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Purification and Characterization of Poliovirus Polypeptide 3CD, a Proteinase and a Precursor for RNA Polymerase

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A cDNA clone encoding the 3CD proteinase ($3CD^{pro}$) of poliovirus type 2 (Sabin), the precursor to proteinase $3C^{pro}$ and RNA polymerase $3D^{pol}$, was expressed in bacteria by using a T7 expression system. Site-specific mutagenesis of the 3C/3D cleavage site was performed to generate active proteolytic precursors impaired in their ability to process themselves to $3C^{pro}$ and $3D^{pol}$. Of these mutations, the exchange of the Thr residue at the *P4* position of the 3C/3D cleavage site for a Lys residue ($3CD^{pro}$ T181K) resulted in a mutant polypeptide exhibiting the smallest amount of autoprocessing. This mutant was purified to 86% homogeneity and used for subsequent proteolytic studies. Purified $3CD^{proM}$ (M designates the cleavage site mutant $3CD^{pro}$ T181K) was capable of cleaving the P1 capsid precursor, a peptide representing the 2BC cleavage site, and the 2BC precursor polypeptide. Purified $3CD^{proM}$ demonstrated the same detergent sensitivity in processing experiments with the capsid precursor as was observed by using P1 and crude extracts of poliovirus-infected HeLa cell lysates. Purified $3CD^{proM}$ did not have any detectable RNA polymerase activity, whereas $3D^{pol}$, separated from $3CD^{proM}$ by gel filtration in the last step of purification, did. We conclude that $3CD^{proM}$ can process both structural and nonstructural precursors of the poliovirus polyprotein and that it is active against a synthetic peptide substrate. Moreover, cleavage of 3CD to $3D^{pol}$ is needed to activate the 3D RNA polymerase.

One of the most intriguing phenomena in picornavirus replication is the use of partial cleavage products of the polyprotein to perform functions distinct from those of the final cleavage products (reviewed in reference 13). Precursor polypeptides with specific functions offer an additional level of genetic economy to the virus. The poliovirus-encoded protein 3CD is a cleavage product of P3 and a precursor to both the viral proteinase, $3C^{pro}$, and the viral RNA-dependent RNA polymerase, $3D^{pol}$. In vitro translation of poliovirus-specific RNAs has implicated 3CD as the obligatory enzyme to proteolytically process the capsid precursor, P1, thereby generating VP0, VP1, and VP3 (16, 33). Hence, 3CD has been called $3CD^{pro}$. Direct evidence for P1 cleavage with purified $3CD^{pro}$ has been lacking. However, $3C^{pro}$ alone cannot process the P1 precursor to completion unless the concentration of the enzyme is increased to 25 μM in vitro (18, 23), an observation supporting the role of $3CD^{pro}$ in morphogenesis. It is assumed that $3CD^{pro}$ interacts with the P1 precursor in such a way as to potentiate the site-specific cleavages.

Recently, $3CD^{pro}$ has been implicated to be involved in the replication of polioviral RNA. Initial evidence came from genetic studies, where a four-base insertion between nucleotides 67 and 68 of the genomic RNA yielded a small-plaque phenotype. Pseudorevertants of the small-plaque phenotype mapped to the $3C^{pro}$ cistron (2). Subsequently, $3CD^{pro}$ and a host protein(s) of unknown nature have been shown to form a ribonucleoprotein complex with an RNA probe representing the first 110 nucleotides of the polioviral genome (1).

To study the role $3CD^{pro}$ plays in poliovirus proliferation, we have expressed cDNA encoding a $3CD^{pro}$ cleavage site mutant in a bacterial expression vector and have purified the proteinase to 86% homogeneity. $3CD^{proM}$ was analyzed with respect to its activity as a proteinase toward P1, a 2BC polypeptide, and synthetic peptides. $3D^{pol}$ that copurified with $3CD^{proM}$ until the last step had the expected primer and RNA-dependent RNA polymerase activity, whereas $3CD^{proM}$ was found to be inactive as a RNA polymerase.

MATERIALS AND METHODS

Plasmid construction. The cDNA encoding the 3CD cistron of poliovirus type 2 (Sabin) was cloned into the T7 expression plasmid pET2A (32) by standard procedures, yielding a plasmid called pMN36. 3CD cDNA was also cloned into the vector pBSSK+, generating plasmid pBSS23CD. pBSS23CD was used to obtain single-stranded uracil-incorporated DNA needed for site-specific mutagenesis by the method of Kunkel (20). The oligonucleotides used for mutagenesis were generated with a Applied Biosystems 380B DNA synthesizer.

Buffers. Buffer A consisted of 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM dithiothreitol (DTT), 1 mM Na_3EDTA, and 5% glycerol. Buffer B consisted of 50 mM Tris-HCl (pH 8.0), 1 M NaCl, 10 mM DTT, 1 mM Na_3EDTA, and 5% glycerol. Buffer C consisted of 50 mM Tris-HCl (pH 8.0), 1 mM DTT, 1 mM Na_3EDTA, and 5% glycerol. Buffer C was used as a base buffer for two ion-exchange chromatographic steps in which only the NaCl concentration was altered. Buffer D consisted of 100 mM sodium phosphate, 100 mM sodium sulfate, 1 mM DTT, 1 mM Na_3EDTA, 100 μM GTP, and 5% glycerol. Buffer E consisted of 20 mM *N*-2-hydroxyethylpiperazine-*N*'2-

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ethanesulfonic acid (HEPES)–NaOH (pH 7.4), 100 mM NaCl, and 1 mM Na_3EDTA. Buffer F (1×) consisted of 12% glycerol, 80 mM Tris-HCl (pH 6.8), 25 mM DTT, 1% sodium dodecyl sulfate, and a trace amount of bromophenol blue. Buffer G consisted of 40 mM Tris-HCl (pH 8.0) and 100 mM NaCl. Buffer H (2×) consisted of 125 mM Tris-HCl (pH 6.8), 4% sodium dodecyl sulfate (SDS), 50% glycerol, 1.3 M β-mercaptoethanol, and a trace amount of bromophenol blue.

Protein concentration determination and electrophoresis of protein samples. Protein concentration was determined by the Bio-Rad protein assay (Bio-Rad Laboratories). SDS–12.5% polyacrylamide gels were used to assess P1 processing (40 parts acrylamide to 1 part methylenebisacrylamide). All other gels were SDS–10 to 20% polyacrylamide gradient gels (40 parts acrylamide to 1 part methylenebisacrylamide). Electrophoresis was performed according to the method of Laemmli (21).

