Effects of Mutations in Poliovirus 3D^{pol} on RNA Polymerase Activity and on Polyprotein Cleavage

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A series of short insertion mutations was introduced into the poliovirus gene for 3D^{pol} at a number of different locations. When substituted for wild-type sequences in a full-length, infectious cDNA and tested for infectivity, all 3D mutants were nonviable. The mutant cDNAs were introduced into a bacterial plasmid designed to direct the expression of poliovirus 3CD, a viral protein composed of contiguous protease and RNA polymerase sequences. Bacteria transformed with these plasmids all expressed similar amounts of 3CD, and all mutant proteins cleaved themselves to generate wild-type 3C^{pro} and mutant 3D^{pol} polypeptides with approximately the same efficiency as wild-type 3CD. The released mutant 3D^{pol} proteins were all defective in RNA-dependent RNA polymerase activity in vitro. Uncleaved 3CD is a protease required for processing the viral capsid protein precursor, P1. In an in vitro assay of P1 cleavage activity, some of the mutant 3CD proteins expressed in *Escherichia coli* showed normal activity, while others were clearly inactive. Thus, alterations in the sequence and/or folding of different regions of the 3D protein have differential effects on its various activities.

Poliovirus RNA is translated into a large polyprotein which is cleaved by viral proteases 2A^{pro} and 3C^{pro} to generate mature viral proteins (16, 47). Most cleavages are accomplished by protease 3C or precursors containing 3C, which cleave between 9 of the 13 glutamine-glycine bonds present in the polyprotein (16). Cleavage at the amino terminus of 3C generates the protein 3CD, a fusion of 3C^{pro} and 3D^{pol}, which is required for processing of the capsid protein precursor P1 (22, 50). In addition, the 72-kilodalton (kDa) 3CD is cleaved to produce 3C^{pro} and 3D^{pol} (17), each of which performs its separate catalytic function. Although this latter cleavage is presumably catalyzed by the 3C^{pro} active site, it is not known whether it occurs intramolecularly in *cis* or intermolecularly in *trans*.

Poliovirus protein 3D^{pol} catalyzes RNA chain elongation in a primer- and template-dependent manner (12, 49). Although the enzyme was first purified in 1979 (49), little is known about its structure or activities. It has a predicted molecular weight of 52,000 (26, 40) and is a neutral (49), hydrophilic protein (45). The 3D sequences are involved in a number of different activities, but no distinguishable structural or functional domains of the protein have been identified.

One region of poliovirus 3D^{pol} (amino acids 284 to 332) is highly conserved among polymerases of picornaviruses and related plant viruses (10, 23). The region includes a core of glycine-aspartate-aspartate which is surrounded by hydrophobic amino acids. Ten of 14 amino acids are identical in this region of poliovirus Mahoney type I and human rhinovirus strain 14 (10), and 12 of 14 amino acids are identical between poliovirus Mahoney type I and human rhinovirus strain 2 (45). (Sequence comparisons are described in Materials and Methods.) Reverse transcriptases of some retroviruses also have a similar core (YXDD) and surrounding hydrophobic amino acids (23). Because this region is highly conserved, it has been proposed to be the active site or a

region which interacts with RNA (23). Limited site-directed mutagenesis of the aspartate and glycine residues in the core of human immunodeficiency virus reverse transcriptase and bacteriophage Q β RNA polymerase drastically reduced polymerase activity and infectivity, respectively (19, 29). However, nothing more is known about the role of this region in the mechanism of RNA polymerization.

To study the activities of 3D^{pol}, we have expressed poliovirus RNA polymerase in *Escherichia coli* from cloned cDNA inserted into bacterial expression vectors (41, 42). Crude bacterial extracts exhibit RNA polymerase activity which is dependent on primer and template. One plasmid (pEXC-3D) directs the expression of poliovirus 3CD, which is cleaved to 3C^{pro} and 3D^{pol} in vivo. The cleavage never goes to completion in *E. coli* (41), and immunoblots of bacterial extracts indicated that approximately equal amounts of 3CD and 3D were produced (11). Since poliovirus polymerase expressed in this manner manifests enzymatic activity in vitro, we can determine the effects of alteration of the amino acid sequence on various activities of 3D^{pol} as an initial step toward defining functional domains of the protein. The 3D sequence is known to encode RNA polymerase activity and to contribute to 3CD protease activity. Other possible activities include uridylylation of VPg (48) and interaction with membranes and other proteins (7).

In this report, we describe a set of mutant proteins containing amino acid insertions in different regions of 3D^{pol}. We analyzed the effects of these mutations on virus viability and the following three activities of 3D or 3CD: (i) RNA polymerase activity, (ii) cleavage at the junction between 3C^{pro} and 3D^{pol}, and (iii) cleavage of capsid protein precursor P1. Our findings indicate that RNA polymerase activity and proteolytic processing activity do not reside in separate functional domains of the protein, but rather, the folded protein contains overlapping structural requirements for these activities.

