JOURNAL OF VIROLOGY, Oct. 1987, p. 3181–3189
0022-538X/87/103181-09$02.00/0

Vol. 61, No. 10

Processing Determinants Required for In Vitro Cleavage of the Poliovirus P1 Precursor to Capsid Proteins

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Received 13 April 1987/Accepted 29 June 1987

We generated defined alterations in poliovirus protein-processing substrates and assayed the effects of these alterations with an in vitro expression system. A complete cDNA copy of the poliovirus genome was inserted into a bacteriophage T7 transcription vector. Using this expression template, we produced RNA transcripts containing defined regions of the poliovirus capsid precursor polypeptide (P1) and RNA transcripts containing mutations in the P1 and P2 regions. In vitro translation of P1-derived transcripts allowed us to characterize the 3C-mediated cleavage of P1 to capsid proteins. We demonstrated that, for either posttranslational or cotranslational cleavage at any of the Q-G amino acid pairs within P1, almost the entire P1 precursor is required. We also examined the sequences involved in 2A-mediated processing of the Y-G site at the P1-P2 junction. We demonstrated that minimal sequences 3′ to the 2A coding sequences are required to generate active 2A proteinase in vitro and that two specific four-amino-acid insertions in protein 2C do not alter 2A- or 3C-mediated processing of the poliovirus polyprotein. In addition, we demonstrated that substantial deletion of P1 sequences does not alter 2A-mediated cleavage of the Y-G site at the P1-P2 junction. These results allowed us to compare the P1 sequences required for 2A- versus 3C-mediated processing of the capsid precursor, and we discuss these results in the context of the three-dimensional structure of the capsid proteins.

Picornavirus RNA is a monocistronic message which is translated into a large precursor polyprotein. This polyprotein is cleaved into smaller, functional proteins during and after translation. For poliovirus, a member of the *Picornaviridae*, there are at least two known proteinases encoded in the genome which are responsible for these cleavages. The 2A proteinase cleaves the capsid precursor protein (P1) from the rest of the polyprotein soon after the ribosomes have translated through the 2A coding sequences (23). The 2A proteinase cleaves the P1-P2 junction specifically at a tyrosine-glycine (Y-G) amino acid pair, as well as at a second Y-G pair in the P3 region. However, the 2A proteinase must recognize additional sequences surrounding the Y-G sites, since only 2 of the 10 Y-G pairs encoded in the polyprotein are cleaved. The second proteinase, 3C, cleaves the polyprotein specifically at glutamine-glycine (Q-G) amino acid pairs to generate functional proteins (5). The production of the mature 3C proteinase is thought to occur by an autocatalytic mechanism (6, 12) which would involve cleavage in *cis* of the Q-G pairs which flank 3C. It has been demonstrated that cleavage of several Q-G pairs by 3C proteinase can occur in *trans* for both poliovirus and encephalomyocarditis virus, another picornavirus (10a, 13, 24). Using the systems described below, we have previously demonstrated that the Q-G cleavage between P2-P3 and the Q-G cleavages which generate 2A, 2BC, 2C, VP0, VP3, and VP1 can all occur in *trans*. In a manner similar to cleavage by the 2A proteinase, the 3C proteinase must recognize additional sequences or conformations surrounding the Q-G pairs, since only 9 of the 13 pairs are cleaved. Specifically, the capsid precursor protein contains three Q-G amino acid pairs. However, only two pairs are cleaved such that VP0, VP3, and VP1 are generated. VP0 is eventually cleaved to give the mature capsid proteins VP2 and VP4 during virion morphogenesis (1, 9). The enzyme responsible for this cleavage event has not been identified. The overlapping format of the poliovirus genome complicates efforts to study in vivo the effect of a mutation introduced into the poliovirus genome. Because of the precursor-to-product relationship, one mutation introduced into a specific site has the potential of altering several gene products. For example, a 3C mutation producing a novel phenotype of protein processing has been recently shown also to affect viral RNA synthesis (3). The in vitro system described below circumvents this complication and allows a detailed study of poliovirus protein processing.

The system we developed for in vitro assays of poliovirus cleavage site mutations takes advantage of infectious cDNA clones of poliovirus genomic RNA (11, 17, 21). The DNA copies of poliovirus RNA retain all of the biological properties required for initiating a productive infection when transfected onto primate cells in culture (11, 18, 21) and offer the advantage of genetic manipulation of the poliovirus genome. To synthesize defined transcripts of poliovirus RNA, wild-type or altered cDNA clones of poliovirus were inserted into a vector containing the bacteriophage T7 promoter. T7 RNA polymerase was used to synthesize poliovirus-specific RNA from the cDNA templates. The in vitro-synthesized RNA was subsequently assayed in an in vitro translation reaction for production of poliovirus-specific proteins. Translation and processing of the wild-type and altered poliovirus precursor polypeptides were then analyzed by polyacrylamide gel electrophoresis. By using 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.

In the present study, we used the in vitro systems described above to generate defined cleavage site mutations in the poliovirus genome and subsequently assay phenotypic expression of such mutations. These systems allowed us to

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characterize 3C-mediated cleavage of the capsid polypeptide precursor (P1) to capsid proteins. We demonstrated that almost all of P1 is required for posttranslational 3C-mediated cleavage at any of the Q-G bonds with P1. We also investigated cleavage of P1 by cotranslating RNA containing capsid precursor sequences and RNA containing proteinase sequences such that P1 was synthesized in the presence of 3C proteinase activity. Our results indicated that individual Q-G sites are not recognized in the context of their immediate environment, (neither posttranslation nor during translation) and a nearly complete carboxyl terminus of P1 is required to allow the precursor to assume a recognizable conformation such that it is cleaved by 3C proteinase. These in vitro systems also allowed us to study 2A-mediated cleavage of P1 from the rest of the polyprotein. Our results suggest that 2A proteinase requires only the carboxy-terminal portion of VP1 to cleave P1 from P2 and minimal sequences downstream of 2A to generate active 2A proteinase. In addition, we generated insertions downstream of the 2A coding region (in the 2C protein) and demonstrated that these alterations do not alter 2A- or 3C-mediated processing of the polyprotein.

MATERIALS AND METHODS

Restriction endonucleases and enzymes. Restriction endonucleases and T4 ligase were purchased from New England BioLabs, Inc., and Boehringer Mannheim Biochemicals. T7 RNA polymerase was purchased from Pharmacia, Inc. RNasin was purchased from Promega Biotech.

Cells and viruses. HeLa S3 cells in suspension were used for cell extract preparation. HeLa cells were infected with poliovirus type I, Mahoney strain, for infected-cell extract preparation (2, 4). Rabbit reticulocyte lysate was purchased from Bethesda Research Laboratories, Inc.

