Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 4002–4006, June 1987
Biochemistry

Poliovirus polypeptide precursors: Expression *in vitro* and processing by exogenous 3C and 2A proteinases

(proteolytic processing)

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Communicated by Michael G. Rossmann, February 17, 1987 (received for review December 10, 1986)

ABSTRACT **Plasmids have been constructed to generate substrates for the study of proteinases 2A and 3C of poliovirus. They contain the P1 (capsomer precursor) region of the poliovirus genome or P1 and part of P2 (a nonstructural precursor), which can be transcribed and translated *in vitro*. A transcript containing the entire 5′ nontranslated region and the P1 region of the viral RNA gave poor translation in a reticulocyte translation system. Truncation of the 5′ nontranslated region to its 3′-most segment gave acceptably good yields of radiolabeled P1. P1 was specifically processed to yield capsomer proteins by enzymes supplied in a postmitochondrial supernatant from poliovirus-infected cells. Thus, proteinase 3C can be supplied exogenously (in *trans*) and effect processing. This system may be used to provide P1 for the assay of proteinase 3C. Precursors that lacked either the 1A or 1D regions were poor substrates for proteinase 3C—observations that demonstrated a stringent structural requirement in processing by 3C. The translation product of a transcript encoding P1 and part of P2 was rapidly cleaved at the P1–P2 site in the absence of infected-cell extract. A transcript that contained a mutated 2A region gave a stable P1–P2 precursor that could be processed specifically by exogenous proteinase from infected-cell fractions. Processing of P1 appeared to require cleavage of the P1–P2 bond. These results support our previous data that 2A is the second polioviral proteinase and also provides a means of assaying proteinase 2A *in vitro*.**

Poliovirus genomic RNA is ≈7.5 kb in length (1) and is not conventionally "capped" but has an oligopeptide, VPg, of viral origin covalently attached to the 5′ end. It has been shown that polysomal poliovirus RNA lacks VPg, and this is the only known difference between poliovirus mRNA and genomic RNA (ref. 2 and references therein).

The RNA of poliovirus type 1 (Mahoney strain) encodes a single continuous reading frame of 2208 codons (1), which is typical of the family Picornaviridae, to which poliovirus belongs. In infected cells the translation product yields mature capsid and noncapsid proteins through proteolytic processing, largely by nonstructural viral protein 3C (3, 4), that cleaves the polyprotein precursor at 9 of 13 glutamine-glycine pairs (ref. 5 and references therein). No proteins other than poliovirus-specific polypeptides are known to serve as substrates for proteinase 3C. Recently, we (6) presented evidence that viral protein 2A is also a proteinase and probably cleaves at two sites, both of which are tyrosine-glycine pairs. Other workers have recently provided genetic evidence suggesting that proteinase 2A can act in *trans in vivo* and, hence, that it is a diffusible protein (7). It has also been speculated (8) that the final maturation cleavage of viral protein VP0 is self-activated. Processing is to some extent cotranslational, insofar as the full-length translation product cannot be found under normal conditions *in vivo* (reviewed in ref. 9).

The 5′ nontranslated regions (NTR) of picornaviral RNAs are exceptionally long (600–1200 nucleotides). In poliovirus, the initiation site for translation is the ninth AUG, at nucleotide 743 in the sequence (10), a result that appears to conflict with the "scanning" model for the initiation of protein synthesis (ref. 11 and references therein). Some RNAs from distant relatives of poliovirus among the Picornaviridae such as encephalomyocarditis virus (EMCV) and foot-and-mouth disease virus are very good messengers in micrococcal nuclease-treated rabbit reticulocyte lysates (RRL) and yield a pattern of protein products that closely resembles those seen *in vivo* in infected cells (12). By contrast, poliovirus RNA is a poor messenger in this system and displays a strong tendency towards initiation at a site in the P3 region of the genome. This has hampered work on proteinase 3C, for which the most obvious substrate is the P1 protein, because both 3C and P1 are translated simultaneously in RRL. Addition of cellular fractions from uninfected HeLa cells can restore a more normal pattern of translation (13), and such extracts must contain factors that either suppress initiation in P3 or enhance initiation in P1 on translation in RRL.

