In vitro molecular genetics as a tool for determining the differential cleavage specificities of the poliovirus 3C proteinase

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ABSTRACT

We describe a completely in vitro system for generating defined poliovirus protein-ase mutations and subsequently assaying the phenotypic expression of such mutations. A complete cDNA copy of the entire poliovirus genome has been inserted into a bacterio-phage T7 transcription vector. We have introduced proteinase and/or cleavage site mutations into this cDNA. Mutant RNA is transcribed from the altered cDNA template and is subsequently translated in vitro. Employing such a system, we provide direct evidence for the bimolecular cleavage events carried out by the 3C proteinase. We show that specific genetically-altered precursor polypeptides containing authentic Q-G cleavage sites will not act as substrates for 3C either in cis or in trans. We also provide evidence that almost the entire P3 region is required to generate 3C proteinase activity capable of cleaving the P1 precursor to capsid proteins. However, only the 3C portion of P3 is required to generate 3C proteinase activity capable of cleaving P2 and its process-ing products.

INTRODUCTION

Picornavirus RNA is translated into a precursor polypeptide which must be com-pletely processed to generate functional proteins. For poliovirus, a member of the Picornaviridae, translation of viral RNA (plus stranded, messenger polarity) in infected cells or in vitro in cell-free extracts produces two known viral proteinases. These pro-teinases, called 2A and 3C, are responsible for nearly all protein processing of polio precursor polypeptides. Protein 2A was recently identified as the enzyme that carries out cleavage at tyrosine–glycine (Y–G) sites in polio proteins (1) and protein 3C has been shown to carry out cleavage at glutamine–glycine (Q–G) bonds (2). The production of the mature 3C proteinase is thought to occur by an autocatalytic mechanism (3, 4) and some data has been reported in support of such a mechanism. However, no clear demonstra-tion of 3C cleavage in trans has been reported. In addition, it has not been determined if 3C, the smallest processing product containing the Q-G-specific proteinase sequences, is responsible for all cleavages at Q-G bonds or if precursors to 3C are responsible for a portion of these cleavages. The overlapping format of the poliovirus genome complicates efforts to study in vivo the effect of a mutation introduced into the polio genome. Because of the precursor-to-product relationship, one mutation introduced into a specific site has the potential of altering several gene products.

The system we have developed for in vitro assays of poliovirus proteinase mutants takes advantage of infectious cDNA clones of polio genomic RNA (5, 6, 7). Plasmid DNA from these clones is capable of producing infectious virus following transfection of primate cells in culture. The virus recovered from such transfection experiments appears to be identical to the original virus stock employed to generate the RNA templates used in cDNA cloning (6, 7, 8). Thus, the DNA copies of polio RNA retain all of the biological properties required for initiating a productive infection and offer the advantage of genetic manipulation of the polio genome. In order to synthesize defined transcripts of polio RNA, wild-type or altered cDNA clones of poliovirus were inserted into a vector containing the T7 promoter. The T7 RNA polymerase was used to synthesize polio specific RNA from the cDNA templates. The in vitro-synthesized RNA was subsequently assayed in an in vitro translation reaction for the production of polio-specific proteins. The translation and processing of the wild-type and altered polio precursor polypeptides was then analyzed using polyacrylamide gel electrophoresis. By employing both an in vitro transcription system and an in vitro translation system, no infectivity of the cDNA clones or the RNA derived from these clones was required. The two in vitro systems allow analysis of poliovirus translation and proteolytic processing independently from other viral processes.

Here we describe the use of the above in vitro system for generating defined poliovirus proteinase mutations and subsequently assaying the phenotypic expression of such mutations. We provide direct evidence for several bimolecular (trans) cleavage events carried out by the poliovirus 3C proteinase. We also show that specific genetically-altered precursor polypeptides containing authentic Q-G cleavage sites will not act as substrates for 3C either in cis or in trans. In addition, we demonstrate that truncated P3 polypeptides containing the complete 3C sequence produced proteins that are only capable of cleaving a limited set of Q-G cleavage sites. Most, if not all, of the P3 precursor appears to be required to generate a proteinase capable of cleaving the P1 precursor to the capsid proteins. In contrast, only the 3C portion of the P3 precursor is required to produce proteinase capable of generating the P2 precursor polypeptide and P2-derived cleavage products.

MATERIALS AND METHODS
Restriction endonucleases and enzymes
Restriction endonucleases, T4 ligase and DNA polymerase I (Klenow fragment) were purchased from New England Biolabs and Boehringer Mannheim. T7 RNA polymerase was purchased from Pharmacia. RNase-free DNase was purchased from Worthington. RNasin was purchased from Promega Biotec.

Cells and Viruses

HeLa S3 cells in suspension were used for cell extract preparation. HeLa cells were infected with poliovirus type I (PVI), Mahoney strain, for infected cell extract preparation (9). Rabbit reticulocyte lysate was purchased from Bethesda Research Labs.

Construction of the general transcription vector

pT7-14 contains the entire poliovirus genome inserted into the pGEM-1 cloning vector (Promega Biotech). This plasmid was constructed by cloning the full-length polio insert from an infectious cDNA clone, PEV104, (6) into the plasmid pGEM-1. The polio cDNA was removed from the vector sequences with restriction endonuclease Eco RI and inserted into the Eco RI site of the polylinker. In the original construction of the 5' end of the poliovirus genome, dG tailing was employed. This resulted in 18 dG residues between the 5' Eco RI site and polio nucleotide 1. Thus the final RNA transcript would be expected to contain 32 additional residues attached to the 5' end. The infectivity of in vitro synthesized polio RNA is increased by reducing the additional 5' sequences of the RNA transcript (10; unpublished observations). Therefore, the majority of the 5' dG tail of the cDNA clone was removed to produce the plasmid pT7-1 (see below).

pT7-1 also contains the entire poliovirus genome inserted into the pGEM-1 cloning vector. However, the 5' additional dG residues have been reduced to 3 from the original 18 dG residues in pT7-14. pT7-1 was constructed by digesting pPV20 with Eco RI to remove the polio cDNA from the vector sequences. pPV20 contains a full-length, infectious cDNA copy of the polio genome and results from the insertion of the cDNA into pNT4 at the Eco RI site (6). After minimal Bal 31 digestion at the 5' and 3' ends of the cDNA, the fragments were made blunt using the Klenow fragment of DNA polymerase I. Eco RI linkers were attached using T4 ligase. Digestion of the DNA with Eco RI and Hind III and subsequent cloning of this population of fragments into vector pNT4 yielded a population of clones (pNT5'-Bal31) with undefined 5' polio sequences. The extent of Bal31 5' removal was determined by digestion with Bgl I; the removal of the Bgl I site at polio 35 would indicate excessive Bal31 digestion. The clones which contained at least polio 35, as determined by the presence of the Bgl I site, were sequenced by the Maxam-Gilbert method (11). One clone was identified (data not shown) that carried a plasmid with only 3 dG residues between the Eco RI linker and polio nucleotide 1. Thus, we had removed 15 non-viral nucleotides upstream of the 5' end of the polio coding sequence. The pNT5'-Bal31 clone with only 3 dG residues was digested with Eco RI and Bgl II to yield a 5.6 kb fragment containing the 5' polio sequences. pT7-14 was digested partially with Eco RI and to completion with Bgl II. The 4.8 kb fragment containing the T7 promoter and polio 3' sequences was ligated to the 5.6 kb fragment using T4 ligase. This plasmid construct, pT7-1, represents the template for in vitro synthesis of wild type (unmodi-

fied) polio RNA as well as the vector into which DNA fragments containing either cleavage site or proteinase mutations were inserted.