Assessment of autocatalysis of $3CD^{pro}$ cleavage site mutants. *Escherichia coli* BL21(DE3), transformed with plasmids encoding 3CD cleavage site mutants, was subjected to pulse-chase experiments. Cultures of *E. coli* harboring these plasmids were grown in M9 medium to an A_{600} of 0.8. At this cell density, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture to a final concentration of 0.4 mM. Thirty minutes after induction, rifampin was added to a final concentration of 50 μg/ml to halt host transcription. The cultures were pulsed for 60 s with Translabel ([^{35}S]Met and [^{35}S]Cys), and then Casamino Acids were added to a final concentration of 2 mg/ml. At specific time points in the chase, the cultures were harvested, and the total cellular lysates were electrophoresed through an SDS–10 to 20% polyacrylamide gel (Fig. 2A, lanes 2 to 6 and 8 to 12). Immunoprecipitations using anti-3C antisera were also done at the 2-h chase time point (Fig. 2A, lanes 7 and 13). The gels were fluorographed with En^{3}Hance (New England Nuclear Corporation), dried, and subjected to autoradiography.

Preparation of the bacterial cell paste. Glycerol stocks of *E. coli* BL21(DE3) transformed with pMN36 T181K (pMN36 with a mutation encoding a T-181→K substitution) were used to inoculate 5 ml of Luria broth (LB) containing 100 μg/ml of ampicillin. This initial culture was then serially diluted in 50-ml cultures of LB containing ampicillin to a final dilution of 10^{-9}, which was grown overnight at 37°C. A culture with an A_{600} of 0.3 was added to 500 ml of M9CA medium (i.e., M9 medium containing 2 g of Casamino Acids per liter) with 100 μg of ampicillin per ml and incubated with shaking at 37°C to an A_{600} of 0.5. This culture was used to inoculate 9.5 liters of M9CA medium and grown at 37°C with stirring (900 rpm) and aeration (16 liters/min) in a 14-liter benchtop fermentor (Microferm II; New Brunswick Scientific Co., Inc.). When the A_{600} of a 1:5 dilution reached 0.5, 40 ml was withdrawn from the culture as an uninduced sample. IPTG was added to the 10-liter culture to a final concentration of 0.4 mM. After 30 min of induction, the temperature of the fermentor was shifted from 37 to 25°C, and induction was continued for 4 more hours. At this time, 40 ml of the culture was withdrawn as an induced sample, and the entire culture was harvested by centrifugation at 3,500 × g for 15 min, washed with 750 ml of buffer G, and recentrifuged. The paste was then transferred to a plastic bag, weighed, and stored at −80°C. The yield ranged from 55 to 70 g of wet cell paste. The extent of induction of the desired polypeptide was determined by electrophoresis of total cell protein of a fraction of the induced and uninduced samples through an SDS–10 to 20% gradient polyacrylamide gel, followed by staining of the gel with Coomassie blue.

Purification of $3CD^{proM}$. All operations except the gel filtration step were performed at 4°C. Fifteen grams of induced cell paste was suspended in buffer A to a total volume of 40 ml. This suspension was then passed through a French pressure cell three times at 10,000 lb/in^2. Forty milliliters of buffer A was added to the lysate (fraction 1) and spun at 10,000 × g (9.5 krpm in a Sorvall SS34 rotor) for 20 min at 4°C. The supernatant from this centrifugation step (fraction 2A) was spun at 100,000 × g (38 krpm in a Beckman TY60Ti rotor) for 1 h at 4°C. Na_3EDTA was added, with stirring, to the supernatant of the 100,000 × g spin to a final concentration of 5 mM (fraction 3A). Ammonium sulfate was slowly added with stirring to fraction 3A to a final saturation of 35% (typically, 15.63 g of ammonium sulfate was added to the 75-ml fraction 3A volume). The ammonium sulfate-precipitated proteins were collected by spinning the suspension at 9.5 krpm in a Sorvall SS34 rotor for 15 min at 4°C. The pellet was dissolved in 15 ml of buffer C containing 30 mM NaCl (fraction 6) and dialyzed against 1 liter of the same buffer for 1 h at 4°C. After the 1-h dialysis, the buffer was changed, and dialysis was continued overnight. The pellet fractions from the 10,000 × g spin (fraction 2B) and the 100,000 × g spin (fraction 3B) were separately suspended in 20 ml of buffer B and mixed by inversion for 30 min at 4°C. The protein suspensions were then spun at 100,000 × g (38 krpm in a TY60Ti rotor). The supernatant from the 100,000 × g spin of the fraction 2B back extraction (fraction 4A) and the supernatant of the 100,000 × g spin of the fraction 3B back extraction (fraction 5A) were dialyzed against 1 liter of buffer C containing 30 mM NaCl for 1 hour at 4°C. After the 1-h dialysis, the buffer was changed, and dialysis was continued overnight. The next day, fractions 4A, 5A, and 6 were combined (fraction 7) and overlaid onto a 70-ml bed of DEAE-cellulose equilibrated with buffer C containing 30 mM NaCl. After a four-column-volume wash with this buffer, the bound proteins were eluted with 140 ml of buffer C containing 300 mM NaCl. This fraction (fraction 8) was dialyzed against 2 liters of buffer C containing 50 mM NaCl at 4°C for 1 h, after which the buffer was changed and dialysis was continued for an additional hour. Fraction 8 was then loaded onto a 15-ml phosphocellulose P11 column equilibrated with buffer C containing 50 mM NaCl. After a wash with four column volumes of this buffer, the bound proteins were eluted with 30 ml of buffer C containing 200 mM NaCl. The eluted protein (fraction 11) was precipitated by the addition of ammonium sulfate to 50% saturation. The precipitated proteins were collected by spinning the suspension at 9.5 krpm in a Sorvall SS34 rotor for 15 min at 4°C. The pellet was dissolved in 2 ml of buffer D and spun at 14 krpm in an Eppendorf Microfuge for 5 min to remove any particulate matter. The supernatant (fraction 12) was loaded onto a TosoHaas G3000SW high-performance liquid chromatography (HPLC) sizing column equilibrated with buffer D at room temperature. Peak fractions were pooled (fraction 13) and used in subsequent proteolytic and polymerase assays.