Construction of transcription vectors. Construction of the general transcription vector has been previously described (24). pT7-1 contains the entire poliovirus genome, which was inserted into the pGEM-1 cloning vector (Promega Biotech) and represents the template for in vitro synthesis of T7-1 (wild type, unmodified) RNA. (Note that RNA transcribed from a template will be referred to by the template name without the p prefix.)

pT7-1 (lin *Pst*I), pT7-1 (lin *Hae*II), pT7-1 (lin *Xba*I), pT7-1 (lin *Sna*BI), pT7-1 (lin *Bst*EII), pT7-1 (lin *Nde*I), and pT7-1 (lin *Hin*cII) are transcription templates resulting from linearizing the pT7-1 template with the respective restriction endonuclease. *Pst*I and *Hae*II linearize the transcription template within the VP3 coding region. *Xba*I, *Sna*BI, *Bst*EII, and *Nde*I linearize the transcription template within the VP1 coding region. *Hin*cII linearizes the transcription template within the 2B coding region, leaving the coding region for 2A proteinase intact (see Fig. 1).

pT7-P3 contains the coding sequences for the P3 region, as well as the amino-terminal portion of VP1 and the carboxy-terminal portion of 2C. pT7-P3 was constructed by digesting pT7-1 with *Sph*I. *Sph*I cuts once in the pGEM-1 vector and at poliovirus nucleotides 1127, 2732, 2923, 4085, and 4154. The 3.5-kilobase fragment containing vector sequences and poliovirus nucleotides 1 to 1127 and the 3.8-kilobase fragment containing the remainder of vector sequences and poliovirus nucleotides 4154 to 7524 were gel purified and ligated with T4 ligase (see Fig. 3).

pT7-1 (2C:*Eco*RI) contains a four-amino-acid insertion (Arg-Asn-Ser-Asp) in 2C. This mutation was constructed by inserting an *Eco*RI linker (G-G-A-A-T-T-C-C) into the *Bam*HI site at poliovirus nucleotide 4600. A subgenomic clone containing the poliovirus sequence 2954 to 6056 was partially digested with *Bam*HI, and the *Eco*RI linker was inserted. The sequence containing this insertion was subsequently introduced into a full-length poliovirus cDNA clone and then cloned into the pT7-1 vector.

pT7-1 (2C:*Sac*I) contains four-amino-acid insertion (Pro-Ser-Ser-Asp) in 2C. This mutation was constructed by inserting a *Sac*I linker (C-G-A-G-C-T-C-G) into the *Bam*HI site at poliovirus nucleotide 4600. This insertion was introduced into a subgenomic poliovirus clone and ultimately cloned into pT7-1 as was pT7-1 (2C:*Eco*RI).

pT7-1 (P1:ΔNS) lacks the carboxy-terminal portion of VP0, all of VP3, and the amino-terminal portion of VP1 but maintains the polyprotein reading frame. This plasmid was constructed by digesting pT7-1 with *Nru*I and *Sna*BI to delete the sequence 1172 to 2954 and then religating with T4 ligase.

In vitro transcription with T7 RNA polymerase. Before transcription, the DNA template was linearized with the appropriate restriction endonuclease. Transcription reactions were carried out, as previously described (24), with T7 RNA polymerase. Transcription reactions were extracted with phenol-chloroform and precipitated by addition of ammonium acetate to 2.5 M and 2 volumes of ethanol.

In vitro translation of transcripts derived from altered cDNA. Translation reactions were carried out at 30°C in rabbit reticulocyte lysate supplemented with an extract from uninfected HeLa cells. Some of the reactions were subsequently incubated with infected HeLa cell extract (see below). HeLa cell extract preparation has been previously described (2, 20). Both uninfected and infected HeLa cell extracts were made mRNA dependent by treatment with micrococcal nuclease (14). Translation reactions were carried out as previously described (24). If the reactions were to be incubated with infected HeLa cell extract, the final RNA concentration in the translation reaction was 10 μg/ml. The translation reaction was terminated after 3 h by addition of cycloheximide and pancreatic RNase at 5 and 10 μg/ml, respectively. Either 3 μl of the lysis buffer used for cell extract preparation or 3 μl of infected-cell extract was added to 10 μl of the translation reaction, and incubation of the mixture was continued at 30°C for 1 h.

In a cotranslation experiment, T7-P3 transcripts were added to a 5-μl translation reaction mixture at a final concentration of 4.0 μg/ml and allowed to program the synthesis of P-3-derived proteins for 1 h. Transcripts derived from the linearized pT7-1 template were added to a 5-μl translation reaction mixture at a final concentration of 20 μg/ml and incubated for 5 min at 30°C before its addition to the translation reaction mixture programmed with T7-P3 RNA at the 1-h time point. The translation reaction containing both transcripts was allowed to continue for 3 h. Translations programmed solely with RNA derived from the linearized pT7-1 template or T7-P3 RNA were incubated for 3 h at 30°C. Translation was terminated by addition of RNase.

All translation reaction mixtures were diluted with Laemmli sample buffer and subjected to electrophoresis on a 10% polyacrylamide gel containing sodium dodecyl sulfate. After fluorography, the gel was exposed to XAR-5 film.

RESULTS

Generation of defined RNAs by in vitro transcription of altered cDNAs. To synthesize defined transcripts of poliovi-

