Poliovirus Protein 3CD Is the Active Protease for Processing of the Precursor Protein P1 in vitro

By JAN JORE, BERNARD DE GEUS, RICHARD J. JACKSON, PETER H. POUWELS AND BETTY E. ENGER-VALK*

Medical Biological Laboratory TNO, P.O. Box 45, 2280 AA Rijswijk, The Netherlands and
1Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K.

(Accepted 29 March 1988)

SUMMARY

A transcription/translation system for generating poliovirus proteins in vitro has been used to assess the proteolytic activity of various polypeptides containing the virus-coded 3C region towards the poliovirus precursor protein P1. Plasmids containing a phage T7 promoter followed by either the complete poliovirus P1 sequence or various sequences containing the 3C region were used for this purpose. We showed that all except one of the 3C-containing polypeptides had a very restricted activity towards P1, generating only a small amount of VP1 and no VP0 or VP3. The only polypeptide capable of fully processing P1 into VP0, VP3 and VP1 in vitro was protein 3CD, consisting of the complete 3C and 3D sequences.

INTRODUCTION

Poliovirus is a member of the enterovirus subgroup of the picornavirus family. All picornavirus genomes encode a single, large precursor polyprotein, which is processed in a series of proteolytic steps to yield the virion capsid proteins and non-structural proteins (reviewed by Nicklin et al., 1986). In the case of poliovirus, evidence has been presented that protease 2A is responsible for the cleavage of a tyrosine-glycine (Y-G) bond generating the capsid precursor protein P1 (Toyoda et al., 1986; Nicklin et al., 1987). A second viral protease is involved in cleavage of glutamine-glycine (Q-G) bonds, including processing of P1 into the viral capsid proteins VP1, VP3 and VP0 (Hanecak et al., 1982). The final processing event, cleavage of VP0 into VP4 and VP2, may occur by an autocatalytic mechanism, in which viral RNA serves as a proton acceptor (Nicklin et al., 1986).

The second viral protease is usually referred to as protein 3C which is the smallest molecule that contains the sequences required for Q-G cleavage (Hanecak et al., 1982). From expression studies in bacterial cells it can be concluded that a protein of the size of mature 3C is generated from a larger precursor by a proteolytic process requiring the 3C protein sequence (Hanecak et al., 1984; Ivanoff et al., 1986). Nevertheless, although data have been presented indicating that 3C alone is sufficient for cleavage at Q-G sites in the P2 region (Ypma-Wong & Semler, 1987), it has been suggested that processing of P1 may require additional sequences, possibly from the 3B and/or 3D regions (Hanecak et al., 1982; Ypma-Wong & Semler, 1987). However, no direct test has been made to discover which polypeptide containing the 3C region is responsible for efficient processing of P1.

Because of our interest in obtaining poliovirus intermediate structures such as 14S particles and/or procapsids (Rombaut et al., 1983 and references mentioned therein) without the use of infectious poliovirus RNA, we wished to know which 3C-containing polypeptide is required to process protein P1 into the capsid proteins VP0, VP1 and VP3. To address this question, we constructed plasmids containing the phage T7 promoter which encoded either the precursor protein P1 or various 3C-containing proteins. The set of constructed plasmids encoded proteins...
harbouring either 3C sequences only (protein 3C), 3C plus part of 3A and the complete 3B sequences (protein 3A'BC), 3C plus part of 3D (protein 3CD'), 3C and the complete 3D sequences (protein 3CD) or 3C with both 5' and 3' sequences (protein 3A'B2CD'). Some of these 3C-containing proteins possess autocatalytic activity and generate a product that comigrates with protein 3C. However, this autocalytic activity appeared to have no correlation with the capacity of these 3C-containing proteins to process P1 into VP0, VP1 and VP3: protein 3A/BC, like mature protein 3C, generated only a small amount of VP1. On the other hand, protein 3CD' was found to be capable of efficiently processing P1 in vitro into the separate viral capsid proteins.