Proteolytic assays. RNA encoding the P1 capsid precursor was transcribed from pMN25 (24) and translated in a HeLa cell lysate according to the specifications of Molla et al. (22). The translation conditions of the polypeptide 2BC substrate have been described previously (12). The 2BC peptide cleavage substrate was a kind gift from Boehringer Ingelheim. The reaction conditions for digestion with $3CD^{proM}$ were similar to those for digestion with $3C^{pro}$ described

previously (23). Briefly, 4 μl of 10× buffer E, 4 μl of 10 mM DTT, and 1 μl of the in vitro-translated substrate were added to the various fractions, and the final reaction volume was adjusted to 40 μl with H_2O. These reaction mixtures were then incubated at 30°C for 1 h, after which 40 μl of 2× buffer H was added to each reaction mixture. The samples were boiled for 5 min before being loaded on an SDS-12.5% polyacrylamide gel. After electrophoresis, the gels were fluorographed by soaking them in En^{3}Hance as described by New England Nuclear and dried. Kodak XAR film was preflashed prior to exposure to the specific activity gel. Densitometry was performed with an LKB gel scanner. Reaction conditions for peptide cleavages and detection of cleavage products have been described previously (12).

RNA-dependent RNA polymerase assay. [^{3}H]UTP incorporation using a poly(A)-dependent, oligo(dT)-primed poly(U) polymerase assay has been described previously (10).

RESULTS

3CD^{pro} cleavage site mutagenesis. A study of 3CD^{pro} requires that, during purification of this protein, the cleavage to 3C^{pro} and 3D^{pol} be inhibited. The cleavage site, TQIQ/GEIQ, between 3C^{pro} and 3D^{pol} is unfavorable (27) yet allows sufficient processing to yield the required cleavage products in the course of viral proliferation. In order to retard 3CD autoprocessing further, mutations targeting the crucial *P1* and *P4* positions were made in the 3C/3D cleavage site (Fig. 1B; see reference 25). The cleavage site nomenclature is that of Berger and Schechter (3); briefly, the *P1* residue corresponds to the newly generated carboxy-terminal residue and the *P1'* residue corresponds to the newly generated amino-terminal residue. The cDNA encoding poliovirus type 2 (Sabin) 3CD was cloned into pBSSK+ and subjected to site-specific mutagenesis by the method of Kunkel (20). The cleavage site mutants were then cloned back into the 3CD expression plasmid pMN36. The extent of residual autocleavage was determined by inducing the expression of this mutated gene in *E. coli* BL21(DE3) and performing pulse-chase experiments in the presence of Translabel ([^{35}S]Met and [^{35}S]Cys) and rifampin (100 μg/ml). The bacterial extracts of wild-type 3CD and the cleavage site mutants were electrophoresed through an SDS-10% to 20% gradient polyacrylamide gel. The results are shown in Fig. 2A for mutant T181K, which showed the least 3C/3D cleavage of all mutants generated. This is demonstrated in the experiment whose results are shown in Fig. 2B, where, after a single time point of a 2-h chase, mutant T181K produced little, if any, detectable 3C^{pro} and 3D^{pol}. Therefore, mutant T181K was chosen as a candidate for purification.

The results of the mutations in the *P1* and *P4* positions confirm and extend previous observations of the importance of these positions for proteolytic processing (4, 7, 8, 17, 19, 25–27, 31). Indeed, the choice of the conversion of the Thr residue at position 181 to Lys was based on the previous observation that a Lys residue at the *P4* position of the 3B/3C cleavage site abolished processing of this site in vitro (19).

Expression and purification of 3CD^{proM}. The cDNA encoding the poliovirus type 2 (Sabin strain) 3CD cistron, or mutants thereof, was cloned into the pET2A vector under control of a T7 promoter. The N-terminal Met codon of T7 gene 10 was fused to the 3CD coding sequence whose open reading frame was terminated by a single UAG stop codon. This plasmid, called pMN36, was transformed into the bacterial strain BL21(DE3) and expressed as described

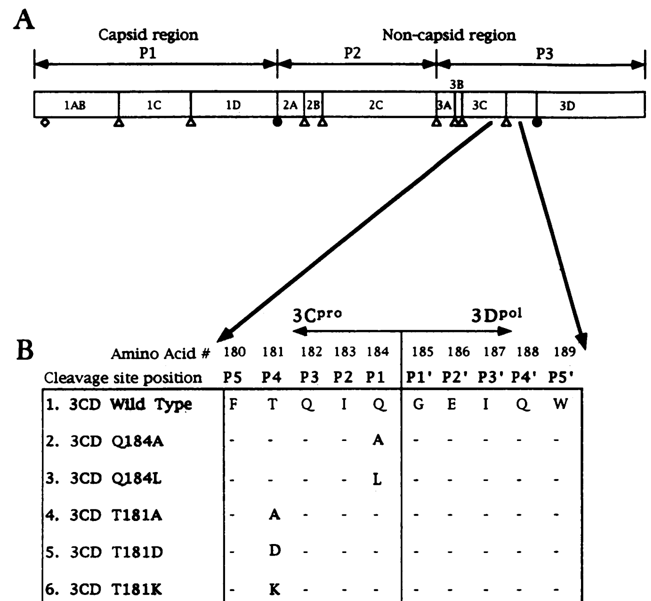

FIG. 1. Cleavage site mutations in 3CD^{pro} within the poliovirus polyprotein. (A) Schematic map of the poliovirus polyprotein. Open triangles indicate 3C^{pro} and 3CD^{pro} cleavage sites mediated at Gln-Gly dipeptides, closed circles indicate 2A^{pro} cleavage sites mediated at Tyr-Gly residues, and the open diamond represents the maturation cleavage site which occurs at an Asn-Ser dipeptide sequence found within 1AB to generate the N-terminal (1A) VP4 and C-terminal (1B) VP2 upon packaging of the viral genomic RNA (reviewed in reference 13). Nomenclature is that of reference 30. (B) Enlargement of the amino acid sequence spanning the 3C/3D cleavage site. The cleavage nomenclature of the site positions is that of Berger and Schechter (3). Dashes indicate identity with the wild-type sequence. Amino Acid # refers to the number of amino acids in 3CD^{pro} of poliovirus, accounting for the fact the recombinant protein most likely possesses an amino-terminal Met residue (23).

previously (23). However, expression at 37°C yielded only insoluble 3CD^{proM}, and attempts to generate soluble, active proteinase from pellets through denaturation with urea or guanidine followed by stepwise renaturation were unsuccessful. On the other hand, expression at 25°C yielded 3CD^{proM} at a level of about 1% of the total cellular protein (Fig. 3). Of this product, about 50% occurred in the soluble form. A handicap in our purification strategy was that we had to avoid detergents because of the sensitivity of P1 cleavage to these reagents (23) (see below).