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MATERIALS AND METHODS

Materials. Klenow fragment of DNA polymerase I, T4 DNA ligase, and restriction enzymes were purchased from Bethesda Research Laboratories, Inc., or New England BioLabs, Inc. T7 RNA polymerase was purified by B. Szewczyk at the University of Utah by the method of Grodberg and Dunn (15). Rabbit reticulocyte lysate, goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase and RNasin were purchased from Promega Biotec. Nitrocellulose BA83 was purchased from Schleicher & Schuell, Inc., and Nitro Blue Tetrazolium and 5-bromo-4-chloroindolyl phosphate were purchased from Sigma Chemical Co.

Sequence comparisons. Mahoney type I poliovirus and human rhinovirus strain 2 sequences were aligned by using the ALIGN program available from BIONET.

Construction of mutations. DNA manipulations and transformations were performed by standard procedures (33). Mutations were introduced into plasmid pMV7-2.9, which is a pBR322-derived plasmid containing poliovirus sequences from nucleotides (nt) 4601 to 7440 and an 84-nt poly(A) tract, as previously described (43). To generate mutations, this plasmid was linearized with *Hinf*I or *Dde*I under conditions favoring partial digestion. Full-length molecules were purified on agarose gels, and the 3-nt 5' overhangs were filled in with the Klenow fragment of *E. coli* DNA polymerase I. Blunt-ended DNA molecules were ligated to form circular plasmids containing the 3-nt insertions, and the ligation mixture was transformed into *E. coli* C600. Plasmid DNA from amp^R transformants was screened for the loss of a single *Hinf*I or *Dde*I site. Mutations generated in pMV7-2.9 were introduced into full-length poliovirus cDNA (plasmid pEV104) by using the *Acc*I site at poliovirus nt 6218, the *Pvu*II site at poliovirus nt 7053, or the *Eco*RI linker present at the end of the 3'-terminal poly(A) tract.

The mutations were introduced into 3CD expression plasmid pEXC-3D (41) by subcloning a *Bgl*II-*Avr*II fragment representing poliovirus nt 5602 to 7206 from pMV7-2.9 or its derivatives containing the various insertion mutations into a large *Bgl*II-*Avr*II fragment of pEXC-3D. Wild-type hybrid 3CD was generated in a similar manner, producing pEXC-3D(MV). This method produced several plasmids containing poliovirus 3CD sequences, each containing a mutation in the 3D portion. Expression is driven by the *trp* promoter and results in 3CD proteins containing an additional methionine residue at the amino terminus (20). pEXC-3D(YG)₂ was derived from plasmid pT7-3D(YG)₂, which was generated by linker insertion mutagenesis (50). Construction of pEXC(H→G)-3D was described previously (42).

Infectivity assays. COS-1 cells were transfected with mutated full-length poliovirus cDNAs by the calcium phosphate precipitation method (14, 44). Plaque assays were performed as described previously (43).

Preparation of bacterial extracts. All plasmids were propagated in *E. coli* C600. Bacterial cultures were grown overnight in L broth supplemented with 20 µg of tryptophan per ml and 50 µg of ampicillin per ml. Overnight cultures were diluted into M9 medium supplemented with 0.5% Casamino Acids and 50 µg of ampicillin per ml to an optical density at 595 nm of approximately 0.07. After growth at 37°C for 1 h, indoleacrylic acid was added to a final concentration of 5 µg/ml to induce the *trp* promoter. Cultures were grown to an optical density at 595 nm of 0.5, collected by centrifugation, washed twice in 10 mM sodium phosphate (pH 6.7), and resuspended to 3 × 10⁹/ml in the same buffer. Cells were

sonicated by two to four 30-s bursts on ice with a 0.5-in. (1 in. = 2.54 cm) probe at 60 W on a 20-kc Sonifier (Heat Systems-Ultrasonics, Plainview, N.J.). To evaluate the extent of cell lysis, the lysates were centrifuged at 16,000 × *g* for 15 min at 4°C and the amount of protein in the supernatant was estimated by measuring the A₂₈₀.

Analysis of mutant proteins. Bacterial extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 10 or 12.5% gel (28). For immunoblotting, proteins were transferred to nitrocellulose (46) and treated with rabbit anti-polymerase serum (41) or with anti-3C^{pro} serum (16) as described previously (41), except that immunological reactivity was visualized by using a secondary antibody coupled to alkaline phosphatase (5, 30). The antiserum was preadsorbed with a crude extract of *E. coli* to remove TrpE-reactive antibodies. Poliovirus-infected cell extracts were prepared as previously described (6). Briefly, poliovirus-infected cells were lysed and centrifuged at 10,000 × *g* for 15 min, and the supernatant (I-S10) was used as a marker in Fig. 2.

Polymerase assays. Poly(U) polymerase assays were performed as previously described (18). Briefly, 10-µl bacterial extract volumes were incubated in 50-µl reactions containing 11.4 µM [³H]UTP (36.9 µCi/µmol); 0.34 µg of oligo(U); 1 µg of poly(A); 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 8.0); 3 mM magnesium acetate; 60 µM ZnCl₂; 500 µM each ATP, GTP, and CTP; 4 mM dithiothreitol; and 4 µg of actinomycin D per ml. Five microliters of poliovirus polymerase purified from infected HeLa cells through the phosphocellulose step (18) was assayed as a positive control in every experiment. Samples were assayed at 30°C for 30 or 60 min. Twenty microliters of the reaction mixture was assayed for acid-precipitable radioactivity at zero time and at 30 or 60 min. Each extract was also assayed for primer-independent activity, which was always less than 1%.