METHODS

Restriction endonucleases, enzymes and linkers. Restriction endonucleases were purchased from New England Biolabs, Boehringer and Bethesda Research Laboratories. T4 DNA ligase, calf intestinal phosphatase and T4 polynucleotide kinase were purchased from Boehringer. RNasin was obtained from Promega Biotec. Cap analogue [mG(5')ppp(5')G] was purchased from Pharmacia. T7 RNA polymerase was purified from an over-producing clone as has been described by Tabor & Richardson (1985). Synthetic oligonucleotides were purchased from Dr J. V. Boom (University of Leiden, The Netherlands).

Bacterial strains and plasmids. Escherichia coli strain GM48 (dam-3, dcm-6, thi-1, leu-6, thi-1, lacY, galK2, galT22, ara-14, tonA31, tsx-78, supE44) (Marinus, 1973) was used for obtaining plasmid DNA that was digestible with SacI.

Plasmids pVR104 (Racaniello & Baltimore, 1981b), pT7-1 (Ypma-Wong & Semler, 1987) and pT7-6 were gifts of Drs D. Baltimore, B. Semler and S. Tabor, respectively. Plasmid pVR104 comprises the cDNA sequence of poliovirus type 1 Mahoney, starting at nucleotide 114, cloned into the Psrl site of pBR322. Plasmid pT7-1 comprises the entire cDNA sequence of poliovirus type 1 Mahoney inserted into the EcoRI site of pGEM-1 (Promega Biotec). Plasmid pT7-6 (Fig. 1a) contains the T7 promoter region followed by a series of unique cloning sites.

Construction of T7 plasmids

The Pl-encoding vector pLOP324 (Fig. 1a). The HgiAl/NdeI fragment (poliovirus nucleotides 747 to 3381; Racaniello & Baltimore, 1981a), isolated from plasmid pVR104, was provided at the 5' end with the synthetic oligonucleotide 5' AGCTTCCACCATGGGTGCT 3', restoring the original 5' sequence up to the translation initiation codon (ATG) of the P1 coding sequence. The translation initiation codon is preceded by a HindIII site and nucleotides that conform to the optimal context for efficient initiation (Kozak, 1984). The NdeI end of the fragment was provided with a synthetic oligonucleotide 5' TATTAGTAAGTT 3' restoring the 3' end of the P1 coding sequence. The latter linker also provides translational stop codons at the 3' end of the P1 sequence. The fragment thus generated was joined to the HindIII/SmaI fragment at the 3' end of the P1 sequence. The resulting vector was designated pLOP324.

The 3C-encoding vectors pLOP311 to 315 (Fig. 1a, b). The parental plasmid of this series, pLOP311, was constructed as follows. A HindIII/MnlI fragment (poliovirus nucleotides 5240 to 6112; Racaniello & Baltimore, 1981a) was inserted into plasmid pT7-6 which had been digested with HindIII and BamHI. The BamHI site was ligated to the oligonucleotide 5' CACACATAAGGTCTATCTAG 3', providing a translational stop codon. The translation start codon ATG is present within the poliovirus 3A sequence (ATG at nucleotides 5288 to 5299; Racaniello & Baltimore, 1981a). The plasmid thus obtained, pLOP311, codes for a polypeptide which is designated 3A'BC; a schematic diagram of it is shown in Fig. 1(a). This 3C-containing plasmid was used for construction of the various 3C vectors of which the polioirus-specific parts are schematically depicted in Fig. 1(b).

Plasmid pLOP312 comprises the 2.75 kb EcoRV/SstI fragment and the 0.17 kb EcoRV/DdeI fragment from plasmid pLOP311; the DdeI end was joined to the SstI end using an oligonucleotide linker with the sequence 5' TCAcGAGTCTATATAAGTAacGAGCT 3'. The polylinker sequence joined the translational stop codons to the C-terminal codon of the 3C sequence. The translation initiation codon is the same as in plasmid pLOP311. Plasmid pLOP312 codes for a polypeptide which is designated 3A'B2CD'.