Proteinase 3C of EMCV has long been shown to be capable of cleaving precursors when added to the products of the arrested translation of EMCV RNA, experiments which would be difficult to perform in the case of poliovirus for the reasons already given. More recently proteinase 3C of EMCV has been shown to be an enzyme that may be purified and is stable in storage at low temperature (ref. 14 and references therein). In the absence of an assay, no clear experiments of this sort have been done with poliovirus. To study proteolytic processing in poliovirus, we have found it convenient to construct plasmids that contain segments of the poliovirus genome and express them—i.e., both transcribe and translate them—entirely *in vitro*. In this paper we investigate the choice of the 5′ NTR for more efficient translation of P1 *in vitro* in RRL, and we use P1 as a substrate for exogenous proteinase 3C provided from infected lysate. By extending transcripts from the P1 into the P2 region, we confirm our previous results (ref. 6; where we used expression in *Escherichia coli*) that intact proteinase 2A is required for cleavage of the P1–P2 tyrosine-glycine bond and by the

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Abbreviations: NTR, nontranslated region(s); RRL, rabbit reticulocyte lysate(s); EMCV, encephalomyocarditis virus; BMV, Brome mosaic virus.

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introduction of mutations into the proteinase 2A-encoding region of cloned P1–P2 sequences, we show that 2A also acts in *trans*.

MATERIALS AND METHODS

Construction of Plasmids. Plasmids were isolated, modified, and sequenced by standard procedures (15). The route of construction of most of the poliovirus constructions mentioned will be described elsewhere. All poliovirus sequences were inserted counterclockwise into pBR322 derivatives (A. Rosenberg, J.J.D., and F. W. Studier, unpublished work) that contained the promotor and upstream sequences of gene *10* of phage T7 inserted anticlockwise into the *Bam*HI site. All poliovirus type 1 (Mahoney strain) cDNA was derived from pT7PV1-5 (16) or its precursor, pJB101 (unpublished work). All poliovirus type 3 (Sabin strain) cDNA was taken from the expression plasmids pVS(3)2801, -2813, and -2820 (6).

Preparation of RNA for Translation *in Vitro*. RNA polymerase of phage T7 was purified from a bacterial expression system by a modification (J.J.D., unpublished results) of the published procedure (17).

Plasmids used in this work were digested with *Dra* I, which cleaves the vector at nucleotide 3943 of pBR322 close to the site of insertion of the poliovirus cDNA (*Eco*RI at position 4363 or *Hin*dIII at 29), with the exception of one experiment in which pMN22 was digested with *Nhe* I, which cleaves at nucleotide 2470 in the poliovirus sequence. The transcript of this was designated "pMN22(N)-RNA." RNA was synthesized as described (16) and purified from the reaction mixture by standard procedures. Transcription of pMN25 gave a full-length transcript [3.8 kilobases (kb)] and a premature termination product (0.6 kb) in roughly equivalent molar yields. The former was isolated by centrifugation on a sucrose density gradient.

***In Vitro* Translation.** Translation *in vitro* was performed using RRL purchased from Promega Biotec (Madison, WI) essentially as described by the supplier. Reaction mixtures contained 140 mM K^+ and 1 mM Mg^{2+}. Translation mixtures contained 1 μCi (1 Ci = 37 GBq) of [^{35}S]methionine per ml (1100 Ci/mmol; New England Nuclear). Incorporation of ^{35}S was determined by the standard procedure (18). Brome mosaic virus (BMV) RNA (Promega Biotec) was used as a standard capped message at 10 μg/ml (final concentration) in the translation reaction mixture and typically gave 20% incorporation of added radioisotope after 60 min.

Preparation of HeLa Cell Postmitochondrial (S10) Fractions. Postmitochondrial supernatants (S10) were prepared essentially as described (19). HeLa S3 spinner cells were harvested at 3.5 hr after infection with 250 plaque-forming units per cell of poliovirus type 1 (Mahoney). After homogenization of the cells and centrifugation, the S10 supernatant was adjusted to 10% (vol/vol) glycerol before storage at −60°C. No difference was found between the results obtained with uninfected and mock-infected cells, so uninfected S10 was used in the preparation of the data presented here.