P1 cleavage assay. A P1 cleavage assay was developed to assay the ability of mutant 3CD proteins to cleave capsid protein precursor P1. Bacterial extracts containing mutant poliovirus 3CD were mixed with [³⁵S]methionine-labeled P1 substrate produced by transcription and translation *in vitro* (see below), and products were analyzed by SDS-PAGE.

In vitro transcription. Transcription *in vitro* was performed with pTPOV-3916 DNA (32), which was linearized with *Hinc*II or *Hind*III, as the template. This produces a transcript containing nt 1 through 3915 of poliovirus RNA. Transcription reactions were performed as previously described (34), except that a small amount of [α-³²P]GTP was included in the reactions, and the reactions were not treated with DNase. Reaction mixtures were extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated by addition of ammonium acetate to 2.5 M and 2 volumes of ethanol. Transcripts were analyzed on methylmercury hydroxide-containing agarose gels (2).

In vitro translation. Poliovirus RNA transcripts were translated in a rabbit reticulocyte lysate (Promega treated translation kit) supplemented with 23% uninfected HeLa cell extract (S10) (6) as previously described (51). Translation was performed as recommended by the manufacturer, with 30 to 50 ng of RNA per 10-µl reaction.

Proteolytic processing reaction. After incubation at 30°C for 3 h, translation was stopped by addition of RNase and cycloheximide to 200 and 100 µg/ml, respectively, and subsequent incubation at 30°C for 30 min. Ten microliters of the translation reaction was mixed with 10 µl of bacterial extract or 3 µl of infected HeLa cell extract (I-S10) prepared

FIG. 1. Locations of mutations in 3D^{pol}. A linear representation of the sequence of poliovirus protein 3CD is shown, and the locations of insertion mutations are indicated. Single-amino-acid insertions are indicated by vertical bars, a four-amino-acid insertion is indicated by the open bar, and the stop codon insertion is indicated by an X. The region which is most highly conserved among picornaviral polymerases is indicated by the cross-hatched rectangle (23).

as described by Brown and Ehrenfeld (6). This mixture was incubated at 30°C for 1 h to allow processing of P1. Processing reactions were diluted 10-fold with Laemmli sample buffer, and 20-μl samples were analyzed by SDS-12.5% PAGE, followed by fluorography.

RESULTS

Generation of mutant proteins. Several mutations were introduced into the 3D sequence (Fig. 1). The mutations included a linker insertion resulting in four extra amino acids inserted between amino acids 147 and 148, a stop codon insertion which produces a truncated protein that lacks the last 62 amino acids from the carboxy terminus, and five single-amino-acid insertion mutations after amino acid residues 149, 241, 257, 290, and 376.

Infectivity. The mutated DNAs were substituted for their corresponding wild-type segments in a full-length poliovirus cDNA. Full-length wild-type cDNA clones of poliovirus are infectious and have been useful for studies of altered regions of poliovirus RNA or protein (9). All of the mutations in the 3D sequences tested abolished the ability of the cDNAs to generate infectious virus (Table 1). Two of the mutations were not tested, including the leucine insertion after amino acid 257 and the stop codon insertion after amino acid 399. The latter mutation would not be expected to result in an infectious plasmid, since it results in a significant deletion of genetic material. The lethal effect on infectivity could be due to failure of the mutant 3D proteins to function in any of numerous steps in RNA replication, i.e., RNA chain elongation, initiation of RNA synthesis, protein-protein interactions, or association with membranes. In addition, the mu-

tation could affect protease activity, since poliovirus 3CD has been demonstrated to mediate P1 processing (22, 50). It is also possible that the changes in nucleotide sequence could affect the ability of the RNA to serve as a template for replication.

Expression of mutant proteins in *E. coli*. The mutated 3D sequences were also introduced into plasmid pEXC-3D, which directs the expression of poliovirus 3CD under the control of the *trp* promoter. Figure 2 shows an immunoblot analysis of crude extracts of *E. coli* carrying expression plasmid pEXC-3D. Anti-3D^{pol} serum was used to probe the blot in Fig. 2A. A poliovirus-infected HeLa cell extract (I-S10) was analyzed in lane 1 for comparison with proteins present in the bacterial extracts. This marker lane shows a 72-kDa protein (3CD), a 52-kDa protein (3D^{pol}), and the alternate cleavage product 3D'. The latter protein is produced by 2A^{pro}-mediated cleavage (16, 47) and is not required for virus replication (31). A bacterial extract which does not contain poliovirus proteins did not react with this anti-3D serum (Fig. 2A, lane 2). The largest immunoreactive protein in extracts containing pEXC-3D (lane 3) is 3CD (41). A slightly smaller protein represents an internal initiation product, previously described by Hanecak et al. and Ivanoff et al. (17, 20). A major immunoreactive band comigrated with the mature 52-kDa 3D^{pol} protein found in infected HeLa cells. We have previously shown that cleavage of 3CD to generate 3D^{pol} in *E. coli* is mediated by the viral sequences themselves (42). For example, a mutation in the putative active site of 3C^{pro} abolished cleavage of 3CD. A pattern of two doublet bands was reproducibly seen in extracts of bacteria containing viral protein expressed from pEXC-3D (Fig. 2A, lane 3), but their relative amounts were variable, and the mechanism of their generation is unknown.