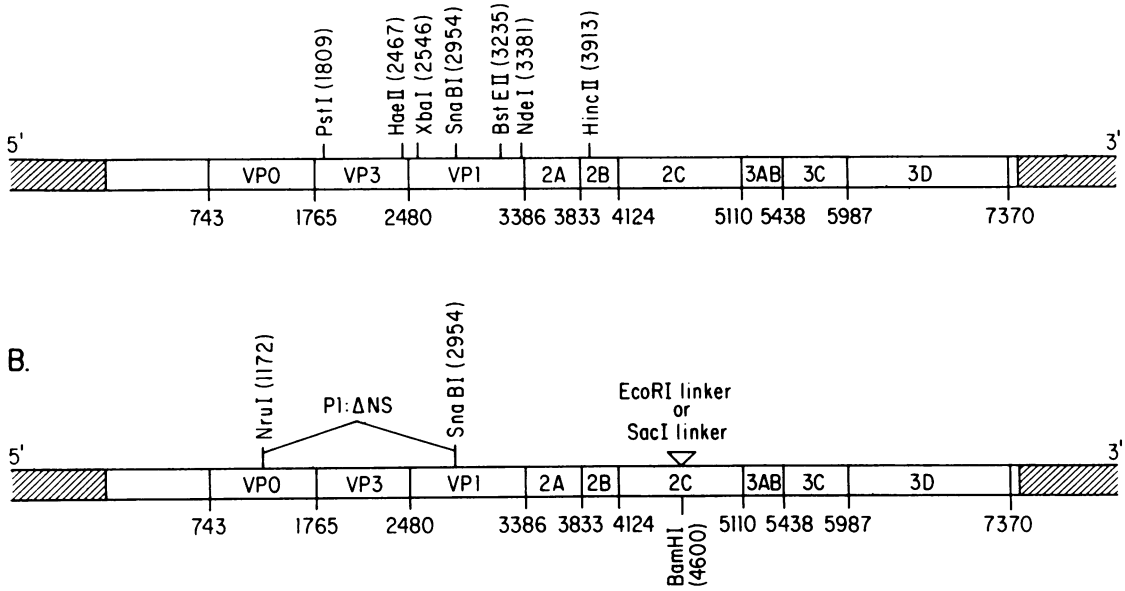

FIG. 1. cDNA templates depicting the simplified genomic structure of poliovirus and relevant restriction endonuclease recognition sites. The coding sequences for the major viral proteins are indicated, and the flanking T7 vector sequences are hatched. The first 742 nucleotides of the poliovirus genome are not translated. The 2A proteinase is responsible for cleavage at the VP1-2A Y-G site. The 3C proteinase cleaves at all of the other sites shown in the diagram. The restriction sites used to linearize the pT7-1 template are shown in panel A. The nucleotide at which the respective restriction endonuclease cleaves is indicated in parentheses. The location of the genomic alterations in templates pT7-1 (P1:ΔNS), pT7-1 (2C:*Eco*RI), and pT7-1 (2C:*Sac*I) are indicated in panel B. pT7-1 (P1:ΔNS) was constructed by deleting the *Nru*I-*Sna*BI fragment from poliovirus nucleotides 1172 to 2954. pT7-1 (2C:*Eco*RI) and pT7-1 (2C:*Sac*I) were both generated by insertion of a linker at the *Bam*HI site at poliovirus nucleotide 4600. The 2C protein resulting from either linker insertion has four additional amino acids.

rus mRNA that can subsequently be used to program an in vitro translation system, we cloned a complete cDNA copy of the poliovirus genome into plasmid pGEM-1 (24). The resulting transcription vector, pT7-1, (see Fig. 3), contained a bacteriophage T7 promoter 5′ to the poliovirus sequences and a polylinker containing a *Sac*I site 3′ to the poliovirus sequences. pT7-1 represents the template used for in vitro synthesis of wild-type (unmodified) poliovirus RNA, as well as the vector which is linearized within the poliovirus coding sequences to produce truncated transcripts. Insertion and deletion of sequences in pT7-1 generated several templates, which are discussed below.

Both wild-type and altered transcripts can be used to program an in vitro translation system to direct synthesis of poliovirus-specific polypeptides. Our interest in these studies was to determine the sequences required for cleavage of the capsid polyprotein precursor to capsid proteins, as well as to determine the sequences required by 2A proteinase to cleave the P1-P2 junction. To study the sequences required for 3C-mediated proteolytic processing of the capsid precursor, pT7-1 was linearized at several sites within the P1 coding region (Fig. 1A). pT7-1 (lin *Pst*I) and pT7-1 (lin *Hae*II) templates contain the coding sequences for VP0 and a portion of VP3. pT7-1 (lin *Xba*I), pT7-1 (lin *Sna*BI), pT7-1 (lin *Bst*EII), and pT7-1 (lin *Nde*I) templates contain the coding sequence for VP0 and VP3 and an increasing amount of the coding sequences for VP1. To observe in vitro 3C-mediated cleavage of an authentic P1 precursor, pT7-1 was linearized at the *Hinc*II site within 2B (Fig. 1A). The pT7-1 (lin *Hinc*II) template contains the coding region for P1, 2A proteinase, and a portion of 2B. We also generated two pT7-1 derivatives which contain four-amino-acid insertions in 2C. Plasmids pT7-1 (2C:*Eco*RI) and pT7-1 (2C:*Sac*I) are shown in Fig. 1B. To study the P1 sequences required for 2A-mediated proteolytic processing at the P1-P2 junction, we generated pT7-1 (P1:ΔNS) (Fig. 1B). T7-1 (P1:ΔNS) RNA contains the coding region for all of the P2 and P3 proteins but only the coding region for the amino-terminal portion of VP0 and the carboxy-terminal portion of VP1.

In vitro translation of P1-derived RNAs defines the sequences required for posttranslational cleavage of P1 to capsid proteins. RNAs were derived from poliovirus cDNA linearized at the restriction endonuclease recognition sites indicated in Fig. 1A. These transcripts were used to program in vitro translation in the presence of [^{35}S]methionine. For in vitro translation of poliovirus RNA, we used rabbit reticulocyte lysate supplemented with an extract (S-10) from uninfected HeLa cells. Supplementation of the lysate with HeLa cell S-10 was necessary because in vitro translation of poliovirus RNA in rabbit reticulocyte lysate results in aberrant polypeptides produced as a result of internal initiation of protein synthesis at several sites on the viral genome (4, 15). Addition of HeLa cell S-10 extract to reticulocyte lysate provides factors that suppress these internal initiation events and/or direct ribosomes to the authentic 5′-proximal initiation codon at nucleotide 743 on the poliovirus genome (2, 4). The virus-specific polypeptides produced by in vitro translation of our in vitro synthesized poliovirus RNAs are shown in Fig. 2. The wild-type T7-1 transcript produced a polypeptide pattern (lane 3) very similar to that seen when purified virion RNA was used to program the in vitro translation reaction (lane 2). The presence of the capsid precursor (P1) indicated that active 2A proteinase, which makes the Y-G cleavage between the P1 and P2 precursor (Fig. 1), was produced in the translation reaction. Production of capsid proteins VP0, VP3, and VP1, as well as proteins P3, 3CD, and 2C, indicated that 3C proteinase activity, which cleaves at Q-G bonds, was also produced in the translation reaction.