Construction of pT7 templates containing altered cDNAs of poliovirus

pT7-1 (SacI:3C) contains a 4 amino acid insertion (Ile-Arg-Ala-Arg) in the amino-terminal end of the 3C proteinase. This plasmid was constructed by inserting a Sac I linker into the Bgl II site at 5601 (4).

pT7-1 (Δ BstEII) lacks the Y-G cleavage site between the P1 and P2 regions, a portion of VP1 and all of the 2A proteinase. This plasmid is the result of deleting the Bst EII fragment from 3235-3925. The original construction of the 690 nucleotide deletion has been described (6).

pT7-1 (BK:3C) contains a 4 base pair insertion in the 3C coding region. The shift in the reading frame creates a stop codon 45 nucleotides downstream from the insertion site such that a truncated 3C protein is produced. This plasmid was constructed by linearizing pT7-1 with Bgl II at 5601, treating the linearized plasmid with DNA polymerase I (Klenow fragment) and religating the plasmid with T4 ligase.

pT7-1 (lin BglII), pT7-1(lin HindIII), pT7-1(lin AccI), and pT7-1(lin PvuII) are transcription templates resulting from linearizing the pT7-1 vector with the respective restriction endonuclease. Linearization of pT7-1 with Bgl II results in a template which lacks the carboxy-terminal sequences of the coding region for 3C. Linearization of pT7-1 with HindIII, AccI, and PvuII results in a template containing the entire coding region of the 3C proteinase as well as additional downstream sequences (refer to Fig. 6).

In vitro transcription with T7 RNA polymerase

Prior to transcription the DNA template was linearized with the appropriate restriction endonuclease. Transcription reactions were carried out as described (12) using T7 RNA polymerase and subsequently treated with RNase-free DNase. To synthesize labeled RNA in vitro, 100 μ Ci/ml α-^{32}P-GTP (Amersham) was included in the transcription reaction. Reaction mixtures containing labeled RNA were extracted with phenol:chloroform (1:1) and precipitated by the addition of NaC1 to .15 $\underline{M}$ and 2.5 volumes ethanol. Unlabeled RNA was used in all of the in vitro translation reactions. Cold transcription reactions were extracted with phenol:chloroform and precipitated by the addition of ammonium acetate to 2.5 $\underline{M}$ and 2 volumes ethanol.

Gel analyses

Precipitated, labeled RNA was glyoxylated (13) prior to electrophoresis on a 1.2% agarose gel. The running buffer was 10 $\underline{mM}$ NaPO$_4$, pH 7.0. The gel was dried and exposed to XAR-5 film. Proteins were separated by electrophoresis on a 10% polyacrylamide gel containing SDS (14). After fluorography (15) the gel was exposed to XAR-5 film.
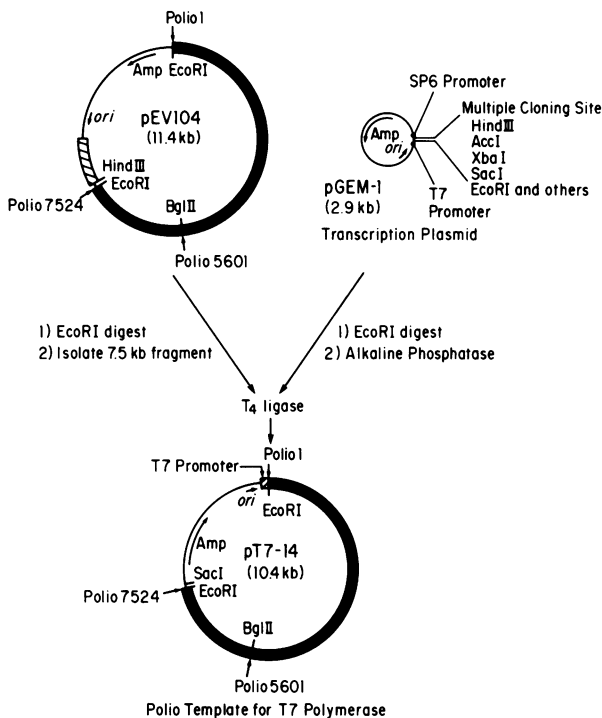
Figure 1 – Strategy for construction of the pT7-14 transcription template contain-
ing the complete poliovirus cDNA clone and the bacteriophage T7 promoter. The cDNA
copy of the poliovirus genome (6) was cloned into pGEM-1 to yield pT7-14.