Figure 2B shows a similar analysis using antiserum specific for 3C^{pro}. The major polypeptides detected in a marker sample of extract from poliovirus-infected HeLa cells are 3CD and 3C^{pro} (lane 1). Anti-3C^{pro} serum reacted with three unidentified proteins present in a bacterial extract that does not contain poliovirus protein (lane 2). In addition, extracts from bacteria that express protein from pEXC-3D contained immunoreactive polypeptides comigrating with 3CD and 3C^{pro}, in addition to the internal initiation product (Fig. 2B, lane 3). Substantially more 3CD protein than 3C^{pro} was present in these extracts. This was surprising, since the same extracts showed approximately equal amounts of 3CD and 3D (e.g., Fig. 2A, lane 3), suggesting that approximately 50% of the 3CD produced in *E. coli* is cleaved. Since it is assumed that cleavage of 3CD yields 3C^{pro} and 3D^{pol} in equimolar amounts, the reduced ratio of 3C^{pro} to 3CD in these extracts

TABLE 1. Effects of mutations in 3D^{pol}

Plasmid	Location of mutation (amino acid)	Type of mutation	Infectious cDNA ^a	Cleavage at 3C/3D junction ^a	Polymerase activity ^a	Cleavage of P1 to VP0, VP3, and VP1 ^a
pEXC-3D			+	+	+	+
pEXC-3D-μ6432	149	Ile insertion	—	+	—	+
pEXC-3D-μ6707	241	Ile insertion	—	+	—	—
pEXC-3D-μ6756	257	Leu insertion	NT ^b	+	+/- ^c	—
pEXC-3D-μ6856	290	Ser insertion	—	+	—	+
pEXC-3D-μ7113	376	Leu insertion	—	+	—	—
pEXC-3D(YG) ₂	147	Tyr-Gly-Ile-Pro insertion	—	+	—	—
pEXC-3D-μ7181	399	Stop codon insertion (truncation)	NT	+	—	—

^a Assays were performed as described in Materials and Methods.

^b NT, Not tested.

^c Extracts containing 3D^{pol} from pEXC-3D-μ6756 exhibited 8% of wild-type polymerase activity (Fig. 4).

FIG. 2. Immunoblot analysis of poliovirus proteins expressed from pEXC-3D. (A) Proteins were resolved on an SDS-10% PAGE gel, transferred to nitrocellulose, and analyzed with serum specific for poliovirus 3D^{pol}. Lanes: 1, poliovirus-infected HeLa cell extract (I-S10); 2, C600 bacterial extract; 3, bacterial extract of C600 harboring pEXC-3D. The internal initiation product is the band immediately below 3CD in lane 3. Poliovirus-infected HeLa cell extract was prepared as described in Materials and Methods. Protein size markers are indicated on the left in kilodaltons. (B) Proteins on an SDS-12.5% PAGE gel were transferred to nitrocellulose and analyzed with serum specific for poliovirus 3C^{pro}. Lanes: 1, poliovirus-infected HeLa cell extract (I-S10); 2, C600 bacterial extract; 3, bacterial extract of C600 harboring pEXC-3D. The internal initiation product is the band immediately below 3CD in lane 3.

suggests that 3C^{pro} is relatively unstable. Reduced accumulation of 3C reactive to 3D^{pol} was observed in several different strains of *E. coli* (unpublished data).

Introduction of mutations into the 3D sequence in the expression plasmids did not significantly alter the levels of protein expression. Figure 3 shows an anti-3D^{pol} immunoblot of extracts containing each of the mutant proteins. Approximately equal amounts of total protein were applied to the lanes, and in several experiments, approximately equal amounts of 3CD protein were recovered from the extracts. Figure 3 also shows that each mutant 3CD and 3D^{pol} protein had a mobility on SDS-PAGE that was slightly faster than that of our original wild type. The differences in the electrophoretic mobilities of the mutant proteins compared with the wild type are attributable to the original full-length poliovirus cDNA clones used in the mutagenesis, since the poliovirus sequences in pMV7-2.9 (the wild-type sequences from which the mutants were derived) and pEXC-3D were derived from separate clones. Although both plasmids were wild type, the 3CD sequences from pMV7-2.9 encode proteins that migrate slightly faster than those from pEXC-3D. This was determined by subcloning the sequences from pMV7-2.9 into pEXC-3D to generate a hybrid wild-type plasmid, pEXC-3D(MV). Hybrid wild-type proteins 3CD and 3D also migrated just ahead of the corresponding proteins from pEXC-3D and exhibited the same

FIG. 3. Immunoblot analysis of poliovirus proteins expressed from plasmids listed in Table 1. Bacterial extracts were prepared as described in Materials and Methods. Proteins on an SDS-10% PAGE gel were transferred to nitrocellulose and analyzed with serum specific for poliovirus 3D^{pol}. Extracts are indicated above the lanes. Protein size markers are indicated on the left in kilodaltons.

mobilities as the mutant proteins derived from pMV7-2.9 (Fig. 3, lane 2). All measured functions and properties of the two wild-type proteins [from pEXC-3D and pEXC-3D(MV)] were identical (see below).