RESULTS

Plasmids Constructed for Expression *in Vitro*. The RNA products of transcription of the plasmids which were constructed for this work are shown in Fig. 1. In plasmids pMN20, pMN22, and pMN25, the 3′ segment of the P1 coding region was modified by the addition of a synthetic linker to place a TGA codon and an *Eco*RI site at its end, which was ligated with the *Eco*RI site of the vector. The sequence of the DNA flanking the *Eco*RI site was determined. The 5′ NTRs were derived from the type 1 Mahoney clone pT7PV1-5 (16), which produces the authentic 5′ NTR of poliovirus mRNA except for the addition of two guanidylate residues to the 5′

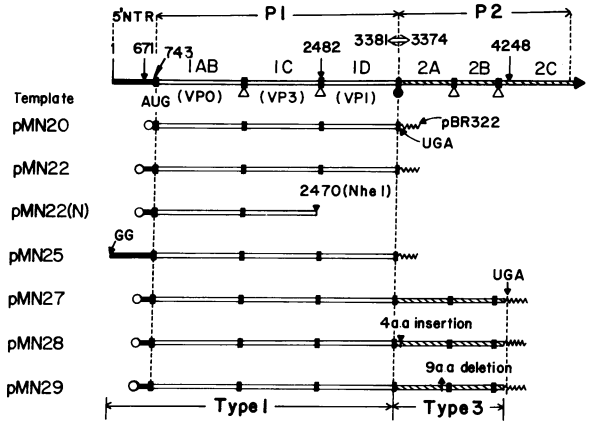

FIG. 1. Map of the sequences contained in the RNAs that are referred to in the paper. The template is indicated on the left. At the top, a segment of a hypothetical hybrid poliovirus genome is represented in which the P2 region (hatched segments) is derived from the type 3 (Sabin) virus and the rest is from type 1 (Mahoney) virus: P1 region (unfilled segment) and 5′ NTR (black segment). Above these segments are indicated the nucleotide numbers that are relevant to the structure and information content of the synthetic RNAs and the map locations of the processed protein products. Indicated below these segments are the traditional designations of the capsomer proteins (in parentheses), the desired initiation codon, and the types of cleavage site: glutamine-glycine (△) and tyrosine-glycine (●). In representations of the transcripts, an open circle represents the short segment of phage T7 sequence. Waved lines represent the segment of the counterclockwise transcript of pBR322 that is included in the sequence. The positions of the desired initiation (AUG) and termination (UGA) codons for protein synthesis are also shown. For clarity, this diagram is not to scale. aa, Amino acid.

terminus. In pMN25, this 5′ NTR was not altered. The 5′ noncoding structure of the RNA transcript from pMN20 was determined by sequencing the template and was predicted to be pppGGGAGACCACAACGGUUUCCCUCUAGU*AUG*-**GGUGCU**. . ., where sequence contributed by the 5′ NTR of T7 gene *10* is underlined, the initiation codon is italic, and the polio P1 sequence is in boldface type.

In pMN22, nucleotides 671–742 of the 5′ NTR of the viral RNA were added downstream of the short T7 sequence shown above. This 5′ NTR was also used in pMN27, -28, and -29.

The P2 segments and the 3′ NTR of pMN27, pMN28, and pMN29 were derived from the Sabin strain type 3 clones described previously (6) and contained all of regions 2A and 2B and 44 codons of 2C, ending at the *Hin*dIII site at nucleotide 4248 of the sequence of the Sabin strain type 3 cDNA. Termination of translation would occur at UGA after translating 17 codons into the pBR322 region, which is included in the transcripts of these plasmids. Poliovirus type 3 (Sabin) and type 1 (Mahoney) cDNAs both contain an *Nde* I site at the junction of their P1 and P2 coding sequences; this site was used to join the P1 region of the Mahoney cDNA to the unmutated P2 segment of pVS(3)2801, to yield pMN27. pMN28 was derived from pVS(3)2813 and contained an insertion of four codons close to the 5′-end of the 2A region, but was otherwise identical to pMN27. Likewise pMN29 was derived from pVS(3)2820 and contained a deletion of a segment coding for nine amino acids close to the 3′ end of 2A, removing the histidine of the putative active site (6).