In vitro translation of transcripts derived from altered cDNA

Translation reactions were carried out in a rabbit reticulocyte lysate supplemented
with an extract from uninfected HeLa cells. Some of the reactions were subsequently
incubated with an infected HeLa cell extract (see below). HeLa cell extract preparation
has been described (9,16). Both the uninfected and infected HeLa cell extracts were
made mRNA dependent by treatment with micrococcal nuclease (17). Translation reac-
tions were carried out as previously described (2) with the following modifications: the
standard reaction mixture (35 μl) contained 90 mM KOAc (instead of KCl), 14.4 μl
rabbit reticulocyte lysate, 7 μl uninfected HeLa cell extract and 10 μg/ml RNA. After
3 hours of incubation at 30°C, cycloheximide and pancreatic RNase were added to 5
μg/ml and 10 μg/ml, respectively. Either 3 μl of lysis buffer used for cell extract
preparation or 3 μl of infected extract was added to 10 μl of the translation reaction
and the mixture incubated at 30°C for the indicated times (see figure legends). Samples
were diluted with Laemmli sample buffer and subjected to electrophoresis.
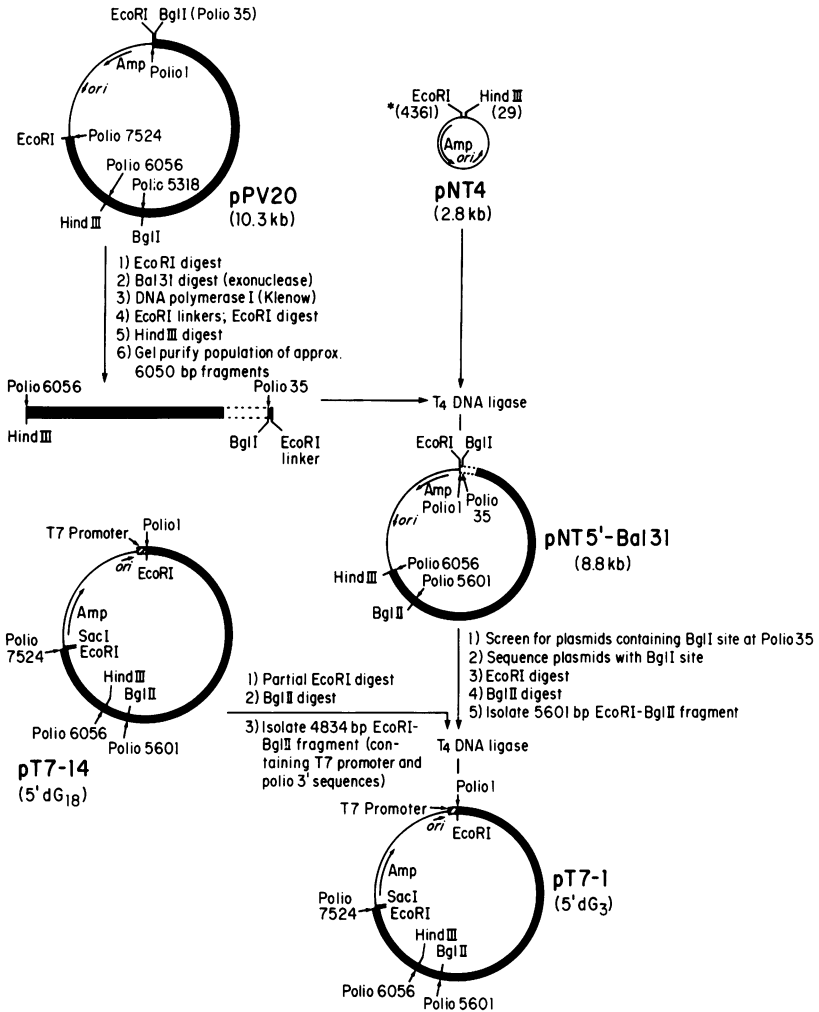
Figure 2 - Strategy for construction of the pT7-1 transcription template containing the complete poliovirus cDNA clone and the bacteriophage T7 promoter. To remove the majority of the 5' dG residues, pPV20 was digested with EcoRI to remove the polio cDNA from vector sequences. Minimal Bal 31 digestion at the 5' and 3' ends of the cDNA and the subsequent steps outlined yielded pT7-1. pT7-1 is the vector into which DNA containing cleavage site or proteinase mutations has been inserted.

RESULTS

Generation of defined mRNAs by in vitro transcription of altered cDNAs

In order to synthesize defined transcripts of polio mRNA that could subsequently be used to program an in vitro translation system, we first cloned the full-length polio insert

from an infectious cDNA clone into the plasmid pGEM-1 (Fig. 1). This commercially available plasmid contains both the bacteriophage T7 and SP6 promoters for transcription located on either side of a multiple cloning site. Depending upon the orientation of the DNA inserted within the multiple cloning site, in vitro transcription can be initiated using either the purified phage T7 RNA polymerase or the phage SP6 RNA polymerase. Although the cloning scheme shown in Fig. 1 yielded polio derivatives of pGEM-1 in both orientations, we have used the plasmid containing the polio sequences inserted downstream from the T7 promoter because we obtain better efficiency and fidelity of transcription as compared to SP6-directed transcription (M. F. Ypma-Wong and B. L. Semler, unpublished observations, see below).

The recombinant plasmid shown at the bottom of Fig. 1 (pT7-14) contains the polio sequences downstream from the T7 promoter. However, between the putative transcriptional start site for the T7 polymerase and the 5' end of the polio genome are additional nucleotides that would result in a transcript containing 32 non-viral nucleotides at its 5' end. These nucleotides are derived from the T7 start site for transcription, the EcoRI linker used for cloning, and the 18 dG residues at the 5' end of the polio clone (resulting from the original cDNA cloning procedure; 6). Because of our concern that these additional sequences might interfere with the polio-specific ribosome binding/protein synthesis initiation events and because a recent report (10) described a higher biological activity (i.e., infectivity) of T7-derived polio transcripts following elimination of additional 5' sequences, we carried out Bal 31 deletions of the 5' flanking sequences of our polio cDNA. The scheme we employed is detailed in Materials and Methods and outlined in Fig. 2. One clone was identified (data not shown) that carried a plasmid with only 3 dG residues between the EcoRI linker and polio nucleotide 1. Thus, we had removed 15 non-viral nucleotides upstream of the 5' end of the polio coding sequence. The final plasmid construct, pT7-1, represents the template for in vitro synthesis of wild type (unmodified) polio RNA as well as the vector into which DNA fragments containing either cleavage site or proteinase mutations were inserted.

The products of in vitro transcription reactions using DNA from pT7-1 as a template for the purified phage T7 RNA polymerase are displayed in Fig 3. Prior to transcription, pT7-1 DNA was linearized by digestion with restriction endonuclease SacI, which cuts immediately downstream from the 3' EcoRI site that flanks the polio poly (A) coding sequence (refer to Fig. 2). The labeled transcription products were denatured by glyoxal treatment and separated by agarose gel electrophoresis. Lane 1 in Figure 3 shows that primarily full-length 7.5 kb transcripts are generated following transcription of pT7-1. Other transcripts of a desired length can also be generated by this method, as shown in Fig. 3, lanes 2 and 3. The 5.6 kb transcript seen in lane 2 is the result of transcription of a pT7-1 template that had been linearized by digestion with BglII, which cuts
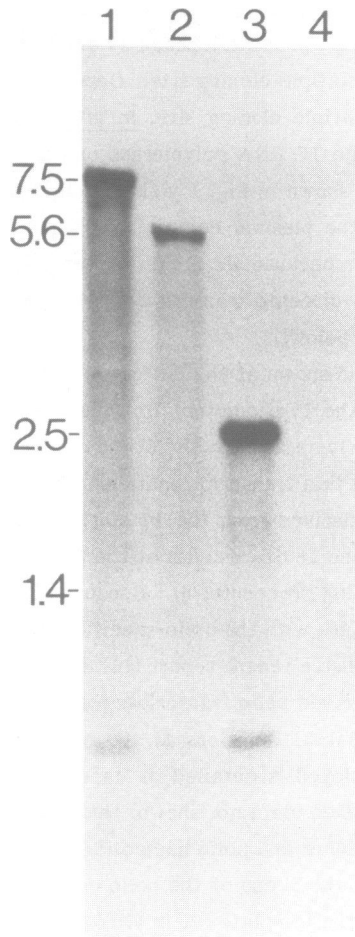
Figure 3 - Agarose gel electrophoresis of glyoxylated poliovirus RNA transcripts synthesized in vitro using T7 RNA polymerase. pT7-1 linearized with SacI produces full-length (7.5 kb) polio transcripts (lane 1); pT7-1 linearized at polio 5601 with BglII and at polio 2546 with XbaI produces truncated transcripts (lanes 2 and 3, respectively). Lane 4 represents a 1.4 kb RNA marker synthesized from a commercially-available plasmid, (Promega Biotec).

