Encephalomyocarditis Virus 3C Protease: Efficient Cell-Free Expression from Clones Which Link Viral 5' Noncoding Sequences to the P3 Region

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All picornaviral peptides are derived by progressive posttranslational cleavage of a giant precursor polyprotein. Translation of encephalomyocarditis virus (EMC) RNA in rabbit reticulocyte extracts produces active viral peptides, including protease 3C, which is responsible for many cleavage reactions within the processing cascade. DNA plasmids containing 5' noncoding sequences of EMC linked to other portions of the viral genome were constructed and transcribed into RNA. Like virion RNA, the clone-derived transcripts directed efficient protein translation in vitro. The 5'-linked constructions may represent examples of a general method for cell-free expression of any cloned gene segment. One construction produced a self-cleaving P3 region precursor, which contained active 3C protease. A genetically engineered insertion within the 3C sequences eliminated endogenous self-cleavage activity without altering the ability of the P3 peptide to serve as substrate in bimolecular reactions with added 3C. Another plasmid encoding the L-VP0 portion of the capsid region was used to demonstrate that scission between the leader peptide (L) and capsid protein VP0 can be catalyzed by 3C. The enzyme responsible for this step was previously unidentified. A rapid purification scheme for isolation of 3C from EMC-infected HeLa cells is also presented.

Encephalomyocarditis virus (EMC) is a member of the cardioviral subgroup of the picornavirus family. All picornaviral genomes encode a single, large precursor polyprotein which represents most of the theoretical coding capacity of the RNA molecule (22). The polyprotein is processed in a series of proteolytic cleavage steps to yield mature virion capsid proteins and other nonstructural proteins (Fig. 1). At least three proteolytic activities are involved in these cleavages (15).

The first proteolytic event within the polyprotein takes place while the peptide is still nascent on a ribosome (12, 28). Recent experiments with poliovirus suggest that viral peptide 2A plays a role in this step (P1-P2 cleavage) (15). However, the primary cleavage event for EMC is not completely analogous, because it occurs at a different site within the polyprotein (2A-2B) and may be catalyzed by a peptide other than 2A. The last step in the proteolytic cascade is maturation processing of VP0 to VP4 and VP2 (13). An agent responsible for this event has never been isolated, but crystallographic data implicate a monomolecular, autocatalytic cleavage mechanism (21).

Most of the remaining polyprotein cleavages are carried out by the viral 3C protease (10, 17, 18). This enzyme is capable of mono- and bimolecular reactions within its precursor substrates (3ABCD to 3AB and 3C and 3D) (11, 19) and is also responsible for several processing steps within capsid precursor peptides (P1 to VP0 and VP3 and VP1) (9, 18). Although not all of the proteolytic sites within the EMC polyprotein have been precisely identified, those known to be cleaved by 3C occur only between glutamine-glycine or glutamine-serine amino acid pairs which are flanked by proline residues (17).

Because of its importance in the viral proteolytic processing scheme, we have directed our attention towards the 3C protease, focusing on the enzyme from EMC. Cardioviral RNA translates with unusually high efficiency in reticulocyte extracts, producing active 3C capable of processing reactions indistinguishable from those observed during viral infection of cells (28). We have taken advantage of this translational activity and constructed DNA plasmids which link EMC 5' noncoding sequences to other segments of the viral genome. Like virion RNA, the clones efficiently expressed viral peptides in cell-free reactions. One construction, encoding the P3 region of EMC, produced active 3C protease in vitro. We have used this plasmid to examine the effects of an engineered insertion into the putative active site of 3C.

We also report a new procedure for the rapid purification of the 3C protease from EMC-infected HeLa cells. Purified enzyme and plasmid-derived 3C have been tested for activity on various segments of the EMC genome. Using a clone which expressed the leader and VP0 peptides, we show that scission of the leader protein from its capsid precursor can be catalyzed by 3C.

MATERIALS AND METHODS

Nomenclature. EMC peptides are named by the L-4-3-4 conventions (24), except that traditional designations are used for the virion capsid proteins VP0, VP4, VP2, VP3, and VP1 (1AB, 1A, 1B, 1C, and 1D, respectively). Previously, peptides L-P1-2A, P3, 3CD, 3D, 3C, and 3AB were referred to as A1, C, D, E, p22, and H, respectively. Peptide ϵ 1 is a charge-altered form of VP0 peculiar to cell-free translation samples programmed with EMC RNA (16). The "large" fragment of RNA includes all heteropolymeric sequences 3'

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FIG. 1. Structure of the picornaviral genome. Viral RNA, encoded peptides, and their protein precursors are illustrated schematically. g' denotes the portion of the genome associated with guanidine resistance, and VPg is the viral protein covalently linked to the 5' end of the viral RNA (7).