Cleavage at the 3C-3D junction. The immunoblot in Fig. 3 shows that all of the mutant 3CD proteins cleaved themselves to generate 3D^{pol} (Table 1). The mutant 3CD proteins described here had 3C-3D cleavage activity equal to that of wild-type 3CD (lanes 3 to 9). None of these 3D mutations is within 100 amino acids of the 3C-3D cleavage site (Fig. 1), and the distant sequence changes did not appear to affect recognition of the cleavage site. Of particular interest is the truncated protein produced from pEXC-3D-u7181 (Fig. 3, lane 9). This protein, although it lacks the carboxy-terminal 62 amino acids, was shown to cleave itself with the same efficiency as wild-type 3CD. Cleavage at the 3C-3D junction in proteins derived from pEXC-3D has been shown to require 3C^{pro} sequences. For example, a mutation which changed the histidine in the putative active site of 3C to a glycine [pEXC(H→G); 20] completely abolished cleavage of 3CD (Fig. 3, lane 10; 42).

RNA polymerase activity. Crude extracts of *E. coli* expressing wild-type 3CD exhibited substantial poliovirus-specific RNA polymerase activity. Activity required cleavage to the 52-kDa 3D protein, which appears to be the only form active as an RNA-dependent RNA polymerase in vitro (42, 49). Since all of the mutants did exhibit comparable levels of 3CD cleavage to 3D^{pol}, extracts were assayed for poly(U) polymerization activity. Bacterial cultures transformed with pEXC-3D, pEXC-3D(MV), or the mutant plasmids were induced, and extracts were prepared as described in Materials and Methods. Immunoblots of bacterial extracts confirmed that similar amounts of 3D^{pol} protein were present in all of the samples. Figure 4 shows the results of a typical

FIG. 4. RNA polymerase activities of wild-type and mutant $3D^{pol}$ proteins. Cultures of *E. coli* harboring the plasmids indicated in Table 1 were induced as described in Materials and Methods. Bacteria were harvested, suspended in sodium phosphate buffer, pH 6.7, and disrupted by sonication. Crude extracts were assayed for poly(U) polymerase activity with a poly(A) template and an oligo(U) primer as described previously (18). Incorporation of trichloroacetic acid-precipitable radioactivity was determined at the indicated times. Symbols: $\square$, wild type (pEXC-3D); $\bullet$, hybrid wild type [pEX-3D(MV)]; $\circ$, pEXC-3D- μ 6756; $\blacksquare$, all other mutants (pEXC-3D- μ).

RNA polymerase assay. Six of the mutant proteins showed no detectable polymerase activity, whereas the protein produced from pEXC-3D and the hybrid wild-type protein [from pEXC-3D(MV)] had indistinguishable levels of activity. Polymerase produced from pEXC-3D- μ 6756 reproducibly showed 8% of wild-type activity under standard growth and assay conditions.

Cleavage of P1 precursor. Since all of the mutations drastically reduced polymerase activity, we analyzed the effects of the mutations on another activity of 3CD which has been identified recently. Several groups of investigators have shown that 3CD is a protease which has a specificity different from that of $3C^{pro}$ alone (22, 36, 50). When poliovirus proteins are synthesized by translation in vitro, $3D^{pol}$ sequences, in addition to $3C^{pro}$, are required to cleave capsid protein precursor P1 to mature capsid proteins VP0, VP3, and VP1 efficiently. In addition, a four-amino-acid insertion after amino acid 147 of $3D^{pol}$ drastically decreases the protease activity of 3CD (50). Because 3D sequences are essential for the protease activity of 3CD, we developed an assay for the ability of mutant 3CD proteins to cleave the P1 precursor (1ABCD). Poliovirus sequences encoding the capsid region P1, $2A^{pro}$, and the first 28 amino acids of 2B were transcribed and translated in vitro to make a labeled precursor polypeptide substrate. The predicted 116-kDa translation product is cleaved between P1 and $2A^{pro}$ during the 3-h translation because of the autocatalytic protease activity of $2A^{pro}$. Cleavage results in the formation of 97-kDa P1 (Fig. 5, lane 4). The products of in vitro translation of full-length poliovirus virion RNA are shown in Fig. 5, lane 3, and lane 1 contains poliovirus proteins labeled with [35 S]methionine from 2 to 4 h postinfection of HeLa cells.

Addition of a poliovirus-infected HeLa cell extract to labeled P1 after translation results in processing of P1 to capsid proteins VP0, VP3, and VP1 (22, 37, 51). Viral protease activity is provided by the infected cell extract in

trans. The results of a processing reaction are shown in Fig. 5, lane 6. (In infected cells, VP0 is eventually processed to VP4 and VP2 during final stages of virion morphogenesis (21), but this cleavage does not occur in vitro.) When crude extracts of *E. coli* containing wild-type 3CD (lane 9) or hybrid wild-type 3CD (lane 10) were incubated with the labeled P1 translation product, protease activity was also provided in trans and cleavage of P1 to capsid proteins VP0, VP3, and VP1 occurred. The extent of P1 processing increased with time and increasing amounts of extract, although nonspecific proteolysis occurred after very long incubation times (data not shown). Incubation with buffer (lane 4) or with a bacterial extract that did not contain poliovirus proteins (lane 5) did not affect P1.