Plasmid pLOP313 was obtained by ligation of the 3A'B2CD' fragment from pLOP311 (obtaining the 5' part of 3C) and the EcoRV/PstI fragment (containing the 3' part of 3C and the pT7-6 vector) of plasmid pLOP312; a PstI/SalI6I linker 5' AGCTCAGTCTATCTAGCT 3', containing a translation initiation codon ATG in an optimal context (Kozak, 1984), was used for joining the fragments at the PstI site. The resulting vector contains 3C sequences only, preceded by a translational start codon and therefore coded for a protein referred to as 3C.

Plasmid pLOP314 comprises the 2.5 kb EcoRV/PstI fragment of plasmid pLOP311 (obtained by partial EcoRV and complete PstI digestion) and the 0.37 kb EcoRV/PstI fragment from plasmid pLOP313. The polypeptide encoded by this plasmid is designated 3CD'.

Plasmid pLOP315 was obtained by ligating the large BglII/SstI fragment from plasmid pLOP313 to the small BglII/SstI fragment from plasmid pT7-1. This plasmid encodes a polypeptide which is denoted 3CD'.

Plasmid pLOP31524 comprises the 2.75 kb EcoRV/SstI fragment from plasmid pLOP311 (obtaining the 5' part of 3C) and the EcoRV/PstI fragment (containing the 3' part of 3C and the pT7-6 vector) of plasmid pLOP312; a PstI/SalI6I linker 5' AGCTCAGTCTATCTAGCT 3', containing a translation initiation codon ATG in an optimal context (Kozak, 1984), was used for joining the fragments at the PstI site. The resulting vector contains 3C sequences only, preceded by a translational start codon and therefore coded for a protein referred to as 3C.
Fig. 1. Schematic presentation of transcription vectors. The plasmid tetracycline and ampicillin resistance genes are marked *tet* and *amp*, the origin of replication *ori*. (a) Parental vectors and strategy for construction of vectors pLOP324 and pLOP311 and (b) diagram of the poliovirus-specific inserts in vectors pLOP312 to -315. Only relevant cleavage sites for restriction enzymes are shown. Non-unique cleavage sites are marked by an asterisk. Open, shaded, and hatched boxes represent poliovirus sequences P1, 3C and other poliovirus sequences respectively.

Plasmid P1-P2-3ABC, a derivative of plasmid pT7-1 which is lacking the 3D sequence, was obtained by exchanging the 3CD-containing *Bgl*II/*Sst*I fragment of pT7-1 for that of the *Bgl*II/*Sst*I fragment of plasmid pLOP313 which harbours the 3C sequence including the translation stop codon.