FIG. 2. Isolation of protease 3C from EMC-infected HeLa cells. (A) Purification fractions. Equal amounts (about 60,000 cpm) of [3 H]leucine-labeled protein from purification fractions F1, F2, F3, and F4 (lanes 1 through 4, respectively) were fractionated by polyacrylamide gel electrophoresis and visualized by fluorography. The arrow indicates the position of the 3C peptide. (B) Activity of fractions. [35 S]methionine-labeled capsid precursor L-P1-2A, produced by in vitro translation (lane S), was incubated for 5 h at 30°C with 5 μ l of mock-infected F1 fraction (lane mock) or 5 μ l of purification fractions F1 through F4 (lanes 1 through 4, respectively) and then subjected to gel electrophoresis and visualized by autoradiography. Lane V contains EMC virion proteins as markers.

to the poly(C) tract. The "small" fragment includes all sequences 5' to the poly(C) tract (4, 5, 25).

Purification of 3C protease. The infection and growth of EMC in HeLa cells has been described previously (23). Cells (2.4×10^9) were infected (10 PFU/cell) with EMC and labeled with [^3H]leucine (Amersham; 131 Ci/mmol) at 0.01 mCi/ml from 3 to 4 h postinfection. The cells were washed once with phosphate-buffered saline (26) and then swollen for 5 min in 25 ml of ice-cold buffer A (10 mM Tris hydrochloride, pH 7.8, 0.1% 2-mercaptoethanol) before Dounce homogenization. Cellular debris was removed by centrifugation (5 min at 5,000 rpm and then 10 min at 10,000 rpm, Sorval type SS34 rotor, 5°C). The resulting supernatant (F1 fraction, 19.1 ml) was made 10 mM in EDTA and centrifuged for 2 h at 39,000 rpm in a type SW41 rotor at 5°C. The fraction 2 supernatant (F2, 19.3 ml) was mixed with 10 ml (packed volume) of buffer A-equilibrated carboxymethyl-Sephadex C-50 (Pharmacia Fine Chemicals) at 22°C for 15 min. After being washed with 60 ml of buffer A and then with 60 ml of buffer B (buffer A containing 80 mM NaCl), the beads were transferred to a column (gel bed 1.5 by 4.5 cm) and further washed with 400 ml of buffer B. The protein was eluted at 0.3 ml/h with buffer C (buffer A containing 0.35 M NaCl). The ^3H -containing fractions were pooled and concentrated with a Centricon-10 (Amicon) microconcentrator (F3 fraction, 440 μl). A portion of the F3 sample (300 μl) was injected into a gel filtration high-pressure liquid chromatography (HPLC) column (TSK-G3000SW, 7.5 by 300 mm; Toyo Soda Manufacturing Co., Ltd.) flowing at 0.5 ml/min with buffer D (50 mM sodium phosphate, pH 7.2, 0.05% 2-mercaptoethanol, 0.1 M NaCl). Fractions containing radiolabeled 3C were pooled, concentrated by Centricon-10 filtration, and stored at -70°C as the F4 fraction (220 μl).

Construction of plasmid DNAs. Restriction enzymes were purchased from New England Biolabs. DNA manipulations were done by standard methods (14). All transformations to ampicillin resistance were performed with *Escherichia coli* HB101. Construction of DNA plasmids pE5T10 and pE3T11 will be described in detail elsewhere. Briefly, EMC RNA was copied into double-stranded DNA. *EcoRI* DNA linkers (New England Biolabs) were ligated to the ends of the double-stranded DNA, and the resulting material was inserted into the *EcoRI* site of the transcription vector pSPT18 (Pharmacia Fine Chemicals). Plasmid pSPT18 is a derivative of plasmid pUC18 containing T7 transcriptional promoter sequences and a polylinker cloning site. The EMC-derived DNA within pE5T10 is approximately 7,500 base pairs in length and encodes the EMC RNA sequences which extend from the 3' side of the viral poly(C) tract (near base 320) through the poly(A) sequence at the extreme 3' end of the genome. Plasmid pE3T11 contains EMC sequences originating from within the poly(C) tract and extending 3' (about 2,300 bases) into the region encoding viral peptide VP3.