Extracts of bacteria carrying plasmid pEXC, which encodes a truncated form of 3CD (designated $3CD^*$; the asterisk denotes an incomplete polypeptide) composed of a complete $3C^{pro}$ sequence plus only 25 amino acids from the amino terminus of $3D^{pol}$, cleaved P1 to form different products, 1ABC and 1D (Fig. 5, lane 7). These extracts contain mature 3C protease generated by self-cleavage of $3CD^*$ (20). Cleavage at the 1C-1D junction, between VP3 and VP1, has been shown to be catalyzed by 3C activity alone, albeit relatively inefficiently (22, 36). This is in contrast to the 1B-1C junction between VP0 and VP3, which appears to require the complete 3CD sequence. The various cleavage products observed to arise from P1 by the activities of $3C^{pro}$ and 3CD proteases are diagrammed in Fig. 6. Thus, active $3C^{pro}$ present in extracts of bacteria carrying pEXC catalyzed cleavage at one but not both cleavage sites in the P1 precursor. When the proteolytic activity was inactivated by mutation of pEXC-3D to substitute a glycine residue for the histidine at position 161 [designated pEXC(H $\rightarrow$ G)-3D], there was no self-cleavage of 3CD to produce $3C^{pro}$ and $3D^{pol}$ (Fig. 3, lane 10) and also no cleavage of P1 at either of the two potential cleavage sites (Fig. 5, lane 8).

Extracts containing each of the seven 3CD proteins with mutations in the 3D moiety (Fig. 1) were assayed for the ability to cleave P1 (Fig. 5, lanes 11 to 17). Some mutated proteins were able to catalyze complete cleavage of P1 to capsid proteins VP0, VP3, and VP1 (Table 1). These included 3CD proteins with single-amino-acid insertions at positions 149 and 290. Ypma-Wong et al. (50) have shown previously that the mutation in pT7-3D(YG)₂ decreases the ability of 3CD to cleave P1. Figure 5, lane 11, shows the same result, except that there is evidence of $3C^{pro}$ activity, which generates 1ABC and 1D (Fig. 6). The products of cleavage by the (YG)₂ extract are those of cleavage by $3C^{pro}$ alone. In addition, insertions following amino acids 241, 257, and 376 abolished the ability of 3CD to cleave P1 into VP0, VP3, and VP1. These extracts also exhibited $3C^{pro}$ cleavage activity between VP3 and VP1, producing 1ABC and 1D. The levels of 3C cleavage activity in these mutant extracts are less than that shown by the extract of pEXC (Fig. 5, lane 7). Anti-3C immunoblot analysis of these extracts showed that wild-type pEXC-3D and all of the mutants contained significantly less 3C protein than did pEXC. Thus, the extent of 3C cleavage activity exerted on the P1 substrate correlated with the amounts of 3C protein present in the extract (data not shown). Neither addition of more mutant extract nor longer incubation resulted in cleavage at the 1B-1C junction. The mutant protein which contains a stop codon insertion at nt 7181 and thus lacks the carboxy-terminal 62 amino acids was not able to cleave the P1 precursor to capsid proteins VP0, VP3, and VP1 (Fig. 5, lane 17). This is similar

FIG. 5. Cleavage of P1 precursor by mutant 3CD proteins. Transcripts encoding the poliovirus P1 capsid precursor were translated in vitro as described in Materials and Methods. After translation, samples of the translation reaction were incubated with bacterial extracts containing poliovirus proteins to allow for processing of the P1 precursor protein. Processing reactions were analyzed by SDS-PAGE on a 12.5% gel, followed by fluorography. Lanes: 1 and 18, [35 S]methionine-labeled proteins from an extract of poliovirus-infected HeLa cells; 2, translation performed without added RNA; 3, translation of poliovirus RNA isolated from virions. Lanes 4 to 17 represent translation of P1 followed by incubation with the following extracts: lane 4, sodium phosphate buffer, pH 6.7; lane 5, C600 bacterial extract; lane 6, poliovirus-infected HeLa cell extract; lane 7, pEXC; lane 8, pEXC(H $\rightarrow$ G)-3D; lane 9, pEXC-3D; lane 10, pEXC-3D(MV); lane 11, pEXC-3D(YG) $_2$; lane 12, pEXC-3D- μ 6432; lane 13, pEXC-3D- μ 6707; lane 14, pEXC-3D- μ 6756; lane 15, pEXC-3D- μ 6856; lane 16, pEXC-3D- μ 7113; lane 17, pEXC-3D- μ 7181.

to the results obtained with a 3CD protein which lacked 104 amino acids from the carboxy terminus (51).

The P1 cleavage activities of all of the mutant 3CD proteins described in this study are presented in Table 1, along with a summary of each of the other 3CD and 3D activities measured.