within the polio sequence at nucleotide 5601. Similarly, a 2.5 kb transcript (shown in lane 3) was generated by transcription of the template plasmid after digestion with XbaI, which makes a 5' proximal cut at polio nucleotide 2546. Thus, full-length transcripts of the polio genome, as well as sub-genomic transcripts of defined length, can be generated in vitro using the plasmid pT7-1 and purified phage T7 RNA polymerase.
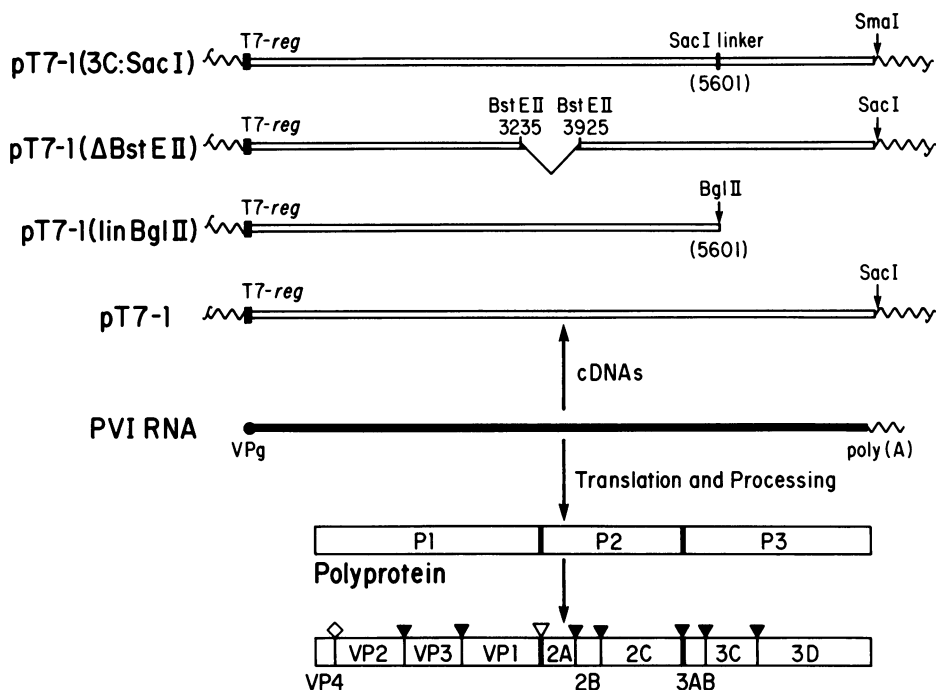
Figure 4 - pT7 constructions containing altered cDNAs of poliovirus and simplified polio processing map depicting cleavage possibilities. A cDNA copy of the entire genomic RNA of poliovirus was inserted into the T7 expression vector. To construct pT7-1 (3C:SacI), an 8 nucleotide SacI linker was inserted at the BglII site (5601). This results in a 4 amino acid insertion in the 3C proteinase. pT7-1 (ΔBstEII) lacks the Y-G cleavage site between the P1 and P2 proteins as well as portions of VP1 and P2 regions. The pT7-1 (lin BglII) template results from a BglII restriction digest of the pT7-1 vector. The bottom half of the diagram shows the cleavage of the wild-type polyprotein into mature polypeptides. The filled triangles (▲) represent cleavage at Q-G sites, the open triangle (△) represents cleavage at a Y-G site. The diamond (◆) represents the morphogenetic cleavage of VP0 to VP4 and VP2.

One unexpected product observed after denaturing agarose gel electrophoresis of in vitro transcripts synthesized from pT7-1 is seen near the bottom of the autoradiograph shown in Fig. 3. A small (~ 600 nucleotides) RNA transcript appears in the products of the in vitro reactions from all three sizes of templates shown in Fig. 3. We assume that this RNA species is a premature termination product produced by the phage polymerase in vitro. A scan of the polio nucleotide sequence (18) reveals a uridine-rich stretch of nucleotides within the non-coding region at approximately 560-580 bases from the 5' end of the genome. Perhaps this sequence is recognized (with a low frequency) as part of a rho-independent transcription terminator (19) by the T7 polymerase. Curiously, we
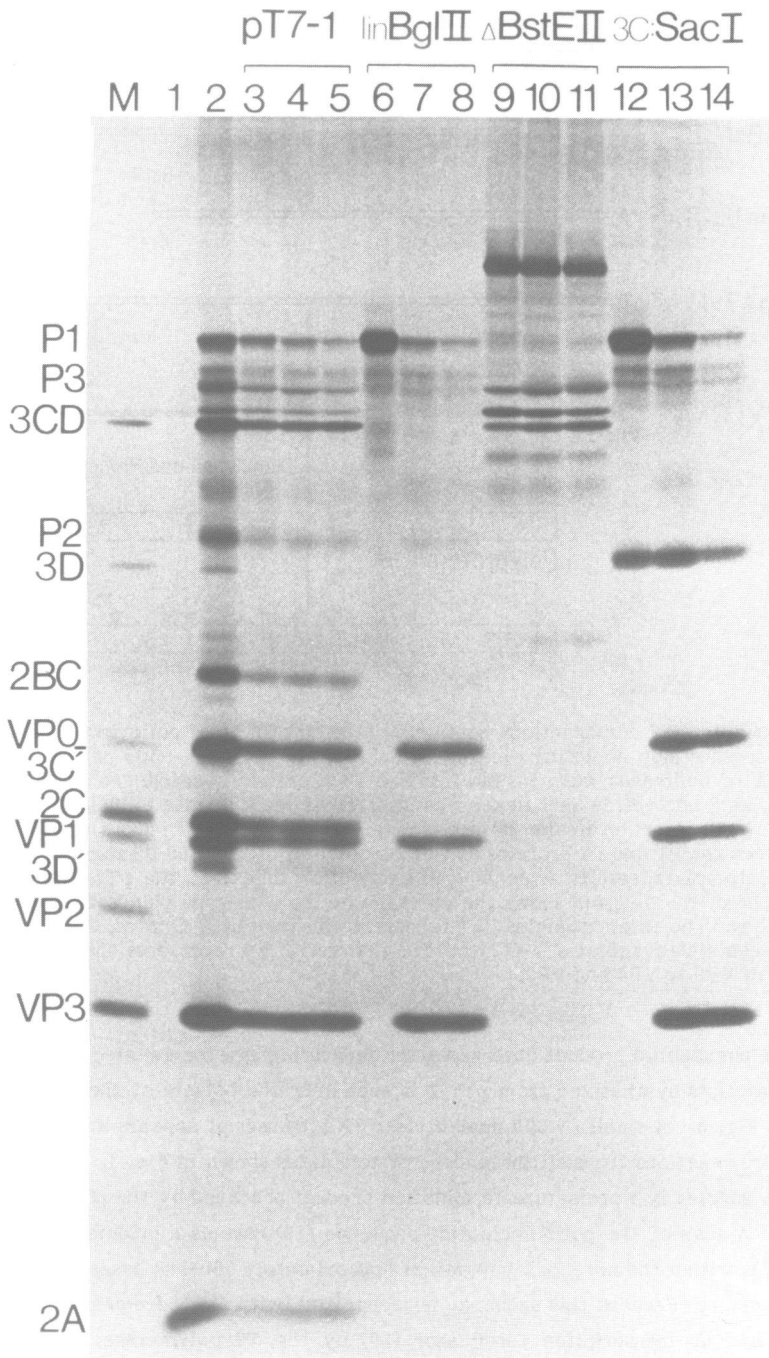
(unpublished observations) and others (20) have observed the same sub-genomic transcript following in vitro synthesis of polio cDNAs using the phage SP6 promoter and purified SP6 polymerase.