To construct plasmid pE5P3, DNA (3.5 μg) from plasmid pEM3 (17) was digested with *NruI* and *MluI*. The resulting 2,242-base-pair fragment, encoding nearly all of the P3 region of EMC, was isolated by preparative agarose gel electrophoresis. The purified fragment was then ligated (T4 DNA ligase; Promega Biotec) into 0.5 μg of pE5T10 DNA which had been previously cut with *BalI* and *MluI*. Samples of the reaction mixture were used to transform bacteria, and colonies were screened by restriction enzyme analysis (*KpnI* and *PstI*) for plasmids containing the appropriate EMC-derived segments. One representative clone was selected and designated pE5P3.

FIG. 3. Construction of plasmid pE5P3. The strategy used to link 5' noncoding sequences of EMC to a segment derived from the P3 region of the genome is outlined. (A) Sequence of EMC at the start of the polyprotein reading frame. The *BalI* restriction sequence is underlined, and the AUG codon which initiates translation is boldfaced. (B) 5' noncoding segment after *BalI* digestion and also the 5' sequence of the P3 segment produced by *NruI* digestion. (C) Completed construction resulting from blunt-end ligation of the segments, including the reconstructed reading frame.

To construct pE5P3c, purified plasmid DNA from pE5P3 (6 μg) was digested to completion with *BglII* and then reacted with 9 U of DNA polymerase Klenow fragment (Bethesda Research Laboratories) in the presence of dCTP, dGTP, dATP, and dTTP (final concentrations, 1 mM each) for 30 min at 20°C. The sample was extracted with an equal volume of phenol-chloroform (1:1) and precipitated with ethanol. The resulting pellet was suspended in buffer (22 μl) containing *ClaI* linker fragments (2.5 μg ; New England Biolabs, catalog no. 1001) which had been previously phosphorylated (14). The reaction mixture was incubated with 40 U of T4 DNA ligase for 3 h at 20°C and then precipitated with ethanol. Excess free linker fragments were removed by gel filtration (Sephacryl S-300; Pharmacia Fine Chemicals), and the plasmid DNA was digested with *ClaI*. Following purification by agarose gel electrophoresis, the plasmid DNA was redigested with *ClaI*, extracted with phenol-chloroform, precipitated with ethanol, and treated with T4 DNA ligase. A sample of the resulting DNA was used to transform bacteria. Colonies were screened for the presence of a unique *ClaI* site within the plasmid DNA. Clone pE5P3c was selected as representative of this construction.

To construct plasmid pE5LVP0, DNA from plasmid pE3T11 (1 μg) was digested with *XbaI* to completion. After

FIG. 4. Key elements of plasmids pE5P3 and pE5LVP0. The EMC genomic segments included within plasmids pE5P3 and pE5LVP0 are illustrated schematically. Important restriction sites used in the constructions are shown. Open boxes (T7 promoter and polylinker) represent sequences from the parental vector (pSPT18). The 5' noncoding sequences (5' NC) contained within the two plasmids were derived from the segment extending from the 3' side of the EMC poly(C) tract up to the AUG codon at the beginning of the polyprotein reading frame. Stippled areas denote viral coding sequences within the plasmids.

extraction with phenol-chloroform and precipitation with ethanol, the DNA was reacted with T4 DNA ligase, and a portion of the mixture was used to transform bacteria. The resulting colonies were screened for the size of the EMC segment. One representative isolate was chosen and designated pE5LVP0.

In vitro transcription of plasmid DNA. Purified plasmid DNA (14) was linearized by digestion with restriction enzyme *Bam*HI (clones pE5P3 and pE5P3c) or *Xba*I (clone pE5LVP0). After extraction with phenol-chloroform and precipitation with ethanol, the samples were suspended in water. Typically, about 1 μ g of linear plasmid DNA was transcribed in reactions (25 μ l) with T7 RNA polymerase as specified by the enzyme manufacturer (Bethesda Research Laboratories), except that the ribonucleotides and dithiothreitol were increased to 1 mM and 25 mM, respectively. RNase inhibitor (RNasin; Promega Biotec) was also included (1.5 U/ μ l). After incubation at 37°C for 1 h, the samples were extracted with phenol-chloroform, precipitated with ethanol, dried under vacuum, and suspended in water (10 μ l; estimated concentration, 1 μ g/ μ l).

Cell-free translation. In vitro translation reactions in reticulocyte extracts were carried out exactly as described previously (28). Typically, 3 to 5 μ l of plasmid transcription product (see above) was used to direct cell-free protein synthesis reactions (30 μ l) radiolabeled with [35 S]methionine

(specific activity, 1,100 Ci/mmol; final concentration, 1 μ Ci/ μ l). After 40 min at 30°C, reactions were stopped by addition of pancreatic RNase and cycloheximide (to 0.3 mg/ml each). EMC virion RNA was used to program reactions for the synthesis of radiolabeled capsid precursor protein L-P1-2A and also for synthesis of the complete polyprotein as described previously (18, 28). Nonradiolabeled reaction products to be used for protease assays were synthesized in lysate mixtures lacking [35 S]methionine but supplemented with 100 μ M unlabeled methionine.