Lysates from 15 g of bacterial pastes were subjected to differential centrifugation to separate soluble from insoluble 3CD^{proM}. Up to 90% of 3CD^{proM} in the pellets could be extracted with buffer B as detailed in Materials and Methods. A multistep purification protocol yielded 86% pure 3CD^{proM}, as determined by laser densitometry (Fig. 4, lane 7). The yields of protein from 15 g of bacterial cell paste are detailed in Table 1.

3CD^{proM} has no polymerase activity. Early studies using crude protein fractions suggested that 3CD^{pro}, which contains the polypeptide chain for 3D^{pol}, has no RNA polymerase activity (11). To analyze 3CD^{pro} for polymerase activity, it was necessary to separate 3CD^{proM} from 3D^{pol}. However, initially we found it impossible to produce only 3CD^{proM} by expression in *E. coli*, as small amounts of 3D^{pol} and 3C^{pro} were consistently generated regardless of the nature of the cleavage site mutation. This phenomenon is probably the

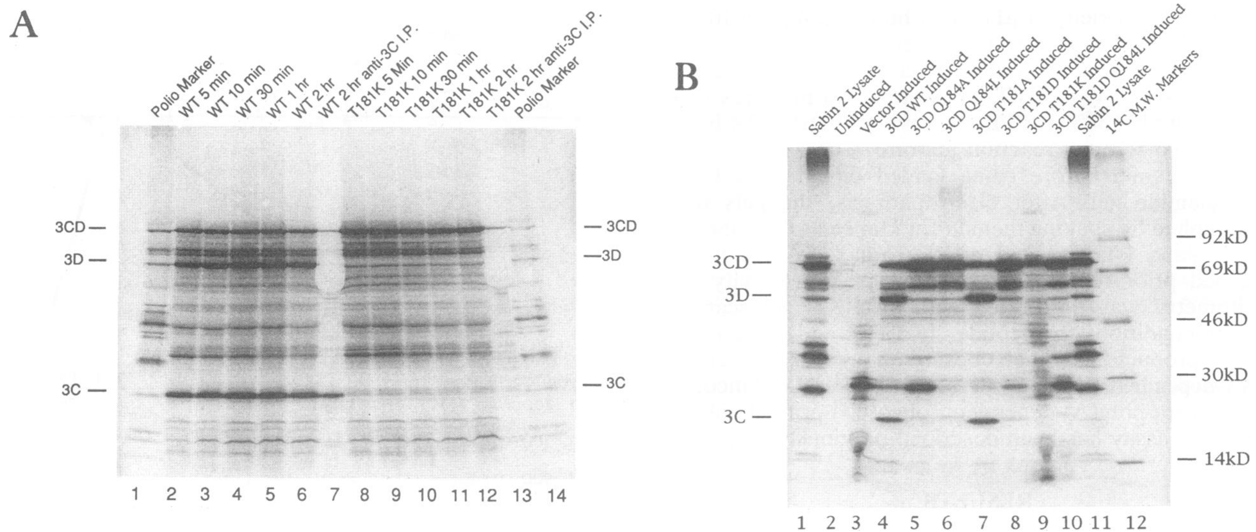

FIG. 2. Processing pattern of cleavage site mutants expressed in *E. coli*. (A) SDS-polyacrylamide gel showing a pulse-chase experiment with *E. coli* BL21(DE3) expressing wild-type (WT) 3CD^{pro} (lanes 2 to 7) and the cleavage site mutant T181K (lanes 8 to 13). Rifampin was added (100-μg/ml final concentration) to shut down host transcription 30 min after induction with IPTG, after which the cultures were pulsed with Translabel for 1 min. For the chase, Casamino Acids were added to a final concentration of 2 mg/ml, and the cultures were allowed to continue growing until the time points shown above each lane, at which time the cultures were harvested. Proteins of the bacterial lysates were electrophoresed through an SDS–10 to 20% gradient polyacrylamide gel, fluorographed with En^{3}Hance, dried, and subjected to autoradiography. Similar analyses were done for the rest of the cleavage site mutants (data not shown). I. P., immunoprecipitation. (B) Pulse-chase experiment similar to those described for Fig. 2A, except that every mutant was analyzed after a 2-h chase. Lanes 1 and 11 are lysates of poliovirus type 2 (Sabin)-infected HeLa cells. M.W., molecular weight.

result of intramolecular (*cis*) cleavage (28). By analogy to studies with 2A^{pro} (14), *cis* cleavages appear less sensitive to perturbation of the cleavage sequence than do intermolecular *trans* cleavages. Therefore, we believe that these *cis* cleavages occur most efficiently while the polypeptides are in statu nascendi. In any event, 3CD^{proM} and 3D^{pol} copurified up to fraction 13 (Fig. 4, lane 7). Separation of 3CD^{proM} from 3D^{pol} was achieved by using an HPLC gel filtration column (Fig. 5) in which fractions 63 to 65 were essentially free of 3D^{pol} and fractions 70 to 72 were essentially free of 3CD^{proM}. These fractions were used in an assay of poly(A)-dependent poly(U) synthesis (10). After dialysis against the polymerase reaction buffer, 30 ng of protein from fraction 13 and 30 ng of protein from the 3D^{pol}-enriched fractions were used in the RNA synthesis assay. Whereas 3D^{pol} separated from 3CD^{proM} by gel filtration was active in incorporating [^{3}H]UTP with a poly(A) template, 3CD^{proM} was unable to show incorporation above background levels (Fig. 6). The 3D^{pol}-enriched fractions were about four times less active than the purified enzyme (kindly provided by S. Plotch). It should be noted that polymerase activity of 3D^{pol} from the column fractions was obtained only if GTP was incorporated into the gel filtration buffer. We have recently observed a stimulating effect of GTP on polymerase activity of 3D^{pol} (29), and furthermore, GTP was found to stabilize the enzyme (29a). The dip in absorption in the elution profile between the 88- and 90-min time points (Fig. 5A) could be used as a criterion of GTP binding by 3D^{pol} and 3CD^{proM}. This phenomenon has been described before (9, 15) as occurring when protein-ligand interactions were studied by gel filtration.