The in vitro transcription system described above can be used to generate transcripts from wild type and site-specifically altered cDNA copies of the polio genome. These transcripts can then be used to program an in vitro translation system to direct synthesis of polio-specific polypeptides. Our interest in these studies is to determine the effects that specific mutations have on the protein processing activity of the 3C proteinase. The site-specifically altered polio cDNAs that we used in our processing studies are shown in the top halves of Fig. 4 and Fig. 6. In addition to the plasmid containing the wild type polio genomic insert (pT7-1), we generated: i) a transcript containing an in-phase SacI linker insertion at the BglII site that results in a 4 amino acid insertion; ii) a transcript containing a four nucleotide insertion at the BglII site that renders the 3C proteinase inactive; iii) a transcript containing a 690 base pair in-phase deletion that eliminates the coding sequences for the carboxy-terminal amino acids of VP1, the P1-P2 cleavage site, and the coding sequence for the 2A proteinase; and iv) transcripts derived from templates linearized within the 3C coding region or downstream from the 3C coding region (within 3CD).

In vitro translation of altered polio mRNA defines the trans proteolytic cleavage reactions

The transcription products from the template cDNAs shown in Fig. 4 were used to program an in vitro translation in the presence of ^{35}S-methionine (Amersham). For in vitro translation of polio RNA, we used a rabbit reticulocyte lysate supplemented with an extract (S-10) from uninfected HeLa cells. Our rationale for employing such a translation system is based upon several observations. Firstly, poliovirus RNA has been previously translated in vitro in the reticulocyte lysate (21,22). Secondly, Brown and Ehren-

Figure 5 - In vitro translation of transcripts derived from altered cDNA. Translation reactions were carried out in a rabbit reticulocyte lysate supplemented with uninfected HeLa cell extract. Some of the reactions were subsequently incubated with an infected HeLa cell extract (see below). Translation reactions were carried out as described by Materials and Methods. The reactions were programmed as follows: Lane 1, no RNA; lane 2, PV1 virion RNA; lanes 3-5, pT7-1 derived RNA; lanes 6-8, pT7-1 (lin BglII)-derived RNA; lanes 9-11, pT7-1 (ΔBstEII)-derived RNA; and lanes 12-14, pT7-1 (3C:SacI)-derived RNA. After 3 hours of incubation at 30°C, cycloheximide and pancreatic RNase were added. 10 µl of the respective reaction mixture was used for each sample shown in the figure and incubated at 30° as follows: lanes 2, 3, 6, 9, 12 received 3 µl lysis buffer used for cell extract preparation and were incubated 2 hours; lanes 4, 7, 10, 13 received 3 µl of infected extract and were incubated for 1 hour; lanes 1, 5, 8, 11, 14 received 3 µl of infected extract and were incubated for 2 hours. Samples were diluted with Laemmli sample buffer and subjected to electrophoresis. The marker (M) lane displays the ^{35}S-methionine-labeled proteins from an extract of poliovirus-infected HeLa cells harvested 5 hours post-infection.
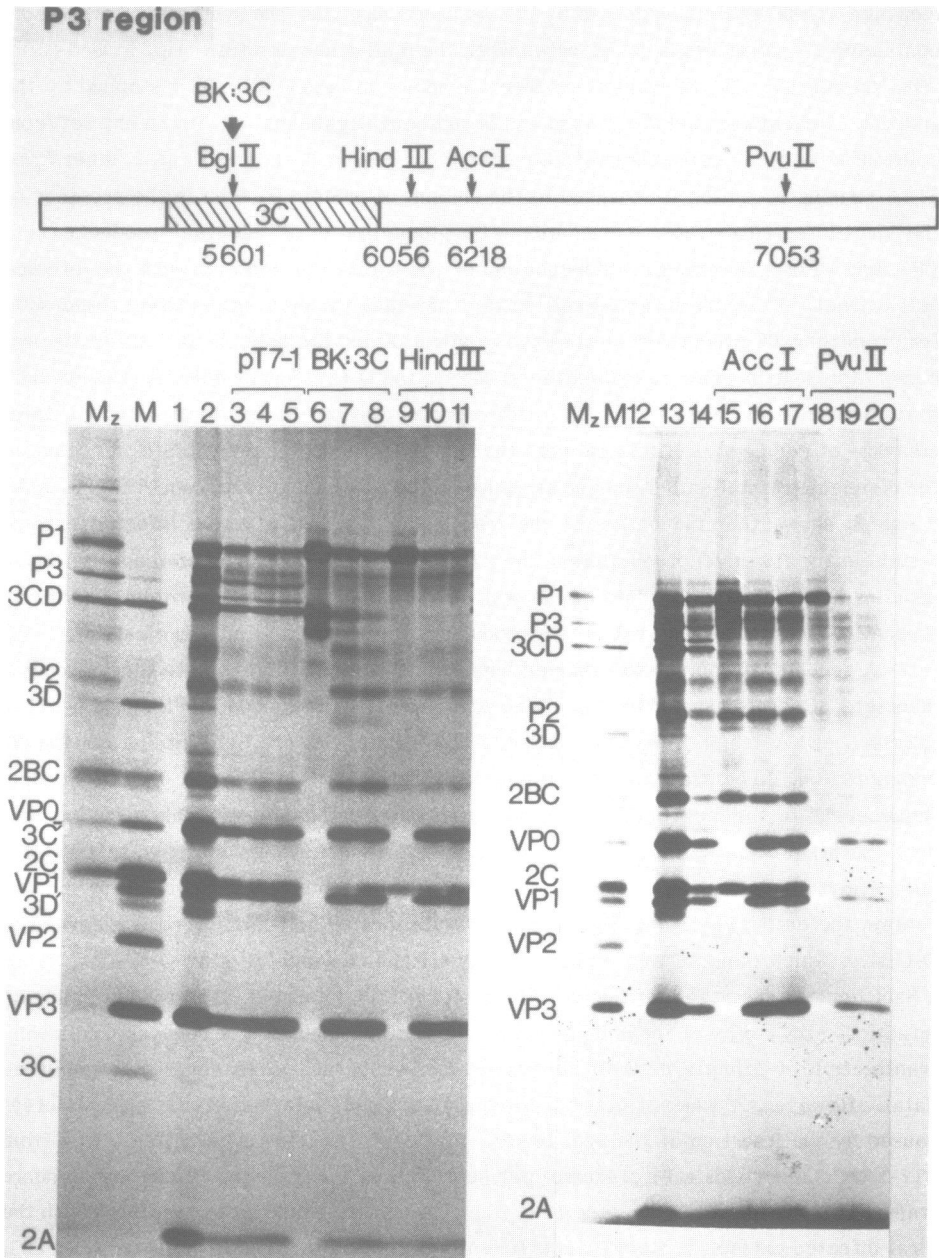
feld (16) reported that addition of a ribosomal salt wash fraction from HeLa cell extracts to a reticulocyte lysate increased the number of in vitro polio translation products that co-migrated with in vivo polio-specific polypeptides during polyacrylamide gel electrophoresis. Finally, in vitro translation of poliovirus RNA in reticulocyte lysates has been shown to produce aberrant polypeptides as a result of initiation of protein synthesis at internal sites on the viral genome (23,24). These internal initiations are eliminated by the addition of an S-10 extract from HeLa cells to the reticulocyte lysate prior to the start of the in vitro translation reactions (23). The extract from HeLa cells apparently provides factors that suppress internal initiation events and/or direct ribosomes to the authentic 5' proximal initiation codon at nucleotide 743 on the polio genome (25).