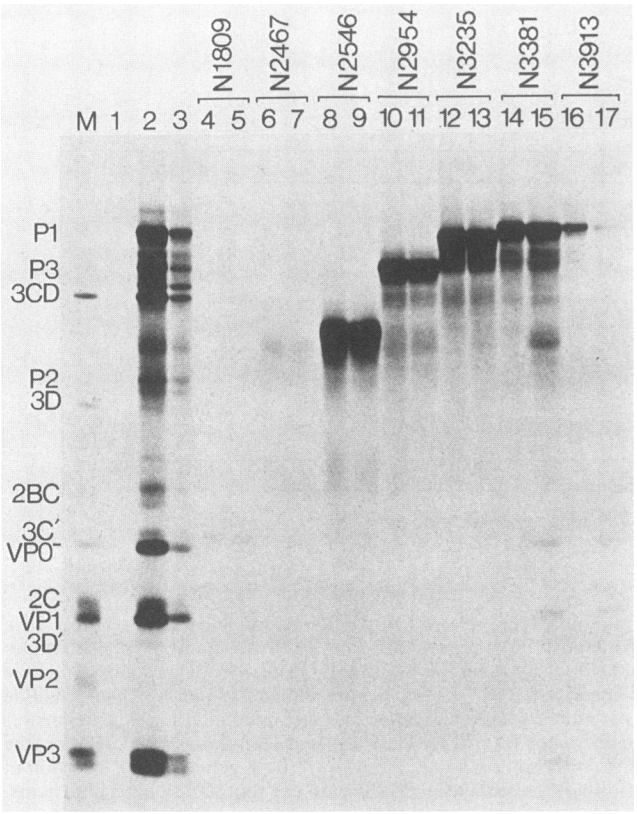

FIG. 2. In vitro translation of P1-derived transcripts and posttranslational addition of 3C proteinase activity. Transcripts containing a defined portion of the P1 coding region were generated by linearizing pT7-1 with restriction enzymes *Pst*I (N1809), *Hae*II (N2467), *Xba*I (N2546), *Sna*BI (N2954), *Bst*EII (N3235), *Nde*I (N3381), and *Hin*cII (N3913). Translation reactions were performed in rabbit reticulocyte lysate supplemented with uninfected HeLa cell extract. Some of the reactions were subsequently incubated with infected HeLa cell extract (see below). The reactions were programmed as follows (lanes): 1, no RNA; 2, PV1 virion RNA; 3, T7-1 RNA; 4 and 5, T7-1 (lin *Pst*I) RNA; 6 and 7, T7-1 (lin *Hae*II) RNA; 8 and 9, T7-1 (lin *Xba*I) RNA; 10 and 11, T7-1 (lin *Sna*BI) RNA; 12 and 13, T7-1 (lin *Bst*EII) RNA; 14 and 15, T7-1 (lin *Nde*I) RNA; 16 and 17, T7-1 (lin *Hin*cII) RNA. After 3 h of incubation at 30°C, cycloheximide and pancreatic RNase were added. A portion (10 μl) of the respective reaction mixture was used for each sample shown in the figure and incubated at 30°C as follows. Lanes 2, 3, 4, 6, 8, 10, 12, 14, and 16 received 3 μl of the lysis buffer used for cell extract preparation and were incubated for 1 h; lanes 1, 5, 7, 9, 11, 13, 15, and 17 received 3 μl of infected-cell extract and were incubated for 1 h. Samples were diluted with Laemmli sample buffer and subjected to electrophoresis. The marker (M) lane displays [^{35}S]methionine-labeled proteins from an extract of poliovirus-infected HeLa cells harvested at 5 h postinfection.

To determine the P1 sequences required for cleavage of P1 to capsid proteins, we linearized the pT7-1 template at several sites within the P1 coding sequence. RNA was transcribed from these templates and used to program in vitro translation reactions. Since the P1-derived transcripts do not contain the coding sequences for any P3-derived proteins, no 3C proteinase activity was produced. Therefore, a transcript containing a defined amount of P1 sequences produces a truncated polyprotein of predictable size. If the translation reaction is terminated by addition of cycloheximide and RNase and an infected HeLa cell extract is added to the reaction mixture, 3C proteinase activity is provided by the infected extract. As a positive control for *trans* proteinase activity in the infected-cell extract, we linearized the pT7-1 template within the 2B coding region. The T7-1 (lin *Hin*cII) transcript contains the coding sequences for all of P1, the complete 2A proteinase, and a portion of 2B (Fig. 1A). A translation reaction programmed with this RNA is shown in Fig. 2, lane 16. An authentic P1 precursor was produced by 2A proteinase, and lane 17 shows that the P1 protein generated in the translation reaction could be cleaved into capsid proteins by the 3C activity present in the infected-cell extract. Figure 2, lane 4, shows the translation product derived from a T7-1 transcript whose template was linearized at poliovirus nucleotide 1809 with restriction endonuclease *Pst*I. T7-1 (lin *Pst*I) RNA produced a 39-kilodalton (kDa) protein containing the entire amino acid sequence of VP0, the Q-G site between VP0 and VP3, and a portion of VP3. Addition of the infected-cell extract (Fig. 2, lane 5) to this translation did not result in cleavage of this 39-kDa protein into VP0 and a truncated VP3. To extend the analysis of sequence requirements for Q-G cleavage of P1-derived proteins, a translation reaction programmed with T7-1 (lin *Hae*II) RNA was carried out. Such a translation produced a 63-kDa protein which contained all of VP0, the VP0-VP3 Q-G cleavage site, and almost the entire VP3 amino acid sequence (Fig. 2, lane 6). Addition of the infected-cell extract (lane 7) did not result in cleavage of the 63-kDa protein into VP0 and VP3. Note that either (i) both the T7-1 (lin *Pst*I) and T7-1 (lin *Hae*II) transcripts translate inefficiently compared with longer transcripts (compare Fig. 2 lanes 4 and 6 with lanes 8, 10, 12, 14, and 16) or (ii) the protein products derived from these transcripts are unstable.

The in vitro translation products of transcripts derived from the pT7-1 template linearized within the coding region of VP1 are shown in Fig. 2. Transcripts derived from pT7-1 (lin *Xba*I), pT7-1 (lin *Sna*BI), and pT7-1 (lin *Bst*EII) produced proteins of approximate molecular masses 66, 81, and 91 kDa, respectively (lanes 8, 10, and 12). These proteins contained all of VP0, all of VP3, both the VP0-VP3 and VP3-VP1 Q-G cleavage sites, and increasing amounts of VP1 amino acid sequences. Addition of the infected-cell extract (lanes 9, 11, and 13) did not result in cleavage of these proteins to VP0, VP3, and a truncated version of VP1. Clearly, even though all of the VP3 sequences were present, the VP0-VP3 Q-G cleavage did not occur. The cleavage between VP3 and VP1 also did not occur when the infected-cell extract was added to the translation reaction mixture. Generation of a recognizable VP3-VP1 cleavage site did occur after translation of a T7-1 (lin *Nde*I) transcript (Fig. 2, lane 14). The protein product was nearly the size of authentic P1. When the infected-cell extract was added to the translation reaction mixture, cleavage of the pT7-1 (lin *Nde*I)-derived protein into capsid proteins VP0, VP3, and VP1 occurred (lane 5). Apparently, the additional VP1 sequences present in this truncated P1 precursor allow it to assume a recognizable conformation such that the 3C proteinase activity present in the infected-cell extract can cleave at both the VP0-VP3 and VP3-VP1 Q-G bonds. We conclude from these results that almost all of the P1 sequences are required for posttranslational cleavage of both of the capsid Q-G sites by 3C proteinase activity.