The sequence of pMN20 differed at position 806 from the published sequence of our stock virus (1) and all other published poliovirus sequences. This mutation, G to A, giving a glycine to serine change in VP4, had no obvious effect on our results and is probably present in all of the constructions used in this work.

Translation of Poliovirus Precursors. Three synthetic RNAs were compared for their efficiency in directing syn-

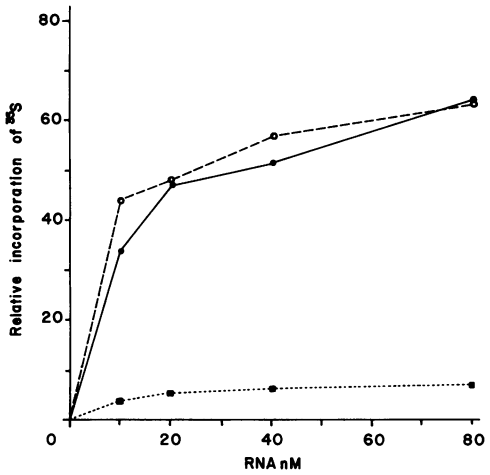

FIG. 2. Graph of [^{35}S]methionine incorporation into alkali-stable, acid-insoluble material after 60 min at 30°C as a function of RNA concentration for synthetic (noncapped) mRNAs with different 5′ NTRs in a cell-free translation system. The positive control, with which each translation mixture was compared, contained 10 μg of BMV RNA per ml, and incorporation of label was set at 100 (arbitrary units). The negative control was not supplemented with RNA, and the amount of radioactivity incorporated in this case was set at 0. All reactions (20 μl) were performed in duplicate; the points given are means. RNAs were pMN20-RNA (○), pMN22-RNA (●), and pMN25-RNA (■).

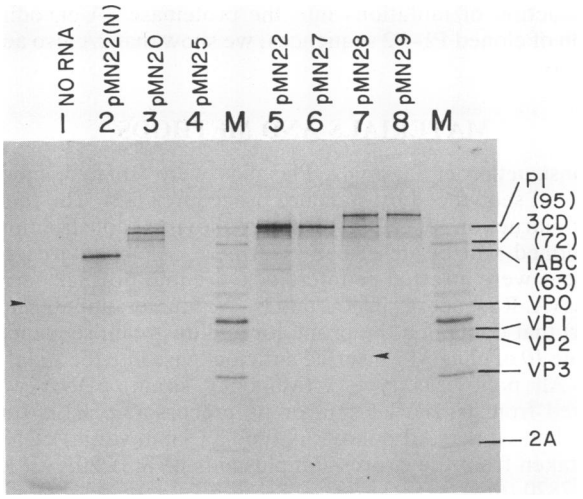

FIG. 3. Products of translation of synthetic RNAs *in vitro*. Samples of translation mixtures (1 μl) were mixed with 0.1 ml of 1% $NaDodSO_4$, and proteins were precipitated with acetone and then resuspended in 50 μl of sample loading buffer (80 mM Tris·HCl, pH 6.8/2% $NaDodSO_4$/25% glycerol); 20 μl was loaded in each lane of a 95 × 1.5 mm 17.5% polyacrylamide gel (15). After stacking at 100 V, gels were run at 200 V. Lanes: 1, no RNA (Note that a protein, indicated by an arrowhead, that has a very similar mobility to VP0 in this system is observed in this lane. This polypeptide has been described previously; see ref. 18); 2, pMN22(N)-RNA; 3, pMN20-RNA; 4, pMN25-RNA; M, marker [cell lysate prepared from cells infected with poliovirus type 1 (Mahoney) in the presence of zinc]; 5, pMN22-RNA; 6, pMN27-RNA; 7, pMN28-RNA (note the faint 28-kDa band indicated by the arrowhead); 8, pMN29-RNA. Gels were impregnated with EN^{3}HANCE (New England Nuclear) according to the manufacturer's instructions and used to expose x-ray film at −50°C for 12 hr. Numbers in parentheses are molecular mass in kDa.