In *vitro* transcription with *T7* polymerase. Prior to transcription, the DNA template was linearized with an appropriate restriction enzyme, extracted with phenol, precipitated with ethanol and dissolved in sterile double-distilled water to a concentration of 0-5 μg DNA/μl. Transcription reactions with T7 RNA polymerase were carried out essentially as described by Verver *et al.* (1987). Briefly, 2-5 μg DNA was transcribed in a reaction mixture (25 μl) containing 40 mM-Tris-HCl pH 8.0, 15 mM-MgCl₂, 10 mM-dithiothreitol (DTT), 1 mM each of ATP, CTP and UTP, 0-2 mM-GTP and 1 mM-m⁷G(5')ppp(5')G to which 100 μg/ml bovine serum albumin, 25 to 50 units T7 RNA polymerase and 20 to 40 units RNasin were added. The GTP concentration was increased to 0-6 mM after 5 min incubation at 37 °C and subsequently to 1 mM, again after 5 min incubation. The incubation was stopped after 20 min at 37 °C by extraction with phenol and precipitation with ethanol. A sample of the RNA thus obtained was glyoxylated and electrophoresed on an agarose gel to check both the size and the yield. The RNA was used in translation experiments without prior DNase treatment.
In vitro translation of transcripts. Messenger RNA-dependent rabbit reticulocyte lysate was prepared according to previously published procedures (Jackson & Hunt, 1983). Conditions for protein synthesis were, excluding the contribution of the lysate, 10 mM-creatine phosphate, 0.1 mM-amino acids (no methionine), 80 mM-KCl, 0.4 mM-MgCl₂, 10 mM-DTT and, if a labelled product was required, [³⁵S]methionine (10 μCi/25 μl reaction mixture). Optimal concentrations of RNA, defined as the concentrations showing a high yield of the authentic product and minimal relative yield of aberrant products (Dorner et al., 1984), were determined by translation of a range of serial RNA dilutions in reaction mixtures containing [³⁵S]methionine and analysis of protein patterns on polyacrylamide–SDS gels. If unlabelled proteins were required, translation was checked by separately incubating a small sample of the reaction mixture in the presence of [³⁵S]methionine. In order to ensure faithful initiation of translation, protein synthesis with viral RNA was performed in the presence of uninfected HeLa cell extracts (Dorner et al., 1984). Translation reactions were carried out at 30 °C for time periods calculated to be sufficient for full-length translation, that is from 20 min for pLOP313 RNA to 75 min for viral RNA. In order to analyse polypeptide processing, samples from the translation reactions were supplemented with cycloheximide and RNase A to final concentrations of 0.5 mM and 10 μg/ml, respectively. These preparations were then incubated at 30 °C for the indicated periods (see legends to figures). Finally, all samples were diluted 1:1 by addition of 100 μg/ml RNase A in 10 mM-EDTA and incubated for 20 min at room temperature.

Analysis of the products synthesized was performed by subjecting the mixtures to electrophoresis in 15% polyacrylamide–SDS gels. Before electrophoresis, the translation mixtures were diluted with Laemmli sample buffer (Laemmli, 1970) and boiled for 2 min. After electrophoresis the gels were fixed in 7% acetic acid, soaked in Amplify (Amersham), dried and exposed to preflashed Fuji RX film at −70 °C.

RESULTS

Generation of defined RNAs by in vitro transcription of various DNAs

In order to synthesize defined transcripts of poliovirus cDNA that can subsequently be used to programme an in vitro translation system, we have cloned various DNA fragments downstream of a T7 promoter region. Plasmid pLOP324, the P1 vector, contains the poliovirus coding sequence P1 from nucleotides 743 to 3386 (Racaniello & Baltimore, 1981 a), preceded at the 5' side by nucleotides that conform to the Kozak rule (Kozak, 1984) and which are expected to be required for efficient translation. The ATG codon proximal to the promoter is the authentic poliovirus precursor start codon. At the 3' end, translation stop codons were introduced allowing the synthesis of precursor P1 without the requirement for proteolytic processing by protease 2A.

The 3C plasmids pLOP311 and pLOP312 both contain the 3B and 3C coding sequences as well as part of the 3A sequences (pLOP312), or parts of both 3A and 3D sequences (pLOP311). In both cases, protein synthesis can start at an in-phase translation initiation codon present in the 3A coding sequence (Racaniello & Baltimore, 1981 a). Experiments with virion RNA in vitro showed that initiation of translation occurred efficiently in the P3 region (Dorner et al., 1984). The other 3C plasmids all contain the 3C coding sequences, with either no additional sequences (pLOP313), part of the 3D sequences (pLOP314), or the whole 3D sequence together with the complete 3' non-translated region (pLOP315). In these plasmids, protein synthesis starts at an ATG codon introduced directly upstream of the 3C coding sequence using a synthetic oligonucleotide that supplies the optimal context for initiation (Kozak, 1984).

On in vitro transcription, all plasmids yielded RNAs of the expected size with similar efficiencies (data not shown).