Translation of P1-derived RNAs in the presence of 3C proteinase activity defines the sequences required for cotranslational cleavage of P1 to capsid proteins. We demonstrated that almost all of the P1 amino acid sequences are required

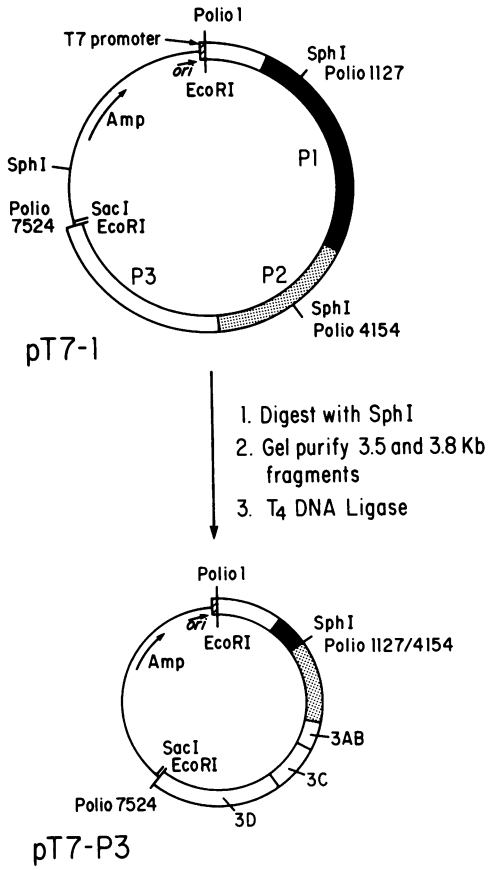

FIG. 3. Strategy for construction of the pT7-P3 template containing the P3 region of the poliovirus cDNA clone and the bacteriophage T7 promoter. Most of the P1 and P2 coding sequences were deleted to produce a template containing primarily P3 coding sequences. Kb, Kilobases; *ori*, origin of replication.

for posttranslational processing of P1 to capsid proteins. These results imply that the carboxy-terminal portion of the P1 precursor is required to allow P1 to assume a conformation that renders both the VP0-VP3 and VP3-VP1 Q-G sites recognizable by the 3C proteinase activity in the infected-cell extract. However, it is possible that processing of P1 proteins occurs most efficiently while the P1 protein is still being synthesized on the ribosomes. Perhaps the cleavage products were not observed because 3C proteinase activity was not present during protein synthesis. To test this possibility, P1-derived transcripts were translated in the presence of 3C proteinase activity. The source of cotranslational 3C activity was generated from a poliovirus cDNA deletion plasmid containing 3C proteinase sequences. This plasmid, pT7-P3 (Fig. 3), contains the coding sequences for all of the P3-derived proteins and a portion of VP0 and 2C. It was constructed by deleting most of the P1 and P2 coding sequences from pT7-1, as detailed in Fig. 3. The protein products from an in vitro translation reaction programmed with pT7-P3-derived RNA are shown in Fig. 4, lane 3. Four polypeptides were observed: P3, 3BCD, 3CD, and the fusion protein of approximate molecular mass 49 kDa containing the amino-terminal sequences of VP0 and the carboxy-terminal sequences of 2C. These results demonstrated that a defined 3C proteinase activity was produced in the translation reaction programmed by T7-P3 RNA and that the proteinase activity cleaved at all of the P3 Q-G bonds normally cleaved in the in vitro translation system.

Translation of P1-derived RNAs in the presence of T7-P3-generated 3C proteinase activity is shown in Fig. 4. In a translation reaction programmed with equimolar amounts of T7-P3 RNA and P1-derived RNAs, T7-P3 RNA translated more efficiently, possibly because of more efficient ribosome binding of this genetically altered mRNA. Therefore, the T7-P3 transcript was incubated in the translation reaction for 1 h to generate 3C proteinase activity, and then the P1-