thesis of P1 *in vitro* (Fig. 2) in commercial translation mixtures based on RRL. Total amounts of translation of pMN20 and pMN22 transcripts were comparable (Fig. 2); but in RRL supplemented with pMN20-RNA, a shorter product predominated over full-length P1 (Fig. 3, lane 3). The former polypeptide was immunoprecipitated by anti-VP2 but not anti-VP4 antiserum (see Fig. 5, lanes 1 and 3, respectively), and its apparent molecular mass (≈90 kDa) corresponded with that calculated for 1BCD, the expected product of initiation at the second AUG of this RNA (position 941 of poliovirus). Internal initiation apparently occurred despite the relatively "good" context of the first AUG of the transcript (ANNAUGG) compared with that of the second (CNNAUGC) (11).

pMN22-RNA gave acceptable translation from the first AUG of the message, specifically incorporating 2.7% of the radioisotope added and giving a maximum approximate yield of one translation product per RNA at low RNA concentrations. In this construction as in pMN20-RNA, the desired initiation site is the first AUG in the sequence, but it is now separated by 98 nucleotides from the 5′ terminus and is preceded by 73 nucleotides of authentic viral sequence. Little internal initiation occurs at the second AUG within this message (Fig. 3, lane 5).

By comparison with pMN20- and pMN22-RNAs, which had truncated 5′ NTRs, pMN25, which had the full 5′ NTR of poliovirus, was poorly translated but yielded P1 (Fig. 2 and Fig. 3, lane 4). To study the effect of this message on the activity of the translation system, we compared the efficiency of translation of a capped messenger (10 μg of RNA from BMV per ml) in the presence and absence of the synthetic transcripts, pMN25- and pMN22-RNA. In the presence of 80 nM pMN25-RNA, specific incorporation of ^{35}S was depressed to 28% of the control reaction (containing only BMV RNA) at 15 min. By contrast, under the same conditions, pMN22-RNA gave a 6% stimulation of ^{35}S incorporation compared to BMV alone. In both cases (data not shown), the translation products of the (capped) BMV RNA were predominant.

Thus, pMN22-RNA was found to contain the best 5′ NTR for the translation of P1 *in vitro* for experiments on processing, and this region was subsequently used in the construction of P1–P2 hybrids.

Processing by Exogenous Poliovirus Proteinases. Mixtures of RRL that had been programmed with 40 nM synthetic RNAs were mixed with equivalent volumes of postmitochondrial supernatants (S10) from infected and uninfected cells that had been stored at −60°C. Nonradioactive methionine was added to 0.5 mM before mixing. After incubation for 30 min at 37°C, protein products were analyzed. Reaction mixtures in which pMN22-RNA had been translated yielded products that comigrated with the capsomer proteins VP0, VP1, and VP3 only after incubation with the infected S10 fraction (compare lanes 5 in Fig. 4 *Upper* and *Lower*). These processing products could be precipitated with the appropriate antisera (Fig. 5, lanes 6, 7, and 8), and they comigrated with the proteins of purified procapsids (data not shown). This observation demonstrated that the enzyme responsible (poliovirus proteinase 3C) is stable in crude mixtures to freezing and acts upon substrate that is synthesized separately. One further protein band of ≈60 kDa appeared specifically in reactions containing infected S10 fraction (compare lanes 5 in Fig. 4 *Upper* and *Lower*) and probably represents both 1ABC and 1CD (63 kDa and 60 kDa, respectively), with 1ABC predominating (compare lanes 6, 7, and 8 of Fig. 5).

We attempted to make a simpler substrate for proteinase 3C (containing only one glutamine-glycine site) by linearizing pMN22 with *Nhe* I. The protein product, corresponding to 1ABC lacking only the terminal three amino acids of 1C (Fig. 3, lane 2), was resistant to processing by 3C (lanes 2 in Fig. 4 *Upper* and *Lower*). The translation products of pMN20-RNA could also be processed when mixed with infected S10 (lanes 3 in Fig. 4 *Upper* and *Lower*), but it was the authentic