DISCUSSION

Infectivity. When five of the seven mutations engineered in this study were inserted into plasmids containing full-length infectious cDNA sequences, no viruses were recovered. Although the infectivity of RNA transcripts synthesized from complete cDNA sequences is greater than that of the cDNA itself, it is unlikely that the increased sensitivity afforded by RNA transfection assays would produce virus, since all of the mutations abolished the ability of the 3D protein to catalyze RNA synthesis. In some of the mutants, failure to cleave P1 would also contribute to loss of infectivity.

FIG. 6. P1 cleavage activities of bacterial extracts containing 3C or 3CD activity. Cleavage of P1 by bacterial extracts containing wild-type and mutant 3CD sequences was assayed as described in the legend to Fig. 5. The products of cleavage by 3C protease activity alone (left side) or in the presence of functional 3CD (right side) are presented schematically. Molecular weights of proteins are indicated in parentheses in kilodaltons.

TABLE 2. Previously identified mutations in 3D^{pol}

Amino acid position	Type of mutation	Phenotype	Reference(s)
4	Gln↔Pro ^a	Wild-type virus	26, 40 ^a
11	Glu↔Asp	Wild-type virus	26, 40
12	Val↔Ala	Wild-type virus	26, 40
92	Thr↔Ile	Wild-type virus	26, 40
93	Glu→Gly	Wild-type virus	3
147	Thr→Ala	Wild-type virus	31
148	Tyr→Phe	Wild-type virus	31
5	<i>trp</i> deletion	No polymerase activity in vitro	39
53	Asp→Asn	Temperature sensitive for VPg-pUpU synthesis	48
73	Tyr→His	Temperature sensitive for VPg-pUpU synthesis	48
170	Gln→His	Actinomycin D sensitivity at 39°C	24
354	Thr insertion	Small plaque, delayed RNA synthesis	4
424	Asn→Asp	Temperature-sensitive virus, defective RNA synthesis	1
409-461	Deletion of 53 amino acids	No polymerase activity in vitro	39

^a Amino acid differences in the two published sequences of Mahoney type I poliovirus are listed in the following order: reference 40↔reference 26.

Cleavage at the 3C-3D junction. The analyses performed in this study were conducted with poliovirus 3CD sequences expressed in *E. coli* from cloned cDNA which had been subjected to in vitro mutagenesis. The resulting 3CD proteins all exhibited approximately 50% cleavage to 3D^{pol} and presumably 3C^{pro}, although less immunoreactive 3C protein accumulated in the bacteria, apparently because of 3C^{pro} instability. The cleavage between 3C and 3D appears to be an intrinsic activity of 3CD sequences and is dependent on at least some specific amino acid residues in the putative 3C active site. However, it is not known whether 3CD cleaves itself autocatalytically in *cis* or whether one molecule of 3CD acts upon another in *trans*. The fact that all mutant 3CD proteins generated in this study cleaved themselves equally well suggests that recognition of the 3C-3D cleavage site does not involve distal 3D sequences. In fact, most of the 3D portion of 3CD is not essential for self-cleavage, as shown by the cleavage of truncated protein from pEXC-3D-μ7181 (Fig. 3), by the self-cleavage of protein from pEXC (20) (which contains 3C^{pro} plus only 25 amino acids of 3D^{pol}), and from a similar construction of Hanecak et al. which generates a protein consisting of 3C^{pro} fused to 25 amino acids of 3D^{pol} (17). These results are consistent with a model in which cleavage occurred cotranslationally in *cis*, since there appears to be no requirement for proper folding of intact 3CD to present the 3C-3D cleavage site. On the other hand, 3C alone can cleave the 3CD bond in *E. coli* in *trans* when it is presented as a fusion protein (TrpE-3C*D) (41). It appears that 3CD is not completely cleaved in bacteria (41, 42), in poliovirus-infected cells (Fig. 2A, lane 1; Fig. 5, lanes 1 and 18), or in reticulocyte lysates during in vitro translation of poliovirus RNA (50; Fig. 5, lane 3). It is not known what determines the extent of cleavage in any case.

The only mutations known to abolish cleavage at the 3C-3D junction are amino acid changes in the putative active site of 3C^{pro} [pEXC(H→G)-3D (20, 42) and pEXC(C→S) (20)] or at a position adjacent to the cleavage site (43). The latter mutation probably abolishes cleavage by alteration of the substrate, rather than the protease activity. Two site-directed mutations which result in reduced cleavage at the 3C-3D junction in infected cells have already been described (8, 25). These mutations are at amino acids 54 and 74 of 3C^{pro}, respectively. In addition, substitution of residue 60 in 3C^{pro} results in increased cleavage at the 3C-3D junction in vitro and in vivo (50; P. G. Dewalt, W. S. Blair, and B. L. Semler, submitted for publication).

