respective proteinases must rely on extended amino acid contacts. No consistent amino acid motifs can be found around the cleavage sites except for the predominance of a nonpolar amino acid residue at the P1 position. Although among the various cleavage sites there is little consensus even within a single virus, between CVB3 and PV1 there exist similarities at specific cleavage sites. This would suggest that the contexts of the cleavage sites are important in substrate recognition, and these contextual aspects of cleavage site presentation are what show some degree of similarity between CVB3 and PV1, allowing interspecific cleavage to occur (Dewalt et al., 1989).

It is concluded from our work and that of others that several steps must occur in the cleavage of the polyprotein. 1) The presentation of a potential cleavage site as a result of its location in the polyprotein. 2) The formation of specific amino acid contacts between the proteinase and a potential cleavage site. 3) Hydrolysis of the specific peptide bond. The manipulations of the CVB3 and PV1 3C proteinase reported here predominantly affect this second step. Such an interpretation may explain the increase in aberrant, 2A-mediated cleavage events we observe in the PV1 polyprotein. We interpret this as a reflection of the slower rates of mutant 3C-mediated cleavage, allowing less common 2A-mediated events to occur at greater frequency. The reduced dependence of 2A-mediated cleavage on extended conformational determinants has been noted (Ypma-Wong and Semler, 1987b; Lee and Wimmer, 1988). The persistence of large molecular weight proteins that are products of aberrant cleavage, but which contain normally utilized cleavage sites, even in the presence of wild-type 3C activity, strengthens previous arguments (Adler et al., 1980; Toyoda et al., 1986; Ypma-Wong et al., 1988) that polyprotein folding is an active determinant in cleavage.

CONCLUSIONS

Without the availability of a crystal structure, the effects of the alterations in the PV1 and CVD3 3C proteinases cannot be fully interpreted. Nevertheless, some conclusions as to the mechanism of substrate interaction of the 3C proteinases of these two viruses can be drawn. It is clear from our data that major substrate binding determinants lie within the carboxy-terminal region of the 3C proteinase, similar to cellular trypsin-like serine proteinases. It is also clear that more extensive contacts with the substrate proteins occur which are important in their efficient use as substrates. Such contacts must involve structural elements throughout the entire 3C proteinase, as no single replacement proved both necessary and sufficient to confer wild-type cleavage activity. This is especially true of the P1 precursor, which additionally requires a specific 3D interaction. A highly conserved three-dimensional structure of 3C has been suggested by amino acid sequence comparisons between many picornaviruses (Werner et al., 1986; Bazan and Fletterick, 1988). Our data demonstrating that the mutant enzymes described here maintain catalytic activity further supports these conclusions. The proteolytic enzymes of clinically and economically important viruses are attractive targets of antiviral chemotherapy. The elucidation of the mechanisms by which specificity is conferred will be important for the design of antiviral agents. More generally, we have shown the utility of suballelic exchange of moderately related proteins in the molecular genetic analysis of protein function.

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