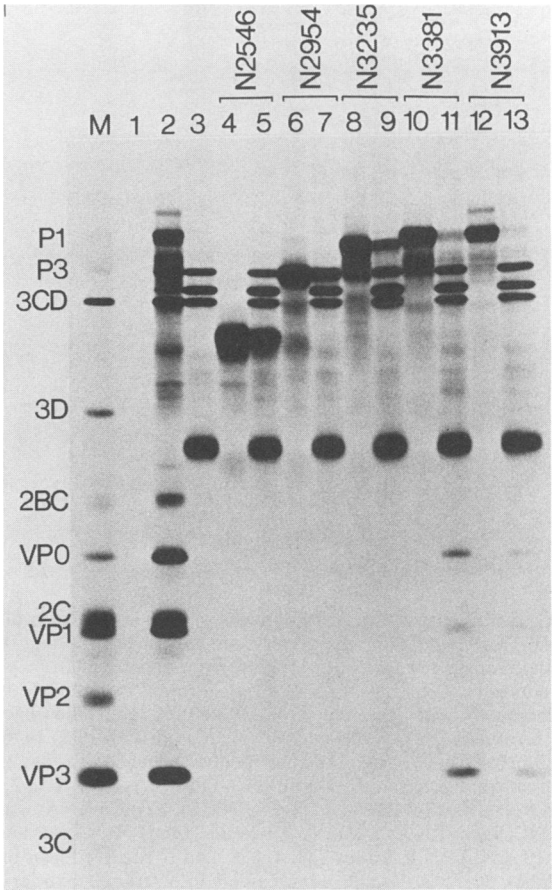

FIG. 4. In vitro translation of P1-derived transcripts in the presence of 3C proteinase activity. Transcripts containing a defined portion of the P1 coding region were generated by linearizing pT7-1 with restriction enzymes *Xba*I (N2546), *Sna*BI (N2954), *Bst*EII (N3235), *Nde*I (N3381), and *Hin*cII (N3913). The T7-P3 transcript contained the coding sequences for the P3 region and produced 3C proteinase activity when translated. All translations were carried out in rabbit reticulocyte lysate supplemented with uninfected HeLa cell extract. Translation and cotranslation reactions were carried out as described in Materials and Methods. The reactions using one species of RNA were programmed as follows (lanes): 1, no RNA; 2, T7-1 RNA; 3, T7-P3 RNA; 4, T7-1 (lin *Xba*I) RNA; 6, T7-1 (lin *Sna*BI) RNA; 8, T7-1 (lin *Bst*EII) RNA; 10, T7-1 (lin *Nde*I) RNA; 12, T7-1 (lin *Hin*cII) RNA. To synthesize P1-derived proteins in the presence of 3C proteinase activity, the translation reaction was programmed with T7-P3 RNA as well as RNA derived from the linearized cDNA template. These cotranslation reactions were programmed as follows (lanes): 5, T7-1 (lin *Xba*I) and T7-P3 RNAs; 7, T7-1 (lin *Sna*BI) and T7-P3 RNAs; 9, T7-1 (lin *Bst*EII) and T7-P3 RNAs; 11, T7-1 (lin *Nde*I) and T7-P3 RNAs; 13, T7-1 (lin *Hin*cII) and T7-P3 RNAs. The samples and markers (M) were prepared for electrophoresis as described in the legend to Fig. 2.

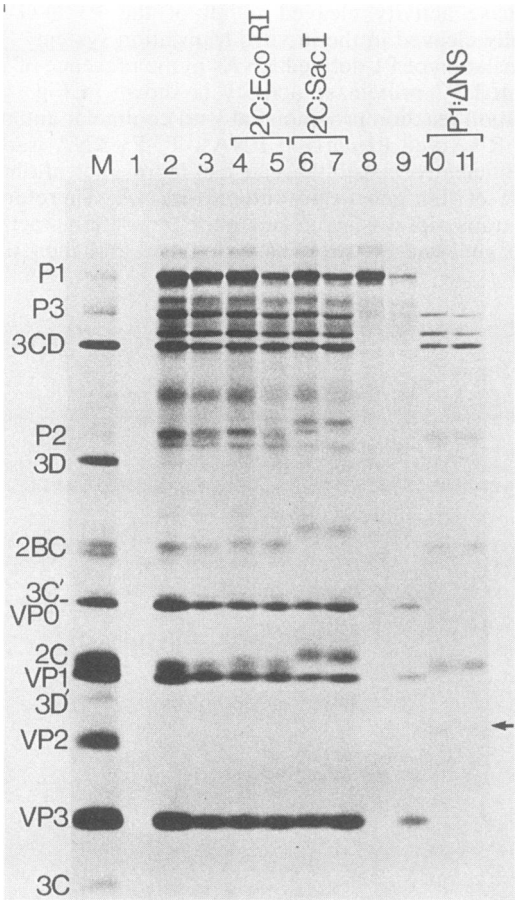

FIG. 5. In vitro translation of transcripts derived from altered cDNA. Transcripts containing insertions into the 2C coding region or a deletion in the P1 coding region were generated from pT7-1. Translation reactions were carried out in rabbit reticulocyte lysate supplemented with uninfected HeLa cell extract. The reactions were terminated and subsequently incubated with lysis buffer or infected HeLa cell extract, as described in the legend to Fig. 2. The reactions were programmed as follows (lanes): 1, no RNA; 2, PV1 virion RNA; 3, T7-1 RNA; 4 and 5, T7-1 (2C:*Eco*RI) RNA; 6 and 7, T7-1 (2C:*Sac*I) RNA; 8 and 9, T7-1 (lin *Hin*cII) RNA; 10 and 11, T7-1 (P1:ΔNS) RNA. Lanes 2, 3, 4, 6, 8, and 10 received lysis buffer, and lanes 1, 5, 7, 9, and 11 received infected-cell extract after translation was terminated. The samples and markers (M) were prepared for electrophoresis as described in the legend to Fig. 2. The arrow indicates a ca. 30-kDa protein immunoprecipitated with antisera specific for VP4 and VP1.

derived transcript was added to the translation reaction mixture at a fivefold molar excess. Both transcripts were then translated simultaneously for an additional 3 h (see Materials and Methods). Figure 4, lane 4, shows the 66-kDa translation product of a reaction programmed with T7-1 (lin *Xba*I) RNA. When both the T7-P3 and T7-1 (lin *Xba*I) RNAs were used to program a single translation reaction, five major protein products appeared. The 66-kDa protein was programmed by the T7-1 (lin *Xba*I) transcript, and the other four bands correspond to proteins programmed by the T7-P3 transcript (lane 5). No cleavage of the 66-kDa translation product to its processing products (VP0, VP3, and a truncated VP1) occurred, despite the presence of 3C proteinase activity in the translation reaction mixture. Similarly when the T7-1 (lin *Sna*BI) and T7-1 (lin *Bst*EII) transcripts were translated in the presence of 3C proteinase activity, no cleavage of the truncated P1 precursor to capsid proteins occurred (lanes 7 and 9). T7-1 (lin *Pst*I) and T7-1 (lin *Hae*II) transcripts were also translated in the presence of 3C proteinase activity. However, no cleavage of the P1 precursor into capsid protein products was observed (unpublished data). The translations of T7-1 (lin *Nde*I) and T7-1 (lin *Hin*cII) transcripts in the presence of 3C proteinase activity are shown in Fig. 4, lanes 11 and 13. As expected, processing of both the truncated P1 precursor and the authentic P1 precursor into capsid proteins occurred. These results clearly demonstrate that the carboxy-terminal portion of VP1 is required to allow P1 to assume a recognizable substrate conformation for 3C proteinase activity and imply that P1 processing to capsid proteins is posttranslational (presumably after P1-P2 cleavage by 2A).