Cleavage of the P1 capsid precursor by purified 3CD^{proM}. Available evidence from in vitro translation suggested that 3CD^{pro} hydrolyzes capsid precursor P1 to the poliovirus protomer consisting of VP0, VP3, and VP1 (16, 33, 34); note

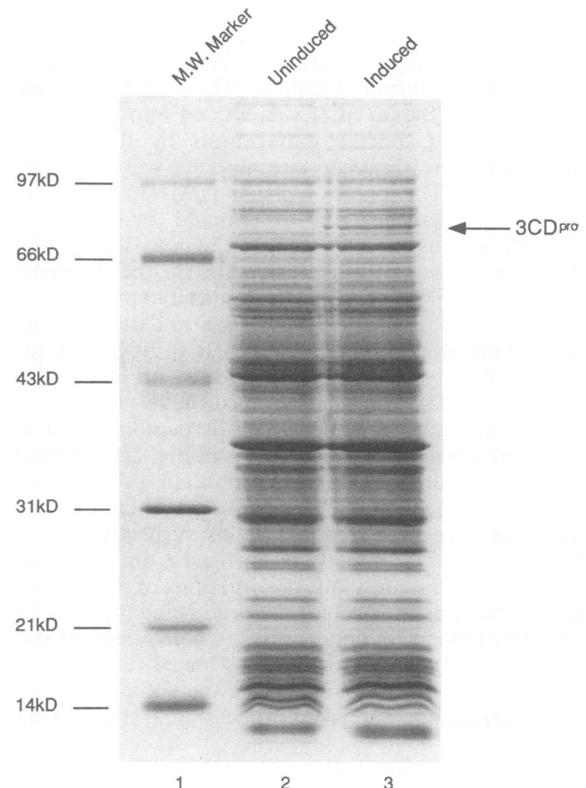

FIG. 3. Induction of pMN36 T181K expressed in a 10-liter culture of *E. coli* BL21(DE3). Samples of the culture were withdrawn before the addition of IPTG (lane 2) and 4 h postinduction (lane 3). These samples were electrophoresed through an SDS–10 to 20% polyacrylamide gel and stained with Coomassie brilliant blue R-250. 3CD^{proM}, with a molecular mass of 72 kDa, is indicated by the arrow.

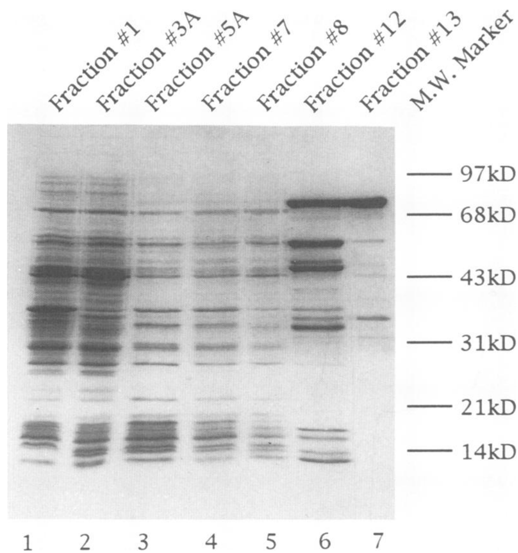

FIG. 4. Purification of $3CD^{proM}$. Twenty-five micrograms of total protein from selected fractions of the $3CD^{proM}$ purification procedure was loaded onto an SDS–10 to 20% gradient polyacrylamide gel and stained with Coomassie brilliant blue R-250. This gel was then subjected to laser densitometry to determine the percent purity of $3CD^{proM}$ in each fraction. M.W., molecular weight.

that the polypeptides P1, VP0, VP3, and VP1 are also referred to as 1ABCD, 1AB, 1C, and 1D, respectively (30). It was therefore of interest to analyze whether our purified $3CD^{pro}$, which contained no detectable $3C^{pro}$ proteinase, was able to cleave P1. The P1 substrate was generated by in vitro translation (22–24) and incubated for 1 h at 30°C with 1 μg of total protein from each purification fraction. The appearance of the 1ABC precursor was used as the basis for the unit definition. The amount of 1ABC released from P1 upon incubation of the substrate mix with crude lysate of bacteria expressing $3CD^{proM}$ (fraction 1) was arbitrarily considered to be 1 U (area of absorbance of 0.082 mm^2 as determined by laser densitometry) (Fig. 7, lane 7). $3CD^{proM}$ of fraction 13 also cleaved P1 to 1AB, 1C, and 1D at a final concentration of 0.44 μM (Fig. 7, lane 13); the whole fraction contained a total of 6 U (Table 1). Purified $3C^{pro}$ processes the P1 precursor to 1AB, 1C, and 1D at a final concentration of 25 μM (18, 23). Thus, purified $3CD^{proM}$, on a molar basis, is about 50 times more efficient in processing the P1 precursor than purified $3C^{pro}$. These results confirm that the purified recombinant enzyme has properties predicted for $3CD^{pro}$ from studies with crude translation extracts.

Two other observations are noteworthy. First, at low concentrations of recombinant $3CD^{proM}$, cleavage occurred predominantly between 1ABC and 1D (Fig. 7, lanes 7 through 12). This unexpected preference for a cleavage site is reminiscent of the cleavage of P1 with $3C^{pro}$ at a high enzyme/substrate ratio (23). Second, by use of crude poliovirus-infected HeLa lysate, the cleavage of 1ABC to 1AB and 1C, but not the cleavage of P1 to 1ABC and 1D, has been reported to be sensitive to 1% Triton X-100 (23), an observation reproduced in Fig. 7, lane 5. We found the same sensitivity to detergent in P1 processing with purified $3CD^{proM}$ (Fig. 7, lane 14). These observations strongly suggest either that specific hydrophobic interactions must be formed between $3CD^{pro}$ and the P1 substrate for complete processing to the protomer to occur or that the detergent perturbs the folding of the P1 substrate in such a manner that $3CD^{pro}$ no longer recognizes one of the Q/G cleavage sites (13).

Cleavage of a nonstructural precursor by $3CD^{proM}$. The precursor for nonstructural poliovirus proteins (P2 and P3) is exclusively cleaved at Q/G pairs, and thus these proteins result from $3C^{pro}$-like activities. Available evidence from in vitro translation studies suggests that $3CD^{pro}$ whose cleavage to $3C^{pro}$ and $3D^{pol}$ is impaired (μ10 Ser insertion mutant of Ypma-Wong et al. [33]) can also cleave the nonstructural precursors. The data shown in Fig. 8 confirm this conclusion. A precursor polypeptide representing 2BC, which we generated by in vitro translation (12), was cleaved to 2B and 2C with purified $3CD^{proM}$. Interestingly, we found this cleavage to be as inefficient as the previously observed cleavage of 2BC with purified $3C^{pro}$ (12).