RNA polymerase activity. Each of the seven mutations described here drastically reduced the polymerase activity of poliovirus 3D^{pol}. This result was surprising, since the mutations are scattered throughout the linear amino acid sequence of 3D^{pol}. The existence of more than one structurally separable domain might be predicted in light of the numerous activities of the 3D sequence. Only one of the mutant proteins (μ6856) was altered in the region that is highly conserved among RNA-dependent polymerases. Most of the insertions are single hydrophobic amino acids (isoleucine or leucine) which might be expected to be tolerated in internal, buried regions of the protein or if exposed in surface loops. Indeed, there are precedents for amino acid insertions or deletions which do not destroy poliovirus enzymatic activities, and these apparently do not cause total misfolding of the protein (4, 43). Although all of the mutations described in this report reduced polymerase activity drastically, mutated 3D^{pol} containing a leucine insertion at amino acid 257 had approximately 8% of wild-type polymerase activity as measured under standard conditions. This mutated 3D^{pol} has not been characterized further; for example, it has not been assayed under varied conditions to test for temperature sensitivity. It is possible that additional mutations would generate altered proteins capable of catalyzing RNA elongation.

Recent studies have resulted in the creation or identification of a number of mutations in the poliovirus 3D gene sequence. The results of these studies are summarized in Table 2. Four amino acid differences in 3D^{pol} are predicted from the reported sequences of two different infectious cDNA clones of the Mahoney strain of poliovirus type 1, both of which generate wild-type virus and thus have functional 3D^{pol}. Three other amino acid substitutions produced by mutagenesis experiments which do not confer mutant phenotypes have been identified in 3D^{pol} (Table 2). Two substitutions at amino acids 147 and 148 were introduced into full-length cDNA clones (31), and the other permissible substitution was identified in a temperature-sensitive strain which contained additional mutations responsible for the temperature sensitivity phenotype (3). All of these permissible amino acid substitutions are located in the amino-terminal portion of the protein. Bernstein et al. engineered a single-amino-acid (threonine) insertion at position 354 of 3D^{pol}, resulting in a mutant virus which produced small plaques and had delayed RNA synthesis in vivo (4). An asparagine-to-aspartic-acid substitution at residue 424 re-

sulted in temperature-sensitive RNA synthesis *in vivo* (1). In neither of these two cases was the biochemical effect on RNA synthesis identified. Recombinant cDNA clones between Mahoney and Sabin type 1 sequences were used by Toyoda et al. (48) to localize two mutations, one or both of which conferred a temperature-sensitive phenotype for formation of VPg-pUpU *in vitro*. The precise involvement of 3D in synthesis of uridylylated VPg was not determined. Finally, deletion of amino acid residue 5 or deletion of the carboxy-terminal 53 amino acids resulted in total loss of polymerase activity *in vitro* (39), similar to the seven mutations described in this report (Table 1). An unusual phenotype is displayed by a glutamine-to-histidine substitution at position 170. Virus containing this mutation manifests actinomycin D-sensitive viral RNA replication at 39°C (24). It should be noted that the effects of mutation on RNA structure (rather than on enzyme activity) were not evaluated.

Expression of active poliovirus RNA polymerase from cloned cDNAs in *E. coli* has been reported by other researchers. Morrow et al. (35) expressed 3A*BCD, which cleaved itself to 3D^{pol}, in a manner similar to that used in our studies. Plotch et al. (39) used a construction which expressed 3D^{pol} with a single methionine residue fused to the amino terminus, producing 3D^{pol} only, in the absence of 3C^{pro}. The resulting polymerase was active in an *in vitro* elongation assay. In addition, a primer-independent activity of purified 3D^{pol} was detected which resulted in incorporation of ATP or UTP into RNA products in the absence of primer. This activity was found in preparations of 3D^{pol} which were purified to near homogeneity. This activity has not been detected in 3D^{pol} purified from infected cells or from 3D^{pol} produced in *E. coli* by other investigators. Further characterization of the reaction will be required to determine whether this activity is truly a property of 3D^{pol}.

Cleavage of P1 precursor. Despite normal cleavage at the 3C-3D junction, the set of altered 3CD proteins studied here showed markedly different protease activities toward the P1 capsid protein precursor. Some appeared fully active, while others were completely inactive. The locations of insertions which destroyed P1 cleavage activity clustered in no particular region along the linear map of the 3D sequence. For example, the mutation in pEXC-3D-μ6432 is very close to that of pEXC-3D(YG)₂, but μ6432 cleaves P1 efficiently and (YG)₂ does not. Likewise, μ6756 and μ6856 are 34 amino acids apart, but the latter is able to cleave P1 and the former is not.

We performed computer-assisted hydrophilicity analyses and secondary-structure predictions as described by Garnier et al. (13) and Kyte and Doolittle (27), but no obvious correlations between the structural properties of the locations of the mutations and the abilities of the proteins to cleave P1 were apparent (data not shown). Additionally, information on the folded structure of 3CD protease will probably be required to understand the functional significance of these structural modifications. Clearly, however, cleavage by 3CD at the 3C-3D junction (discussed above) and cleavage at the 1B-1C junction have different requirements and characteristics. It has been pointed out previously (38) that an alanine residue occupies the position four amino acids upstream of many 3C cleavage sites. This amino acid is not present near the 1B-1C cleavage site between VP0 and VP3 or at the -4 position from the 3C-3D junction.

Our experimental approach of analyzing two different biological activities (i.e., RNA polymerase activity and protein processing) provides an important *in vitro* counter-

part to mutagenesis studies which produce lethal lesions in poliovirus genomes that may have pleiotropic effects on viral functions.

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