Effects of specific insertions and deletions on 2A- and 3C-mediated processing. In a further attempt to define the structural requirements of substrates for 2A- and 3C-mediated protein processing, we analyzed the products of in vitro translation of transcripts derived from two poliovirus templates, each containing a four-amino-acid insertion in protein 2C. The translation products derived from a T7-1 (2C:*Eco*RI) transcript are shown in Fig. 5, lane 4. Note that the pattern of translation products produced by T7-1 (2C:*Eco*RI) RNA was virtually identical to the pattern of products produced by virion-derived and T7-1 RNA transcripts. The amino acid insertion (Arg-Asn-Ser-Asp) did not alter production of P1 by 2A proteinase or cleavage of Q-G sites by 3C proteinase. However, the insertion did result in a minor shift in the electrophoretic mobility of the P2 proteins. The altered P2, 2BC, and 2C proteins (lane 4) migrated more slowly than the authentic proteins (lane 3). Addition of the infected-cell extract to the translation reaction programmed by T7-1 (2C:*Eco*RI) RNA (lane 5) did not result in production of any new proteins. We also carried out in vitro translation of RNA derived from a cDNA clone containing a *Sac*I linker insertion in the 2C coding region. Note that the pattern of translation products produced by T7-1 (2C:*Sac*I) RNA (Fig. 5, lane 6) was nearly identical to the pattern of products produced by wild-type transcripts. However, there was a large electrophoretic mobility shift for the proteins containing 2C sequences (P2, 2BC, and 2C), indicating that the amino acid insertion (Pro-Ser-Ser-Asp) significantly altered the conformation of these proteins. Such an alteration did not affect 2A-mediated processing of the P1-P2 junction or any of the Q-G cleavages performed by 3C proteinase. Addition of the infected-cell extract to the translation reaction did not generate additional cleavage products (Fig. 5, lane 7). The complete processing of polypeptides containing the amino acid insertions in 2C indicates that these alterations of 2C did not alter 2A function or the ability of 3C to recognize the Q-G sites within P1, the Q-G sites flanking 2C, or any other Q-G sites in the polyprotein.

To determine the P1 sequence required for 2A-mediated cleavage at the P1-P2 junction, we analyzed the products of in vitro translation of transcripts derived from pT7-1 (P1:ΔNS). The coding sequences for the carboxy-terminal portion of VP0, all of VP3, and the amino-terminal portion of VP1 were deleted in this construct (including all three Q-G pairs present in P1). Figure 5, lane 10, shows the protein products derived from a T7-1 (P1:ΔNS) RNA. Production of P2- and P3-derived proteins which comigrate with the analogous proteins produced in a translation reaction programmed with T7-1 RNA demonstrated that the P1 deletion did not affect proteolytic processing of these proteins. The

presence of authentic P2 and 2A (seen in a longer exposure of Fig. 5) indicated that 2A cleaved the Y-G site between P1 and P2. The protein of approximate molecular mass 30 kDa (Fig. 5, lanes 10 and 11, arrow), which was produced in the T7-1 (P1:ΔNS) translation reaction, was immunoprecipitated with antisera specific for VP4 and VP1 (unpublished data) and was the size expected for a P1 protein containing the *Nru*I-*Sna*BI deletion. If the P1-P2 cleavage had not occurred during translation, a fusion protein containing the 2A and altered P1 sequences would have been produced. The absence of this fusion protein (estimated molecular mass, 50 kDa) indicated that the P1-P2 cleavage was made efficiently by 2A proteinase. Addition of the infected-cell extract did not produce new cleavage products in the T7-1 (P1:ΔNS) translation reaction (Fig. 4, lane 11). Complete processing of the polypeptide produced by T7-1 (P1:ΔNS) RNA also demonstrated that an alteration in P1 did not affect the 3C-mediated cleavages of the P2 and P3 precursors. It has been suggested that the P1-P2 cleavage occurs before the entire poliovirus transcript has been translated and perhaps immediately after the ribosome has trasversed the coding sequences for the amino-terminal portion of P2 (23). Our observation that a large deletion in P1 does not alter P2 or P3 processing supports these suggestions. In addition, cleavage of P1 from P2 by 2A proteinase in the translation programmed by T7-1 (P1:ΔNS) RNA suggests that the sequences encoded upstream of the *Sna*BI restriction site at poliovirus nucleotide 2954 are not required to generate a recognizable Y-G site at the P1-P2 junction.

DISCUSSION

We used in vitro translation of defined poliovirus mRNAs to determine some of the features of protein processing that regulate cleavage site recognition by 2A and 3C proteinases. To study how 3C proteinase selects appropriate cleavage sites within the capsid precursor protein (P1), we linearized a T7 expression vector containing poliovirus cDNA at several points within P1. The transcripts derived from these truncated templates were translated, and after translation, an infected-cell extract containing 3C proteinase activity was added to the reaction mixture. Using this approach, we demonstrated that almost all of the P1 precursor is required for cleavage of P1 to capsid proteins. We found that a truncated P1 protein which contained intact VP0 and VP3 sequences was not cleaved to VP0 and VP3 by posttranslational addition of the infected-cell extract. Thus, VP0 and VP3 do not in themselves contain the sequences required to present a recognizable Q-G site at the VP0-VP3 junction. In addition, a truncated P1 protein which contained all of VP0 and VP3 and greater than 80% of VP1 was not cleaved by addition of the infected-cell extract. This truncated protein must not contain the information to present a recognizable Q-G site at either the VP0-VP3 or VP3-VP1 junction such that posttranslational cleavage occurs. These studies indicate that, if 3C proteinase activity is added after the translation reaction is terminated, the carboxy-terminal portion of VP1 is required for cleavage of P1 to capsid proteins.

It is likely that 2A proteinase must liberate P1 from the rest of the polyprotein for cleavage of P1 to capsid proteins to occur. P1-P2 cleavage occurs while the polyprotein is still on the ribosomes and occurs so quickly that a P1-P2 protein is not normally observed (8, 22). Early in infection, the P1 precursor is liberated into an environment devoid of 3C proteinase activity, as was the case in our experiments when the infected-cell extract was added after translation was terminated. During the later stages of infection, it is likely that the P1 region is translated in the presence of 3C proteinase activity. It is possible that, in this circumstance, 3C proteinase encounters the P1 protein before it has folded into a final conformation. To test the possibility that the mechanism of P1 cleavage is different under such conditions, we cotranslated the truncated P1-derived transcripts with a transcript (T7-P3) containing proteinase sequences. The T7-P3 transcript produces a defined 3C proteinase activity which is virtually identical to that of the infected-cell extract. When the truncated P1 polypeptides were translated in the presence of T7-P3-derived proteinase activity, the results were identical to those obtained when the infected-cell extract was added posttranslationally. Thus, the mechanism of cleavage site recognition was the same in both cases, suggesting that the carboxy-terminal sequences of VP1 are essential for 3C-mediated cleavage of P1 to capsid proteins throughout infection.