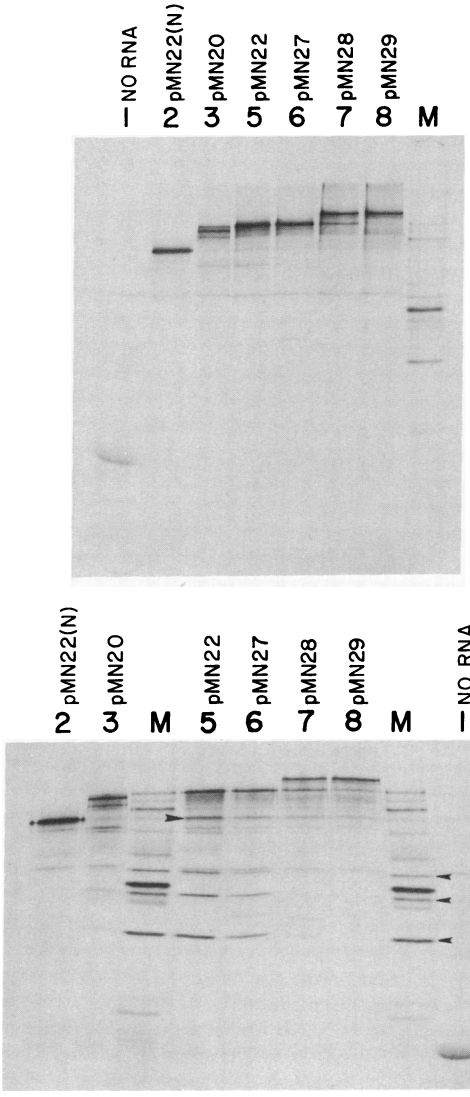

FIG. 4. Effect of incubation of the translation mixtures with uninfected (*Upper*) and infected (*Lower*) S10 fractions. Lanes are labeled as in Fig. 3 according to the messenger added. (*Lower*) The three capsomer proteins VP0, VP1, and VP3 are indicated by arrowhead in the marker (M) lane. Note the band (indicated by an arrowhead) of ≈60 kDa (lanes 5–8). Note also the nonspecific band in lane 1 close to VP0. Proteins were run, the gels were processed as in Fig. 3, and the film was exposed for 24 hr.

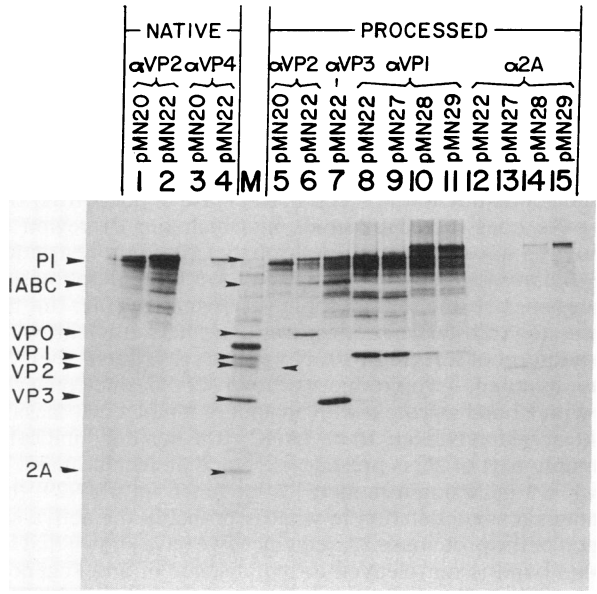

FIG. 5. Immunoprecipitation of proteins from processed and unprocessed translation mixtures. Samples that contained 10 μl of RRL reaction mixture were diluted to 0.1 ml with 1% $NaDodSO_4$/50 mM dithiothreitol/20 mM sodium phosphate, pH 7.0, and heated at 100°C for 5 min before dilution to 1.0 ml with immunoprecipitation buffer (1% Triton X-100/0.1% $NaDodSO_4$/1% sodium deoxycholate/5 mM dithiothreitol/10 mM EDTA/20 mM sodium phosphate, pH 7.0). Aliquots (0.2 ml) were taken for each immunoprecipitation. Fixed *Staphylococcus aureus* was used as the immunoadsorbant. All steps were performed in immunoprecipitation buffer at 22°C for only 30 min. During adsorption and preadsorption steps, mixtures were shaken continuously. After immunoabsorption of proteins with 10 μl of antiserum and ≈2 μl (packed volume) of adsorbant, the adsorbant was washed vigorously with immunoprecipitation buffer (three times), with phosphate-buffered 0.5 M NaCl (pH 7.0)/0.1% Triton X-100 and with 20 mM sodium phosphate (pH 7.0). Adsorbed proteins were released by heating the absorbant at 100°C for 5 min with 40 μl of sample loading buffer, and 20 μl was applied to the gel. The plasmid template (cleaved with *Dra* I) that was expressed *in vitro* is indicated above each lane, as is the specificity of the antiserum used. Lane M is a marker prepared as in Fig. 3. In lane 5, the arrowhead indicates a faint band of approximately the same mobility as VP2. Lane 12 is included as a control. Proteins were run, and the gel was processed as described in Fig. 3. Film was exposed for 48 hr.