In vitro translation of P1 RNA and cleavage by a virion RNA-directed lysate

Run-off transcripts of plasmid pLOP324 were translated in vitro with rabbit reticulocyte lysate in the presence of [³⁵S]methionine. Optimal concentrations of RNA, determined from a serial dilution range, provided synthesis of a specific protein of the expected size (Fig. 2, lanes 2 and 3). In order to assess whether this plasmid-coded P1 has properties similar to those of the natural capsid precursor protein P1, that is, whether it can be processed to the viral capsid proteins VP0, VP3 and VP1, the protein P1 was subjected to processing by virion RNA-encoded proteases. To this end, a sample of the in vitro translated ³⁵S-labelled P1 preparation was mixed with an equal volume of an unlabelled translation mixture which had been incubated with virion RNA. In a separate in vitro translation experiment in the presence of [³⁵S]methionine (Fig. 2, lane 1) this
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Fig. 2. Analysis of processing of in vitro synthesized pLOP324-P1 protein by virion RNA-encoded proteins. Translation reactions were carried out as described in Methods. After translation cycloheximide and RNase were added. Incubation was resumed and samples, taken at various time points and subsequently incubated with RNase, were added to Laemmli sample buffer. Samples taken at time points 0 min (lanes 2 and 4), 15 min (lane 5) and 80 min (lanes 3 and 6) were subjected to electrophoresis. The reactions were programmed as follows: lane 1, virion RNA; lanes 2 to 6, pLOP324 RNA. Lanes 2 and 3 received 10 μl control lysate; lanes 4, 5 and 6 received 10 μl virion RNA-coded lysate. Control lysate contains no added RNAs.

Fig. 3. Analysis of autocatalytic activity of 3C-containing polypeptides. Translation reactions were carried out and stopped as described in Methods and in legend to Fig. 2. Samples taken at time points 15 min (lanes 1, 3, 6 and 8) and 80 min (lanes 2, 4, 5, 7 and 9) were subjected to electrophoresis. The reactions were programmed as follows: lanes 1 and 2, pLOP311 RNA; lanes 3 and 4, pLOP312 RNA; lane 5, pLOP313 RNA; lanes 6 and 7, pLOP314 RNA; lanes 8 and 9, pLOP315 RNA. The Mr values (×10^-3) on the left side refer to protein markers. The arrowhead indicates the position of mature protein 3C.

virion RNA was shown to direct synthesis of the viral capsid proteins VP0, VP1 and VP3, indicating that the virion RNA-directed lysate contained proteins that were capable of proteolytic processing of P1 synthesized in vitro. From the data in Fig. 2 it can be concluded that the plasmid-encoded protein P1 is cleaved quite efficiently into proteins with molecular weights corresponding to those of the viral capsid proteins only upon addition of the virion RNA-directed lysate.
Fig. 4. Analysis of processing activity of proteins 3A'BC and 3C towards P1 encoded by pLOP324. Translation reactions and subsequent incubations were carried out as described in Methods and in legend to Fig. 2. Samples taken at time points 0 min (lanes 3 and 5) and 80 min (lanes 2, 4 and 6) were subjected to electrophoresis. The reactions were programmed as follows: lane 1, virion RNA; lanes 2, 3, 4, 5 and 6, pLOP324 RNA. Lane 2 received an equal volume of control lysate (no added RNA); lanes 3 and 4 received protein 3A'BC (pLOP312); lanes 5 and 6 received protein 3C (pLOP313). It should be mentioned that the fluorogram (except lane 1) has been exposed for a prolonged period to allow detection of protein VP1.

Fig. 5. Analysis of processing activity of proteins 3A'BC and 3C towards P1 encoded by pT7-1. Translation reactions and subsequent incubations were carried out as described in Methods and in legend to Fig. 2. Samples taken at time points 0 min (lanes 3 and 5) and 80 min (lanes 2, 4 and 6) were subjected to electrophoresis. The reactions were programmed as follows: lane 1, virion RNA; lanes 2, 3, 4, 5 and 6, pT7-1 RNA. Lane 2 received an equal volume of control lysate (no added RNA); lanes 3 and 4 received protein 3A'BC (pLOP312); lanes 5 and 6 received protein 3C (plasmid pLOP313). Plasmid pT7-1 was digested with BgII before use as template for RNA synthesis.