The viral-specific polypeptides produced by in vitro translation of our in vitro-synthesized polio RNAs are shown in Fig. 5. The wild-type, T7-derived transcript (from pT7-1) produces a polypeptide pattern (shown in lane 3) that is very similar to the pattern seen when purified virion RNA is used to program the in vitro translation reaction (lane 2). In particular, the presence of the capsid protein precursor (P1) indicates that the polio in vitro transcripts program the synthesis of an active 2A proteinase that makes the Y-G cleavage between the P1 and P2 precursor polypeptides (refer to Fig. 4). The presence of proteins P3, 3CD, and 2C indicates that the 3C proteinase activity is cleaving Q-G bonds, since all of these proteins are generated by Q-G cleavages. Polio proteins 3D and 3D' are not produced in readily detectable quantities following translation of RNA from pT7-1 (compare lanes 2 and 3 in Fig. 5). However, a longer exposure of the gel shown in Fig. 5 clearly shows the production of 3D and 3D' (data not shown). The low level production of these polypeptides may reflect a reduced efficiency of in vitro cleavage of 3CD produced by translation of the synthetic mRNA. As is typically observed of in vitro translations of exogenous polio RNA, the production of 3C is difficult to detect. The production of capsid proteins VP0, VP3, and VP1 is further evidence of 3C cleavage activity at Q-G sites. The equivalent production of the majority of polio polypeptides by in vitro translation from virion-derived RNAs and in vitro transcription derived RNAs demonstrates that the T7 RNA polymerase synthesizes transcripts that are fully functional polio mRNAs.

To test the ability of the 3C proteinase to carry out Q-G cleavages in trans, we analyzed the products of in vitro translation in both the absence and presence of an extract from poliovirus-infected HeLa cells. Fig. 5, lane 6, shows the translation products derived from a pT7-1 transcript whose template had been linearized with restriction endonuclease BglII [shown in Fig. 4 as pT7-1 (lin BglII)]. Because the truncated transcript does not contain the complete 3C coding region, the P1 translation product was not cleaved into VP0, VP3, and VP1 at authentic Q-G sites. The generation of P1 itself (i.e., cleavage between the P1 and P2 precursor polypeptides) occurs as a result of

cleavage at the Y–G cleavage site by the 2A proteinase (1). The synthesis of precursors containing 2A sequences is not affected when the BglII-truncated transcript is used for in vitro translation. If the translation reaction shown in lane 6 (Fig. 5) is stopped by the addition of cycloheximide and RNase and is subsequently incubated with an extract from polio-infected HeLa cells, the protein pattern that results is shown in Fig. 5, lanes 7 and 8. A striking reduction is observed in the amount of protein P1 seen in the presence of the virus-infected extract. Concomittantly, production of P1 cleavage products (VP0, VP3, and VP1) is observed after incubation of the translation reaction with the infected cell extract. Thus, the infected-cell extract provides the cleavage activity responsible for processing P1 precursors synthesized in vitro. Since the polio 3C protein or its precursors (present in infected cell extracts) carries the Q–G cleavage activity, we conclude that this protein is responsible for in vitro production of the capsid proteins via trans cleavage of polypeptide P1. (Also note that the presence of a poly(A) tail is not required for efficient translation.) A longer exposure of the autoradiograph shown in Fig. 5, lanes 7 and 8, shows that incubation of the translation reaction with the infected extract results in the low level production of the P2 precursor polypeptide as well as P2-derived proteins (2A, 2BC, and 2C; data not shown). We conclude that the 3C proteinase activity present in the infected-cell extracts is producing these proteins via trans cleavage.

A second experiment was carried out using a polio cDNA template that encodes a complete 3C proteinase with a 4 amino acid insertion. As seen in Fig. 4, the pT7-1 (3C:SacI) cDNA template contains a SacI linker inserted at the BglII site within the 3C coding region. In contrast to pT7-1 (lin BglII), this template includes all of the P3 coding sequences 3' to the mutation site. It has previously been shown that this mutation renders the 3C proteinase inactive in a bacterial expression system designed to assay for autocatalytic cleavage of 3C (4). However, the bacterial expression vector did not contain the entire P3 coding region and the system could only assay for the cleavage of Q–G sites which flank 3C and could not assay for the cleavage of other Q–G sites. When a SacI linker is inserted into the same BglII site within a plasmid (pEV104) containing an infectious cDNA copy of the polio genome, no infectious virus can be recovered following transfection of primate cells in culture (B. L. Semler and V. H. Johnson, unpublished data). However, it was not possible to characterize the enzymatic/biochemical defect caused by the insertion of the SacI linker. In vitro translation of mRNAs derived from pT7-1 (3C:SacI) yields a P1 precursor polypeptide that is not cleaved under our standard translation conditions (Fig. 5, lane 12). When this reaction mixture is incubated with the viral-infected extract, protein P1 is efficiently processed in trans to generate VP0, VP1, and VP3 (lanes 13 and 14). Note that no production of P2 or P3-derived proteins is observed. Translation of mRNA from pT7-1 (3C:SacI) also produces a polypeptide of $M_r \sim$ 60,000 daltons that migrates between viral proteins 3D and P2 during SDS-polyacryla-

mide gel electrophoresis (refer to Fig. 5, lanes 12-14). This protein does not co-migrate with any known poliovirus-specific polypeptides and does not appear to be a substrate for 3C cleavage in trans. Such a protein may represent a cleavage product generated as a result of cleavage by the altered 3C proteinase, perhaps at sites not normally utilized during polio protein processing. This ~ 60,000 dalton polypeptide is immunoprecipitated with 2C-specific antiserum and we are presently determining its exact origin.