P1 band (Fig. 5, lane 3) that was preferentially processed (Fig. 5, lane 5). Some cleavage of the major translation product (1BCD; Fig. 3, lane 3) could be detected by the appearance of a faint band, particularly after immunoprecipitation with anti-VP2 antiserum, that comigrated with VP2 (Fig. 5, lane 5).

P1 (Mahoney)–P2 (Sabin type 3) hybrid RNAs were translated *in vitro*. Three RNAs (Fig. 1) were compared. Translation of the unmutated pMN27-RNA yielded P1 and a small protein of apparent molecular mass 28 kDa (Fig. 3, lane 6). This latter protein could be immunoprecipitated with anti-2A antiserum (Fig. 5, lane 13). On incubation for 30 min with infected S10, the P1 was seen to be processed (as was observed with the translation products of pMN22-RNA) into proteins of the same size as VP0, VP1, and VP3 (Fig. 4 *Lower*, lane 6). No such effect was seen with uninfected S10 (Fig. 4 *Upper*, lane 6). Translation of pMN29-RNA, carrying a deletion at the putative active site of 2A, gave a polypeptide product that was larger than P1 (Fig. 3, lane 8). Upon mixing with infected S10 (Fig. 4 *Lower*, lane 8), this polypeptide yielded P1, the capsid proteins, and a 28-kDa protein that could be immunoprecipitated with anti-2A antibodies (Fig. 5, lane 15). pMN28-RNA (Fig. 1) yielded a polypeptide larger than P1 but also, without addition of infected S10 (lanes 7 in Figs. 3 and 4 *Upper*), a trace of P1, and a protein of slightly greater mobility than that of the 28-kDa protein already observed in translations of pMN27-RNA (lanes 6 in Figs. 3 and 4 *Upper*). Neither the tyrosine-glycine cleavage site itself nor the two putative catalytic residues of 2A were affected in this mutant. On incubation with infected S10, the yield of the 28-kDa protein increased and the capsomer proteins appeared (Fig. 4 *Lower*, lane 7).

In neither mutant was the yield of capsomer proteins, upon treatment of the translation mixtures with infected S10, nearly so high as was seen when the products of pMN22- or pMN27-RNA were processed. Processing of both mutant precursors and immunoprecipitation of the products of processing with anti-VP1 and -2A antisera, yielded no indication that any intermediate product contained both the sequences of 1D and 2A (Fig. 5).

DISCUSSION

The study of the enzymes responsible for processing in poliovirus has been inhibited by the lack of assay systems.

Our objective was to produce viral precursor proteins *in vitro* as substrates for exogenous proteinases 3C and 2A.

We have synthesized RNAs that encode the capsomer precursor alone (P1) and have shown that one of these messengers efficiently directed the synthesis of P1. This product could be processed by addition of proteinase 3C from the postmitochondrial supernatant of infected cells. In extending our study to the second proteinase of poliovirus, 2A, we have constructed plasmids in which the P1 region of poliovirus has been attached through a common restriction site to part of the P2 region of the type 3 (Sabin) poliovirus. Processing of the P1–P2 bond (tyrosine-glycine) in the unmutated translation product was extremely efficient, without addition of infected S10. No precursor is seen when 2A is not mutated. Two products were observed: one comigrating with P1 and a protein with an apparent molecular mass of 28 kDa, which is likely to be $2ABC^{\Delta}$ (the symbol $^{\Delta}$ indicates that only part of 2C is present; expected molecular mass, 32 kDa). By including a mutant P2 sequence (in pMN29) that contains a small deletion in what is probably the active-site region of the proteinase 2A sequence, we have shown that the P1–P2 bond is not cleaved in the absence of an exogenous source of proteinase. The precursor polypeptide, $P1\text{-}2ABC^{\Delta}$, can be processed to yield P1 (which is further processed to the capsomer proteins) and $2ABC^{\Delta}$ by addition of infected S10. It is clear that the proteinase responsible, 2A, can be provided exogenously.