Fig. 6. Analysis of processing activity of protein 3CD towards P1 encoded by pLOP324. Translation reactions and subsequent incubations were carried out as described in Methods and in legend to Fig. 2. Samples taken at time points 15 min (lane 4) and 80 min incubation (lanes 2, 3 and 5) were subjected to electrophoresis. The reactions were programmed as follows: lane 1, virion RNA; lanes 2, 3, 4 and 5, pLOP324 RNA. Lane 2 received an equal volume of control lysate (no added RNA); lane 3 received protein 3C (pLOP313); lanes 4 and 5 received protein 3CD (pLOP315). It should be noted that, in contrast to the results shown in Fig. 4, protein VP1 is barely detectable in lane 3, because of the much shorter exposure time of the fluorogram.

In vitro translation and autocatalytic cleavage of various 3C-containing polypeptides

Transcripts of plasmids pLOP311, -312, -313, -314 and -315 were translated in a rabbit reticulocyte lysate in the presence of [$^{35}$S]methionine. The data in Fig. 3 show that all transcripts directed the efficient synthesis of proteins of the expected molecular weights. In the case of pLOP311 (protein 3A'BCD') and pLOP312 (protein 3A'BC) a smaller product was also observed; on prolonged incubation the yield of the smaller protein increased and that of full-length product decreased. In the case of pLOP312 (protein 3A'BC) the smaller processed product comigrated with the protein 3C encoded by pLOP313 (Fig. 3). On prolonged incubation (overnight) of the 3A'BCD' preparation some 3C could also be detected (data not shown).
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However, only very low amounts of 3C were generated, making the data irreproducible. No protein of the size of mature 3C could be detected in pLOP314 (protein 3CD') and pLOP315 (protein 3CD) preparations.

Cleavage of precursor protein P1 by the various 3C-containing polypeptides

To determine the proteolytic activity of the 3C-containing polypeptides towards the P1 molecule, in vitro translated 35S-labelled P1 was incubated with in vitro prepared unlabelled 3C-containing proteins encoded by the plasmids pLOP312 and pLOP313. The data in Fig. 4 (lanes 4 and 6) show that incubation of P1 with proteins 3A'BC or 3C generated a very low amount of VP1. This processing seemed to be very inefficient and the further cleavage products of P1, that is VP0 and VP3, could not be detected even on prolonged exposure of the fluorogram. In order to check the correctness of the active site (C-terminal portion; Hanecak et al., 1984; Ivanoff et al., 1986) of the 3C encoded by pLOP313 (and the related plasmids pLOP311, -312 and -314), plasmid P1-P2-3ABC was constructed. In vitro translation of the RNA from this plasmid (data not shown) indicated a processing activity similar to that of the pT7-1 RNA (Ypma-Wong & Semler, 1987), that is only traces of the processed products VP1 and high yields of the proteins 2A, 2C and 2BC. This demonstrated that the 3C entity from the plasmid pLOP313 and its derivatives harbours the correct poliovirus sequence.

To assess whether the presence of other poliovirus proteins might render these 3C-containing proteins more active, processing of the precursor protein P1 was assayed in the presence of the products from the poliovirus P2 region. Plasmid pT7-1, linearized at a restriction site (BglII) in the 3C sequence, has been found to be a suitable template for such a study. In vitro translation with this truncated template gave proteins from the P1 and the P2 region, but without the appearance of processing at the Q-G bonds in the P1 region (Ypma-Wong & Semler, 1987; Fig. 5, lane 2). This pT7-1-encoded 35S-labelled protein preparation was mixed with the in vitro synthesized proteins 3A'BC (pLOP312) or 3C (pLOP313). The results in Fig. 5 (lanes 3 to 6) showed that similarly to the experiments with P1 encoded by plasmid pLOP324, VP1 could be detected, but no VP0 or VP3. These data show that the 3C proteins are equally inefficient at processing P1 generated from a longer precursor protein (P1-P2), and that they are not activated by P2-encoded proteins.