A third experiment was carried out to determine if authentic cleavage sites of a structurally-altered precursor polypeptide would still be recognized and cleaved by the wild type 3C proteinase. Altered precursor polypeptides were generated by translation of the in vitro transcripts derived from clone pT7-1 (Δ BstEII). Plasmid DNA from this clone contains a 690 base pair deletion of a BstEII fragment in the polio genome (nucleotides 3235-3925). The deleted cDNA is missing the coding region for part of VP1, all of protein 2A, and a portion of 2B, but maintains the original open reading frame of the polio polyprotein. The labeled in vitro translation products from the pT7-1 (Δ BstEII) transcripts are displayed in Fig. 5, lane 9. The major polypeptide species observed in these products is a large precursor polypeptide that migrates with an approximate M_r of 135,000-140,000 daltons during SDS-polyacrylamide gel electrophoresis. Such a translation product is the size expected from a P1-P2 fusion in which ~ 25,000 daltons of protein coding region has been removed. In addition, we would predict that no cleavage between P1 and P2 should occur (refer to Fig. 4) because we have removed not only the Y-G cleavage site but also the coding region for the protein (2A) responsible for cleavage at that site. Note also that proteins co-migrating with authentic P3 and 3CD polypeptides are observed in the translation products shown in lane 9. These proteins have been previously shown to be products of Q-G cleavage (2,9) and their presence demonstrates that

Figure 6 - Diagram of the P3 coding region of poliovirus and in vitro translation of transcripts derived from altered cDNA. The coding region of the 3C protein is hatched. pT7-1 (BK:3C) results from a 4 bp insertion into the BglII site of pT7-1. The shift in reading frame creates a stop codon such that a truncated polyprotein is produced. Transcripts containing a defined portion of the P3 coding region were generated by linearizing pT7-1 with restriction enzymes HindIII, AccI, and PvuII. Translation reactions and subsequent incubations were carried out essentially as described in Fig. 5. The reactions were programmed as follows: Lane 1 and 12, no RNA; lane 2 and 13, PV1 virion RNA; lanes 3-5 and 14, pT7-1-derived RNA; lanes 6-8, pT7-1 (BK:3C)-derived RNA; lanes 9-11, pT7-1 (lin HindIII)-derived RNA; lanes 15-17, pT7-1 (lin AccI-derived RNA; and lanes 18-20, pT7-1 (lin PvuII)-derived RNA. Lanes 2, 3, 6, 9, 13, 14, 15, and 18 received 3 µl lysis buffer used for cell extract preparation and were incubated 2 hours; lanes 4, 7, 10, 16, and 19 received 3 µl of infected extract and were incubated for 1 hour; lanes 1, 5, 8, 11, 12, 17, and 20 received 3 µl of infected extract and were incubated for 2 hours. Samples were diluted with Laemmli sample buffer and subjected to electrophoresis. The marker (M) lane displays the ^{35}S-methionine-labeled proteins from an extract of poliovirus-infected HeLa cells harvested 5 hours post-infection. To generate a marker (M_z) which contains large amounts of unprocessed polio-specific proteins, $ZnCl_2$ is added prior to labeling the infected HeLa cells (28).

3C cleavage activity on P3-related proteins has not been affected significantly by the upstream deletion. Curiously, the protein that migrates slightly slower than 3CD is enriched in translations of RNAs derived from pT7-(ΔBstEII). Its molecular weight and the observation that it immunoprecipitates with anti-VPg sera suggest that this polypeptide is the equivalent of 3BCD (Ypma-Wong and Semler, unpublished). Protein 3BCD (formerly called P3-1c) is a polypeptide (apparent MW of 76,000 daltons) containing VPg sequences as well as those of 3CD (26). The ability of the P1-P2 fusion precursor to serve as a substrate for cleavage by 3C has been severely impaired, especially for the generation of capsid proteins. As seen in Fig. 5, lane 9, no detectable amounts of either VP0 or VP3 are observed, even though the cleavage sites that are used to generate these proteins are still intact. This inability of the large precursor polypeptide to be cleaved cannot be overcome by the addition of more 3C proteinase since the infected-cell extract does not cause the production of any detectable amounts of VP0 or VP3 when it is incubated with the translation reaction from pT7-1 (Δ BstEII) transcripts (Fig. 5, lanes 10 and 11). Moreover, incubation of the translation reaction with the infected-cell extract does not result in the cleavage of 3CD or 3BCD into processing products. In our experiments, we have never detected the production of P3-derived products via the addition of the infected cell extract. It is possible these cleavages occur primarily in cis.

It is notable that in all three lanes (Fig. 5, 9-11), a small but detectable amount of a polypeptide that co-migrates with protein 2C (and immunoprecipitates with anti-2C serum, unpublished observations) is observed following in vitro translation of transcripts from pT7-1 (Δ BstEII). It appears that the altered structure/folding of the P1-P2 fusion precursor polypeptide allows cleavage at some Q-G sites (to generate 2C) and not at others. For the capsid portion of such a precursor polypeptide, "correct" folding might never occur because the deletion does not allow the cleavage and generation of an authentic P1. Generation of the 5S protomer consisting of VP0, VP3, and VP1 during polio morphogenesis (27) may be strictly dependent on a free (and properly-folded) P1 polypeptide that contains, in addition to authentic Q-G cleavage sites, the secondary signals required for the recognition and accessibility of the active site of 3C.

A final demonstration that the virus-infected extract can provide proteinase activity in trans which can cleave the Q-G site between the P2-P3 regions and cleave P2 into its processing products (2A, 2BC, 2C) is shown in Fig. 6. Lane 6 shows the translation products derived from pT7-BK:3C. pT7-BK:3C contains a four base pair insertion at the BglII site and the resulting reading frame shift produces a stop codon 45 nucleotides downstream from the BglII site. Since this mutation severely truncates 3C, cleavage of the precursor polypeptide to capsid or P2-derived proteins does not occur. However, lane 7 demonstrates that incubation of the translation reaction with the infected cell extract results in the cleavage of P1 to capsid proteins as well as the production of P2, 2A, 2BC,

and 2C. Lane 8 demonstrates that prolonged incubation of the translation products results in the reduction in the amount of the P2 precursor and the parallel increase in the amounts of 2A, 2BC, and 2C. These results support our conclusion that the polio 3C protein (or other polypeptides containing 3C sequences) present in the virus extract is capable of in vitro cleavage of both the P1 and P2 precursors in trans.