Our results provide methods for assaying both proteinases 3C and 2A, clearly demonstrating the stability of these enzymes to storage at −60°C in crude mixtures and showing their activity on exogenous polioviral precursors.

Our previous observation (6) that unmutated precursors containing uncleaved P1–P2 bonds tend to persist when expressed in *E. coli* is therefore peculiar to that system and may be indicative of the presence of inhibitors or an environment generally unfavorable to the activity of the enzyme. Indeed, we observed that the protein products of the translation of pMN28-RNA (containing an insertion of four codons close to the amino terminus within the 2A segment) underwent partial self-processing in the RRL systems. By contrast, on expression in bacteria, this 2A was effectively inactive.

Two lines of evidence suggest that there are stringent structural requirements for processing by proteinase 3C. First, when precursors containing the uncleaved P1–P2 site were synthesized by translation of the mutant RNA product of pMN29 and were processed by the addition of infected cell extract, no intermediate containing both VP1 and proteinase 2A was detected; moreover, the yield of the capsomer proteins VP0, VP1, and VP3 was reduced in comparison with the products of pMN27-RNA (unmutated). It appears likely, therefore, that the P1–P2 cleavage is necessarily the first step in processing of the polyprotein, even when proteinase 2A is supplied exogenously, and must occur before the glutamine-glycine sites of the capsid can be cleaved. Second, the major translation product of pMN20-RNA lacked almost all of the 1A segment and was only weakly processed by infected S10 to yield a barely detectable protein of mobility similar to VP2. Detection of this product implies that 1A is not essential for, but greatly improves the susceptible presentation of the processing sites between 1B, 1C, and 1D. On the other hand, the protein product of pMN22(N)-RNA, which should lack only the carboxyl-terminal three amino acids of 1ABC, proved completely refractory to processing, although it contains an unaltered glutamine-glycine site. Given the intimate contact between the capsid subunits within the capsomer unit of the virus particle (20), it seems likely that in the absence of the 1A (VP4) or 1D region (VP1), the truncated protein does not fold correctly to expose the scissile glutamine-glycine sites.

Other workers (21) have recently obtained data on the activity of proteinase 3C by a similar approach which has led them to broadly similar conclusions.

For the most efficient translation with correct initiation, a short 5′ region from nucleotides 671–742 was included in the transcript (pMN22-RNA). Inclusion of the full 5′ NTR of poliovirus (in pMN25) vastly reduces translational efficiency in unsupplemented RRL. Furthermore, the purified full-length pMN25-RNA, but not the pMN22 transcript, was shown to inhibit translation of a good capped messenger. However, this effect is unlikely to have any relevance *in vivo*, where shut-off of host protein synthesis has been shown to be specific to capped messengers (in RRL the "viral" message also inhibits its own translation) and correlates with the cleavage of a component of the cap-binding complex p220 (22). These results strengthen the case that some factor deficient in RRL is required to bind and alter the conformation of the 5′ NTR in order to allow translation of the virus from the correct initiation site.

We thank Klaus Wiegers and Rudolf Dernick for providing anti-VP1 antiserum; Richard Jackson, Carl Anderson, and Michael Matthews for their helpful advice; Willy Crockett for technical assistance; and Sylvie van der Werf for suggesting this approach. This work was supported by grants AI15122 and CA28146 from the National Institutes of Health. M.J.H.N. is fellow DRG-848 of the Damon Runyon–Walter Winchell cancer fund; H.G.K. is supported by fellowship Kr 906/1-1 of the Deutsche Forschungsgemeinschaft.

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