Cleavage of a synthetic peptide with $3CD^{proM}$. The data presented so far showed that purified $3CD^{proM}$ could catalyze Q/G-specific proteolysis of polypeptides in a fashion similar to, and in the case of P1 even more efficiently than, $3C^{pro}$. We were interested to see whether purified $3CD^{proM}$ has the ability, like $3C^{pro}$, to cleave a synthetic substrate. The peptide acetyl-EIPYAIEQ/GDSWLKKF-NH_2 (Fig. 9A), representing the cleavage site in 2BC, was incubated overnight with purified $3CD^{proM}$ at 30°C, and the products of the reaction were analyzed by fast protein liquid chromatog-

TABLE 1. Purification of 3CD from 15 g of cell paste of *E. coli* BL21(DE3) transformed with pMN36 T181K

Fraction	Vol (ml)	Concn (mg/ml)[a]	Total protein (mg)	U of 1ABC[b]	U/mg	% Yield	% Purity[c]
1. Lysate	80	28	2,080	1	4.8×10^{-4}	100	1
3A. S100[d]	75	20	1,380	0.73	5.3×10^{-4}	73	1
5A. S100 back extraction	22	5.6	120	1.13	9.4×10^{-3}	113	2
7. DEAE load	64	8.5	442	0.89	2×10^{-3}	89	2
8. DEAE–0.3 M NaCl eluent	150	0.6	100	2.47	2.5×10^{-2}	247	2
12. Phosphocellulose–0.2 M NaCl eluent	2	1.0	2	5.83	2.9	583	30
13. Gel filtration pool	8	0.05	0.41	6.1	14.9	610	86

[a] Concentration of protein as determined by Bio-Rad protein assay (dye binding).
[b] One unit is defined as the release of 1ABC from the P1 substrate to yield an absorbance area of 0.082 mm^2 upon laser densitometry.
[c] Determined by laser densitometry of an SDS–10 to 20% gradient polyacrylamide gel stained with Coomassie brilliant blue R-250.
[d] S100, supernatant from centrifugation at 100,000 × *g*.

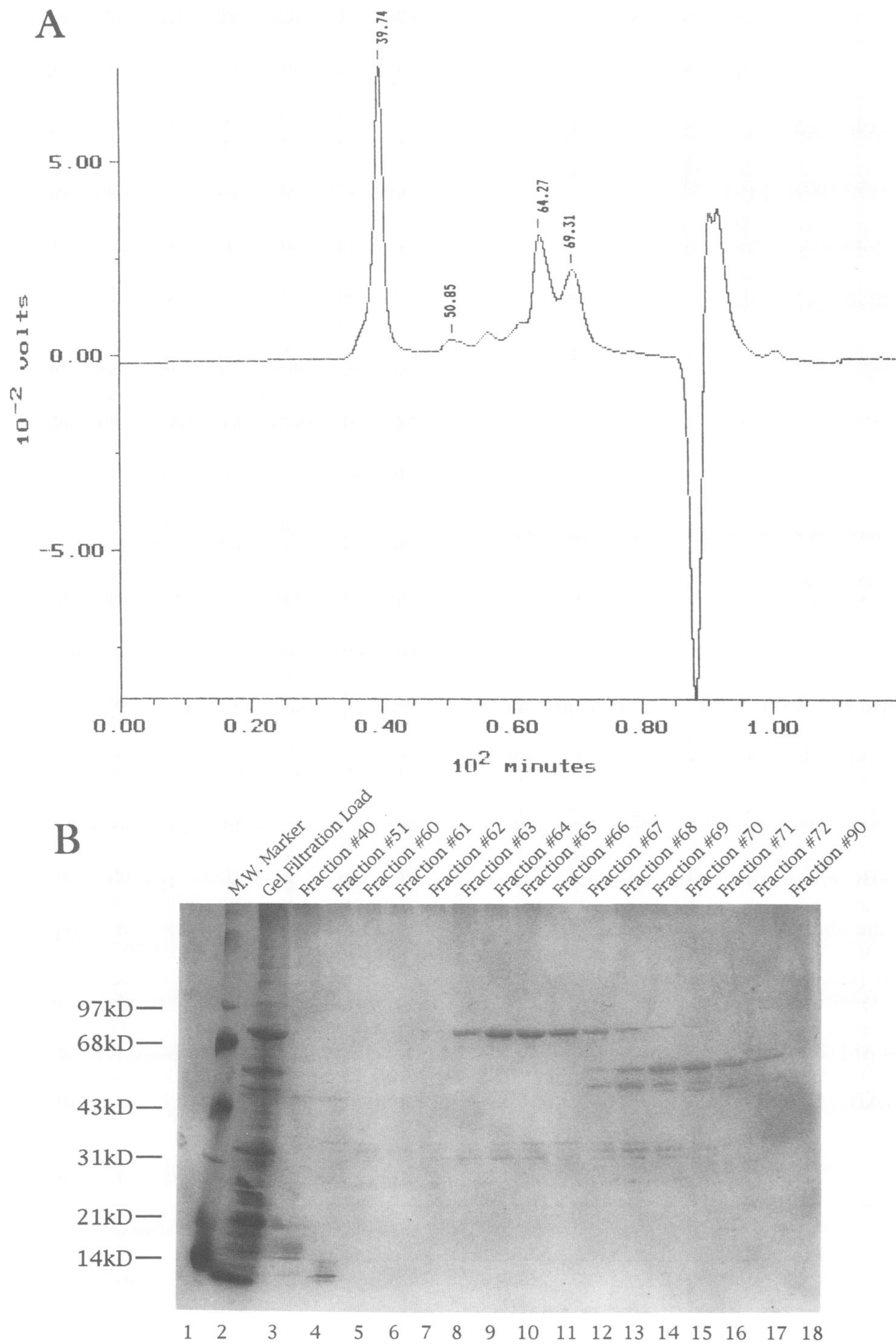

FIG. 5. Details of the last step in the purification of 3CD^{proM}. (A) A volt chromatogram of the HPLC gel filtration run after fraction 12 was loaded onto the column (note that volts correspond to A_{280} absorbance units). 3CD^{proM} eluted at the 64.27-min time point, and 3D^{pol} eluted at the 69.31-min time point. The gel filtration buffer contains 100 μM GTP, which is a ligand for 3CD^{pro} and 3D^{pol} (see text). A reproducible drop in absorbance was observed at 88 to 90 min. A dip in the absorbance indicates a lack of GTP present at that particular time point, since the UV monitor was blanked with buffer D, which contains 100 μM GTP. This dip provides a criterion of GTP binding by 3D^{pol} and 3CD^{proM}. This phenomenon has been described before (9, 15) as occurring in studies in which protein-ligand interactions were studied by gel filtration. (B) Peak fractions from the gel filtration column were electrophoresed through an SDS–10 to 20% gradient polyacrylamide gel and silver stained. 3CD^{proM} eluted between fractions 63 and 66, whereas 3D^{pol} mainly eluted mainly between fractions 69 and 72. M.W., molecular weight.