Differential requirements for P3 region amino acid sequences in cleavage of P1 and P2 precursor polypeptides

To determine the P3 sequences required for the production of an active proteinase, we analyzed the products of in vitro translation of transcripts derived from the polio template which was linearized downstream from the 3C proteinase coding region. Fig. 6, lane 9 shows the translation products derived from a pT7-1 transcript whose template had been linearized with restriction endonuclease HindIII. Note that while there is production of P1, no cleavage of the P1 precursor into capsid proteins (VP0, VP3, and VP1) occurs. In contrast, the production of the P2 precursor and subsequent processing of the P2 precursor into cleavage products (2A, 2BC, and 2C) does occur. We conclude that sequences beyond the actual 3C coding region are required for the production of an active proteinase capable of processing P1 into its cleavage products. In addition, it is clear that sequences beyond polio nucleotide 6056 are not required for the production of P2 or the processing of P2 into its cleavage products. Specifically, 3CD is not responsible for the processing of P2 proteins, since it is not generated following cleavage of pT7-1 with HindIII. The production of capsid proteins in the translation reactions programmed with HindIII-truncated transcripts when the reactions are incubated with virus-infected extract indicates that P1 is a recognizable substrate (refer to Fig. 6, lanes 10 and 11) and has not been altered by generating truncated proteins downstream.

To test whether a polypeptide containing more, but not all of the P3 region would have complete proteinase activity, transcripts were synthesized from the T7-1 template which was linearized with either restriction endonuclease AccI or PvuII. Lane 15 shows the translation products derived from a pT7-1. transcript linearized with restriction endonuclease AccI. While there is production of the P1 translation product, no cleavage of the P1 precursor into capsid cleavage products occurs. Note, however, the production of the P2 precursor and subsequent processing of the P2 precursor into cleavage products does occur. Apparently, the additional sequences downstream from 3C do not restore complete proteinase activity. Incubation of these translation products with the virus-infected extract again demonstrates that P1 is a proper substrate (lanes 16 and 17). Lane 18 shows the translation products derived from a pT7-1 transcript produced when pT7-1 was linearized with restriction endonuclease PvuII. Even though the majority of the P3 region is present, no cleavage of P1 into capsid cleavage products occurs. However, the production of P2 and subsequent processing of the P2 precursor into 2BC and 2C does

occur. It appears that in vitro cleavage of the P2 precursors requires only an intact coding region for the 3C polypeptide, whereas cleavage of the P1 precursor into capsid proteins requires most, if not all, of the P3 amino acid sequences.

DISCUSSION

The data we have presented here show that in vitro translation of defined polio mRNAs can be used to determine some of the features of protein processing that regulate cleavage site recognition by the 3C proteinase. We have used simple modifications of template cDNAs to conclusively demonstrate the trans nature of the 3C-mediated cleavage of the P1 precursor to the capsid proteins. In addition, our studies utilizing several altered transcripts suggest that the Q-G cleavage between P2-P3 and the cleavages which generate 2A, 2BC, and 2C may also occur in trans. In the in vitro translation experiments of polio RNAs containing lethal alterations in the 3C coding region, the cleavage of P1 occurred only as a result of addition of an exogenous source of authentic viral proteinase. It is clear, therefore, that P1 cleavage does not require the co-translational synthesis of the 3C proteinase on the same polyribosomes. In fact, our studies with the in vitro translation of transcripts from pT7-1 (Δ BstEII) suggest that 2A cleavage at the carboxy-terminal Y-G bond of P1 may be necessary before this region of the polyprotein can serve as a "proper" substrate for 3C. The separation of the P1 polypeptide from the rest of the polyprotein during polio translation may ensure the generation of cleavable capsid precursors whose biological activity (as substrates) can be maintained independently from that of the non-structural polypeptides involved in protein processing and RNA replication.

The in vitro translation of defined polio mRNAs has also been used to determine some of the features of proteolytic processing that regulate proteinase activity and specificity. Modification of the template cDNA demonstrates that most, if not all, of the P3 region is required to produce a proteinase capable of cleaving P1 to capsid proteins. However, only the 3C protein sequences (and perhaps a few amino acids downstream from the 3C Q-G carboxy-terminal cleavage site) are required to produce a proteinase capable of generating P2, 2A, 2BC, and 2C. A fusion protein containing bacterial, 3C, and 3C flanking sequences has been expressed in E. coli (4). In this system, the 3C amino-terminal Q-G cleavage occurred immediately and efficiently following translation of the fusion protein. In vitro translation of polio RNA typically does not produce detectable amounts of the 3C protein and we cannot detect an abberant protein containing 3C sequences which is specific to translations programmed with altered RNA transcripts. Therefore, we cannot determine if the 3C amino or carboxy-terminal Q-G cleavages are accomplished in the precursor polypeptides generated in these translations. By extrapolation from the bacterial expression data, it is likely that the amino-

terminal cleavage of 3C does occur during in vitro translation and may result in a 3C polypeptide with additional amino acid residues at its carboxy terminus. In the translations programmed with truncated transcripts, this "tail" may only reduce (but not eliminate) the ability of the proteinase to cleave at P2 Q–G sites. Such a result may indicate that the enzyme–substrate interaction is "loose". However, generation of the 5S protomer consisting of VP0, VP3, and VP1 during morphogenesis may require a very stringent enzyme–substrate interaction. This would not be surprising considering the close proximity of the capsid proteins in the protomer complex. An intact P3 region may be required for the generation of P1 cleavages because only an intact P3 precursor can assume a cleavable configuration such that the cleavage of the carboxy-terminal site of 3C occurs. Perhaps the cleavage of P1 requires the generation of a free 3C polypeptide. If the 3C carboxy-terminal Q–G cleavage is accomplished in the altered precursor polypeptides and mature 3C proteinase is generated, it is unclear why P1 is not cleaved into capsid proteins. Sequences beyond the 3C coding region are not required to generate proteinase capable of cleaving P2 Q–G sites, indicating that neither 3CD nor a 3C protein containing additional P3 sequences is the responsible proteinase. Therefore, the cleavage at P1 Q–G sites must involve a different mechanism of proteinase activation and/or interaction with the substrate.

In conclusion, we have described an in vitro molecular genetic approach to the study of poliovirus proteolytic processing. All polio proteins are translated in equimolar amounts as a giant precursor polypeptide, suggesting that the kinetics and order of protein processing may be the major means of controlling the differential production of poliovirus proteins at specific times during the course of an infection. The in vitro transcription of altered polio RNA transcripts from cDNA using the T7 polymerase and subsequent in vitro translation of these transcripts will allow further detailed studies of these events.

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