Since the other 3C-containing proteins 3A'BCD' and 3CD' showed essentially similar inefficient processing activities (data not shown), it is likely that poliovirus sequences other than the P2 or 3AB sequences are required for cleavage of the Q-G bonds in the precursor protein P1. As the 3D protein sequence downstream of nucleotide 6112 was the only sequence not yet included in our study, plasmid pLOP315 was constructed and used for synthesis of the longest form of the 3C protein, that is protein 3CD. The data in Fig. 6 (lanes 4 and 5) clearly show that this form of the 3C protein efficiently generates all three capsid proteins, that is VP0, VP1 and VP3, in proportions similar to those obtained with virion RNA translated in vitro.

DISCUSSION

Formation of picornavirus virions is dependent on proper proteolytic processing of a polyprotein to yield mature structural and non-structural proteins. In the case of encephalomyocarditis virus proteolytic activity towards P1 has been unequivocally assigned to the 3C protein (see Parks et al., 1986 and references mentioned therein). In contrast, data for poliovirus suggest that whereas 3C is required for proteolytic processing of P1 (Hanecak et al., 1982) this sequence in itself is not sufficient (Ypma-Wong & Semler, 1987). The present study using in vitro transcription and translation of defined poliovirus sequences has been carried out to investigate which 3C-containing protein is necessary for proper processing of P1. To exclude the influence of other poliovirus proteins we constructed a vector, pLOP324, whose run-off transcripts yield only P1 amino acid sequences. This P1 preparation was used as a substrate for analysing proteolytic activity of the various 3C-containing polypeptides described in this paper.

Our study with the various 3C-encoding templates shows that 3C and 3C-containing polypeptides do have proteolytic activity in vitro for a number of Q-G bonds in the poliovirus precursor polyprotein. As deduced from the data in Fig. 3, a protein of the size of mature 3C is
formed with template pLOP312 encoding protein 3A'BC. This indicates that the B–C junction is prone to autocatalytic cleavage. In addition, the appearance of a low amount of mature 3C with template pLOP311, encoding protein 3A'BCD', indicates that some proteolytic cleavage of the C–D junction also takes place, although less efficiently than at the B–C junction. The requirement for two cleavages might render formation of mature 3C from protein 3A'BCD' less efficient. Alternatively, the low amount of mature 3C obtained with the latter template might indicate that the C–D junction is less susceptible to cleavage than the B–C junction.

Finally, these data might be explained by assuming inhibition or even prevention of C–D cleavage once the B–C junction has been cleaved. The absence of detectable amounts of mature protein 3C with templates pLOP314 (encoding protein 3CD') and pLOP315 (encoding protein 3CD) favours the latter hypothesis. The high yield of protein 3CD and very low yield of protein 3C on translation of viral RNA both in vivo and in vitro (Ypma-Wong & Semler, 1987) might also result from this impaired C–D cleavage.

In addition to autocatalytic activity, the 3C-containing polypeptides, except protein 3CD, also possess a proteolytic activity towards the precursor protein P1. However, in contrast to the protein patterns of poliovirus RNA translated in vivo (Dorner et al., 1984) and in vitro (Ypma-Wong & Semler, 1987), and to those of P1 processed in vitro with a preparation of virion RNA-encoded protease (Fig. 4), only small amounts of protein VP1 are formed. Even after prolonged incubations of the protein P1 with the various 3C-containing preparations and after long exposure of the fluorogram no VP0 and/or VP3 can be detected. Taking into account that protein VP1 contains fewer methionine residues than VP0 and VP3 it is to be expected that, under normal processing conditions, VP0 and VP3 would certainly be detectable.