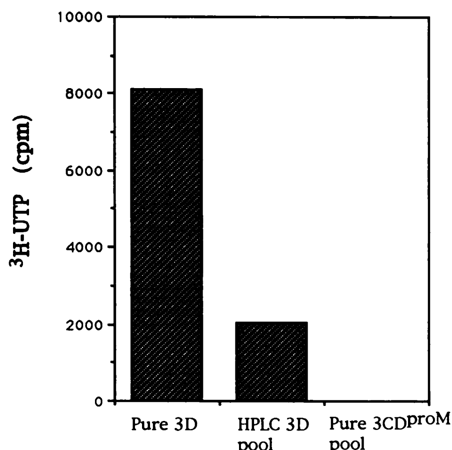

FIG. 6. [^{3}H]poly(U) synthesis with poly(A) as a template and with an oligo(dT)$_{15}$ primer. Thirty nanograms of total protein of purified 3D^{pol} (kindly provided by S. Plotch) or the 3D^{pol}- or 3CD^{proM}-enriched fraction from the HPLC gel filtration column was used in the polymerase reactions. The background counts (i.e., no protein added to the reaction) were subtracted from the incorporated [^{3}H]UTP counts obtained in reactions in which protein was added.

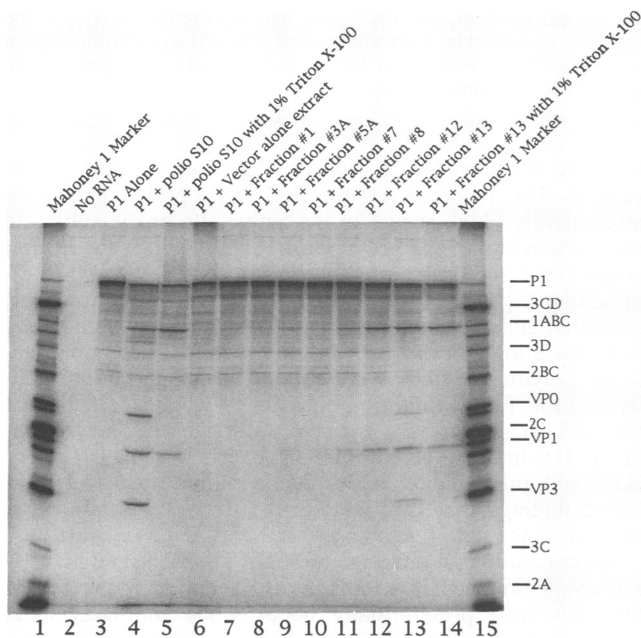

FIG. 7. Specific activity of P1 cleavage. One microliter of in vitro-translated P1 substrate was incubated with 1 μg of total protein from each fraction of the purification (as shown in Fig. 4) for 60 min at 30°C. An equal volume of buffer H was then added to the reaction mixtures. The reaction mixtures were then electrophoresed on an SDS-12.5% polyacrylamide gel, which was then dried, fluorographed, and subjected to autoradiography on preflashed film. The film was then scanned by laser densitometry to estimate the amount of 1ABC product generated by 3CD^{proM} cleavage. The final concentration of 3CD^{proM} in lanes 13 and 14 is 0.44 μM. S10, supernatant from centrifugation at 10,000 × *g*.

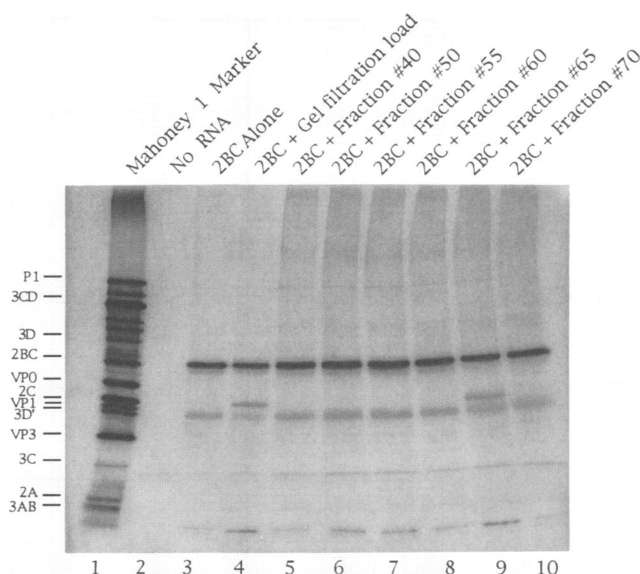

FIG. 8. Cleavage of the 2BC precursor polypeptide by purified 3CD^{proM}. Two microliters of the in vitro-translated 2BC substrate was incubated with fractions from the gel filtration column (Fig. 5B) for 1 h at 30°C. After the incubation, an equal volume of buffer H was added to the reaction mixtures, and the mixtures were boiled for 5 min. The samples were then electrophoresed through an SDS-10 to 20% gradient polyacrylamide gel. The gel was then fluorographed, dried, and subjected to autoradiography. The proteolytic activity coincided with the presence of 3CD^{proM} (lanes 4 and 9). 2B, which contains only one methionine residue, runs to the bottom of the gel and under these conditions was not detected.

raphy. As can be seen in Fig. 9B, the peptide was cleaved to completion under these conditions. The kinetics of the cleavage were similar to those observed with purified 3C^{pro} (data not shown).

DISCUSSION

We have confirmed and extended the results of previous studies of 3CD^{pro}, the precursor to the poliovirus proteinase 3C^{pro} and RNA polymerase 3D^{pol}. Proteolysis at most bona fide Q/G signals of the viral polyprotein in vivo generally occurs quite rapidly, whereas that of 3CD^{pro} is very slow. This suggests that 3CD^{pro} is needed for functions distinct from those of its cleavage products. However, all attempts to entirely abolish cleavage at the Q/G site between 3C^{pro} and 3D^{pol} failed, perhaps because of the unique mechanism of intramolecular interaction between the active site and the substrate sequence (14).