X-ray crystallographic methods have determined that the three major capsid proteins (VP1, VP2, and VP3) of picornaviruses have several three-dimensional characteristics in common (7, 10, 19). Each contains a core consisting of an eight-stranded antiparallel β-barrel structure with two flanking helices. VP4 does not contain this structure. However, the VP4 sequences do not prevent formation of the β-barrel structure contributed by VP2 sequences in VP0. Cleavage of P1 to VP0, VP3, and VP1 forms the 6S protomer structure. The VP0-VP3 and VP3-VP1 Q-G sites are positioned between the three β-barrel domains, whereas the uncleaved Q-G amino acid pair in VP2 is located within the β-barrel structure and is shielded within the VP2 protein. After capsid protein cleavage, the amino-terminal arms of VP0, VP3, and VP1 are closely intertwined within one protomer unit, and it is probable this arrangement corresponds to a 6S assembly unit (7, 16). In our studies, the only truncated P1-derived protein which was cleaved into capsid proteins was produced by T7-1 (lin *Nde*I) RNA. The T7-1 (lin *Nde*I)-derived P1 precursor contains all of the P1 sequences involved in forming the three β-barrel structures (corresponding to VP0, VP3, and VP1) and only lacks the carboxy-terminal amino acid of VP1. In contrast, the truncated P1 proteins produced by RNAs derived from templates linearized at sites in the middle of the VP1 coding region [pT7-1 (lin *Sna*BI) and pT7-1 (lin *Bst*EII)] do not contain the sequences required to complete the β-barrel arrangement of VP1. It is possible that the complete VP1 β-barrel structure is required to generate a recognizable Q-G cleavage site at the VP0-VP1 junction. The T7-1 (lin *Hae*II) and T7-1 (lin *Xba*I) RNAs produce truncated P1 polyproteins which terminate in the region between the VP3 and VP1 β-barrel structures. Thus, the VP3 β-barrel structure is complete. Lack of cleavage by 3C proteinase at the VP0-VP3 junction suggests that a complete VP3 β-barrel is not sufficient to allow the VP0-VP3 Q-G site to assume a recognizable conformation. Perhaps it is the interaction of the VP1 β-barrel structure with VP3 moieties that generates a recognizable 3C cleavage site at the VP0-VP3 junction. Our results are in agreement with recent work by Nicklin et al. (10a), who also showed that a P1 protein linearized within the VP3 coding region was not a substrate for 3C proteinase activity. Construction of T7 templates containing deletions of the amino-terminal coding sequences of VP0, as well as generation of templates containing small insertions and deletions throughout the P1 coding region will allow further characterization of the cleavage of P1 to capsid proteins. We have previously observed that 3C-mediated cleavage of the

capsid precursor to capsid proteins requires additional proteinase sequences compared with cleavage of other noncapsid precursors to their respective processing products (24). Whereas the specific differences between these two mechanisms are unclear, it is attractive to speculate that one mechanism produces a proteinase containing 3C sequences which recognizes epitopes of the capsid proteins produced by the β-barrel structure, whereas the other mechanism produces a proteinase which also contains 3C sequences but does not recognize Q-G sites in the context of the β-barrel structure.

In the present study, we also examined the sequences involved in 2A-mediated processing of the Y-G site at the P1-P2 junction by in vitro translation of RNA derived from templates with alterations in sequences flanking the 2A proteinase. We demonstrated that an RNA containing poliovirus nucleotides 1 to 3913 [pT7-1 (lin *Hin*cII)] contains the sequences required to generate active 2A proteinase during in vitro translation. These results are in agreement with those reported previously by Toyoda et al., who showed that bacterial expression of active 2A proteinase required little, if any, of the coding sequences downstream from 2A (23). Thus, minimal carboxyl sequences flanking 2A are required to produce an active proteinase.

We have also presented data that analyzed the effects of both downstream and upstream mutations on 2A and 3C cleavage activities. For the downstream mutations, we generated templates with four-amino-acid insertions in 2C, [pT7-1 (2C:*Eco*RI) and pT7-1 (2C:*Sac*I)]. Translations of RNAs derived from these templates showed that 2A proteinase recognizes its cleavage sites despite the alteration in the conformation of P2 and the P2-derived proteins produced in the translations. Such conformational alterations can be clearly seen by the shift in the electrophoretic mobility of these proteins on a sodium dodecyl sulfate-polyacrylamide gel and must interfere with the in vivo function(s) of 2C, since full-length cDNA clones containing the above insertions do not produce infectious virus after transfection of primate cells in culture (L. J. Haywood and B. L. Semler, unpublished data). It is interesting that the *Eco*RI and *Sac*I linker insertions in 2C also had no effect on 3C processing of any of the P1 or P2 region polypeptides. These results demonstrate that a major alteration in P2 does not alter P1 processing and support data which indicate that P1 is processed after it is liberated from the rest of the polyprotein. Our results also suggest that both the 2A and 3C proteinases can accommodate these particular conformational alterations in P2 and still carry out their respective functions. As we have previously suggested, perhaps P2 region substrate-3C proteinase interactions are more flexible than those that occur with P1 precursor polypeptides (24).

Our studies aimed at determining the effects of upstream mutations on 2A-mediated cleavage of P1 from P2 focused on a template containing a deletion of poliovirus sequences 1172 to 2954 [pT7-1 (P1:ΔNS)]. RNA derived from this template produced a polyprotein which was correctly processed by 2A as well as 3C, suggesting that the entire P1 precursor is not required to generate a recognizable site for 2A. Specifically, it appears that VP0 and VP3 are not required for P1-P2 cleavage and, at most, the carboxy-terminal half of VP1 is required to generate the P1-P2 cleavage site. These conclusions are further supported by bacterial expression experiments in which the P1-P2 cleavage was made efficiently on a truncated P1 polypeptide that lacked 48 amino-terminal amino acids of VP4 (23). Thus, in contrast to 3C-mediated cleavage of the P1 precursor to capsid proteins, 2A-mediated cleavage of P1 from P2 does not require the sequences 5′ to poliovirus nucleotide 2954. The deletion in the T7-1 (P1:ΔNS)-derived P1 precursor eliminates the possibility that the VP1 β-barrel structure can form, suggesting that 2A proteinase does not recognize the β-barrel structure as part of the P1-P2 cleavage site. It is possible that a recognizable P1-P2 cleavage site is generated by VP1 sequences which have not yet folded into the β-barrel structure and as yet undetermined 2A sequences. However, because of the stability of the β-barrel structure, the VP1 β-barrel structure may be formed before cleavage of P1 from P2. In this case, 2A would recognize the P1-P2 cleavage site in the context of the "tail" of VP1 and 2A sequences. Further investigation into these and other poliovirus proteinase-substrate interactions should be possible by additional manipulations of the in vitro system described in this report.

ACKNOWLEDGMENTS

We thank J. M. Hogle and D. J. Filman for discussion concerning capsid protein structure and Lori Haywood for help with plasmid constructions.

M.F.Y.-W. is a predoctoral trainee of the Public Health Service (CA 09054), and B.L.S. is supported by a research career development award from the National Institutes of Health (AI00721). This work was supported by Public Health Service grant AI 22693 from the National Institutes of Health.

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