These results may suggest the possibility that the Q–G cleavage sites in the precursor protein P1 are not accessible. However, the results with a protease preparation encoded by virion RNA show that the protein P1 synthesized with template pLOP324 can be processed normally (Fig. 2). Moreover, the results with plasmid pT7-1 indicate that similar processing patterns were obtained with another P1 substrate. Therefore, incorrect folding of the precursor protein P1 encoded by pLOP324 and a consequent inaccessibility of the Q–G bond between VP2 and VP3 cannot explain the incomplete processing.

Instability of protein 3C as suggested by Thomas et al. (1983) might possibly be responsible for the observed partial proteolysis. However, co-translation of the various 3C-containing proteins in vitro in the presence of protein P1 does not alter processing of P1 (data not shown). In addition, the protease activity in in vitro translated virion RNA-encoded preparations appears to be stable enough to be used as a source of the proteolytic activity.

On the other hand, the 3C-containing proteins encoded by plasmids pLOP311 to -314 might show an abnormal proteolytic activity. However, the low proteolytic activities of our 3C-containing proteins appear to be similar to that of partially purified 3C isolated from bacteria (E. Wimmer, personal communication) indicating that 3C proteins from different origins have similar activities. It therefore seems legitimate to conclude that in vitro synthesized mature 3C or the 3C-containing proteins encoded by plasmids pLOP311 to -314 are not capable of efficiently cleaving the Q–G bonds in the P1 sequence. The data obtained using template pT7-1 (Fig. 5) show that P2-encoded poliovirus proteins are not involved in rendering protein 3C more active towards Q–G bonds in the precursor protein P1. Therefore, it should be concluded that another 3C-containing protein is the active protease for cleavage of the precursor protein P1.

Our data clearly show that the only polypeptide capable of fully processing P1 in vitro is protein 3CD. The absence of detectable amounts of mature 3C both before (Fig. 3, lanes 8 and 9) and after (data not shown) in vitro processing of P1 by protein 3CD suggest that 3CD itself rather than 3C is the active form of the protease for processing P1 into VP0, VP3 and VP1. This argument is supported by the fact that in vitro translation of virion RNA gives an activity that processes P1, and a high yield of 3CD but undetectable amounts of 3C (Ypma-Wong & Semler, 1987). Whether the presence of the 3D sequence has an indirect effect, such as conversion of the 3C sequence into a more optimal conformation, or a direct effect by being itself involved in proteolytic processing, cannot be determined from the present study. Further experiments with these or other 3C-containing proteins should reveal clues to the answers of these questions. The
in vitro approach used in this study is useful for further research on the influence of other genomic sequences on proteolysis in poliovirus.

It remains an open question whether 3CD is also the active protease for cleavage of the Q-G bonds in the precursor protein P1 in vivo in infected cells. However, one might argue that formation of capsid proteins can occur quickly if only one processing step (B-C junction) is required to obtain a protease which is active towards the precursor protein P1. Assuming efficient and fast autocatalysis of this bond, as might be indicated by data obtained with protein 3A'BC (Fig. 3), processing of Q-G bonds in the P1 region might take place before any processing in the P2 region.

In conclusion, this study shows that both the P1 and P3–CD regions of the poliovirus genome are required for formation of the separate capsid proteins VP0, VP1 and VP3 such as are present in empty viral capsids (Rombaut et al., 1983), opening the possibility of studying the synthesis of these structures without the use of the complete viral genome.

We thank J. M. A. Verbakel for helpful discussions, Dr E. Wimmer for providing virion RNA and Drs B. Semler and M. F. Ypma-Wong for the generous gift of plasmid pT7-1. We also thank Dr P. Vos for providing details for in vitro transcription before publication. This work was supported by a short-term grant from the Wellcome Foundation in the U.K.

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(Received 16 February 1988)