It has been reported that a mutant 3CD^{pro} carrying a Ser insertion between the *P2* and *P3* amino acids of the Q/G cleavage signal showed very little, if any, processing at this site (33). We have made and expressed this Ser insertion mutant in *E. coli* and found proteolysis greatly diminished but not entirely abolished, a phenomenon similar to the properties of the T181K mutant studied here. This may be due to the residue present in the *P4* position of the Ser insertion mutant (Gln instead of Thr). The importance of the *P4* position, which has been previously recognized (4, 19, 25, 27, 31, 33), is apparent from mutants T181K and T181D. Mutations made at the *P1* site, generating mutants Q184A and Q184L, also resulted in diminished autoprocessing. These 3CD^{pro} mutations however, gave rise to alternative

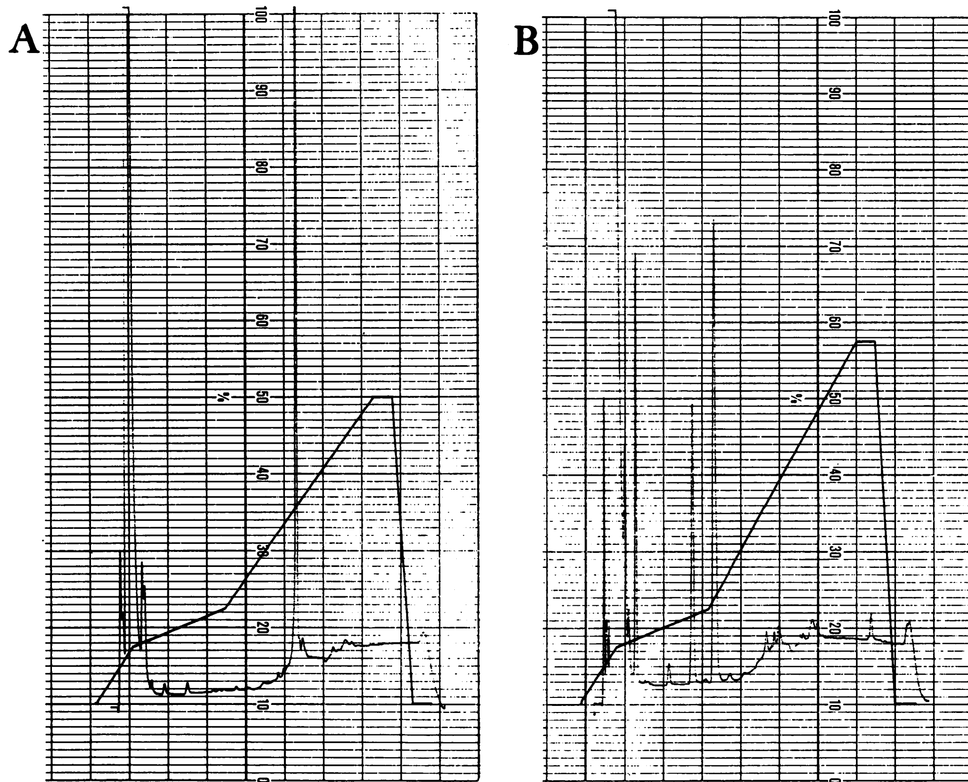

FIG. 9. Cleavage of a peptide by 3CD^proM. (A) Reverse-phase column A_{214} chromatogram of the 2BC substrate peptide after incubation in reaction buffer alone overnight at 30°C. The heavier line represents the solvent gradient, and the lighter line represents the A_{214} of the peptide. The peak occurring in the middle of the gradient is the uncleaved peptide, as judged by its retention time (12). (B) A_{214} chromatogram of the 2BC substrate peptide after incubation with 0.1 μM (final concentration) 3CD^proM overnight at 30°C. The two peaks eluting earliest in the elution gradient represent the 2BC peptide cleavage products, as judged by their retention times.

cleavage products (Fig. 2B, lanes 5, 6, and 10), similar to mutations in which the *P1'* Gly residue was targeted (17). These observations are also reminiscent of some cleavage site mutants of proteinase 2A^pro (14). In contrast, processing was increased when the Thr residue in *P4* of the cleavage site was changed to Ala (Fig. 2B, lane 7), accurately reflecting the cleavages of corresponding synthetic peptides with 3C^pro (27).

Considering our data on the proteolytic activity of 3CD^pro, it is conceivable that this enzyme can carry out all Gln-Gly-specific proteolytic cleavages involved in the processing of the poliovirus polyprotein. The same cannot be stated for 3C^pro because of its inefficiency in P1 cleavage (23). One could envision, however, the possibility that 3C^pro carries out specialized functions in viral proliferation distinct from those of 3CD^pro, but we have so far found no evidence to support this possibility. The interaction between 3CD^pro and P1 is intriguing, as it requires most of the 3D-specific polypeptide chain (5, 6, 34). We believe that there is an additional interaction required in order for 3CD^pro to cleave 1ABC to generate 1AB and 1C. This interaction (3CD^pro-1ABC) and subsequent cleavage are abrogated if detergent is present in the cleavage reaction. The sensitivity of 1ABC cleavage to nonionic detergent suggests hydrophobic interaction, but in fact, the nature of P1 recognition by 3CD^pro is still obscure.

The amino acid chain of the 3C moiety in 3CD^pro must be folded in a native state to allow proteolysis, whereas the structure of the polymerase contained in the 3CD^pro precursor may be altered such that its catalytic function is prevented. The presence of the 3C polypeptide chain may alternatively block access to the active site or the substrate recognition site of the polymerase. 3CD^proM did not exhibit polymerase activity above the background level, as determined by a [^{3}H]UTP incorporation assay. 3D^pol, however, exhibited activity similar to that of the purified polymerase, demonstrating that the purification procedure does not inactivate polymerase activity. We cannot exclude the possibility that the T181K mutation in 3CD^proM was responsible for the lack of polymerase activity, although this seems unlikely. It is interesting to note the apparent binding of GTP to 3D^pol and, presumably, 3CD^pro. This is supported by unpublished findings that GTP stabilizes 3D^pol (29a) and that it stimulates the catalytic activity of 3D^pol (29).

We conclude that cleavage of 3CD^pro to 3C^pro and 3D^pol is a prerequisite for RNA replication. It has been speculated that this may preferentially occur when the enzyme is complexed, together with a cellular protein factor(s), to the 5' end of the viral RNA (1). Our data indicate, however, that 3CD^pro can be processed to its cleavage products in the absence of 5'-terminal RNA sequences (Fig. 2B, lane 4). Therefore, the significance of the formation of a ribonucleoprotein complex at the 5' end of the viral RNA remains to be elucidated.

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