Gel electrophoresis. The method for analytical polyacrylamide slab gel electrophoresis has been described previously (18). Urea (5 M) was included in the gels to aid in the separation of EMC-specific proteins. Protein bands labeled with [35 S]methionine were visualized by autoradiography of the dried gels. Protein bands labeled with [3 H]leucine were visualized by fluorography (2). 35 S-labeled virion proteins used as markers were produced as described previously (23).

RESULTS

Purification of the EMC 3C protease from infected HeLa cells. EMC-infected HeLa cells were labeled with [3 H]leucine to facilitate detection of viral peptides during purification. Clarified supernatant from lysed cells was used

as starting material for 3C protease preparations (F1 fraction). After high-speed centrifugation, the resulting supernatant, F2, was mixed with carboxymethyl-Sephadex. Bound protein was eluted by a high-salt wash (F3 fraction) and further fractionated by HPLC gel filtration. Samples containing ^3H -labeled 3C peptide were pooled and concentrated (F4 fraction).

Figure 2A shows the ^3H -labeled proteins present in F1, F2, F3, and F4. Approximately equal amounts of radioactivity were applied to each sample lane. The intensity of the 3C band relative to those of other viral proteins increased throughout the purification scheme, with the greatest enrichment in the cation exchange chromatography step (F3 fraction). HPLC of the F3 fraction resulted in a 3C preparation which was substantially free of other viral proteins (lane 4, Fig. 2A). The gel depicted in Fig 2A was reexposed for a longer time (10 days), but ^3H -labeled bands other than 3C were still not detectable. Coomassie blue staining of similar preparations revealed small quantities of unlabeled cellular protein in the F4 samples. Based on densitometric tracings of fluorograms, we estimate that at least a 100-fold purification of peptide 3C was achieved between the F1 and F4 samples (not shown).

Fractions F1 through F4 were assayed for the ability to cleave EMC capsid precursor L-P1-2A. The [^{35}S]methionine-labeled substrate was synthesized in a limited cell-free translation reaction directed by EMC virion RNA (18). An autoradiogram showing the peptides produced after reaction of the substrate with enzyme fractions is presented in Fig. 2B. The substrate (lane S) was unchanged by incubation with extract from uninfected HeLa cells (mock lane). However, incubation with samples from any of the four enzyme fractions resulted in cleavage of the substrate into mature capsid proteins (VP0, VP3, and VP1) and their processing intermediates (P1-2A, P1, and 1ABC). Peptides L (leader), 2A, and ϵ 1 were also evident. These peptide profiles are exactly as expected for normal processing of the L-P1-2A precursor by the 3C protease (9, 18, 27).

During typical cell-free protease assays, it is often difficult to detect VP0 and VP3, as these proteins represent the last 3C-catalyzed cleavage step in the capsid region processing cascade (12, 18). Therefore, the VP0 and VP3 bands in Fig. 2B are indicative of a high level of 3C protease activity in the enzyme fractions. Although we do not yet have a method which precisely quantifies the relative specific activity of 3C enzyme samples, we conclude that our rapid isolation procedure yields preparations of active, radiochemically pure 3C protease. Purified F4 samples retained cleavage activity after storage at -70°C (not shown).

Cloning and expression of the P3 region of EMC. The RNA of EMC, unlike that of poliovirus or rhinovirus, translates with unusually high efficiency in cell-free reactions (28). We believe this property to be a function of the 5' noncoding region of the genome, specifically the segment extending from the 3' side of the poly(C) tract up to the AUG initiation codon for the polyprotein (4, 5). The ability of clone-derived transcripts to serve as mRNA in cell-free reactions can be destroyed by alterations or deletions in these sequences (manuscript in preparation).

The complete nucleotide sequence of EMC has now been determined (17; unpublished data). The fortuitous position of a *BalI* restriction enzyme recognition sequence (UGGCCA) as part of the polyprotein translation initiation codon suggested a general method for the cell-free expression of viral coding sequences by linking them to the EMC 5' noncoding region. We have used this technique to express the peptides

FIG. 5. Translational time course of pE5P3-derived RNA in reticulocyte extracts. Plasmid DNA was transcribed *in vitro*, and the products were used to program cell-free protein synthesis. Samples ($2\ \mu\text{l}$) were removed at the indicated times (in minutes), made 2% in sodium dodecyl sulfate and 1% in 2-mercaptoethanol, and heated to 90°C (5 min). Acetone precipitation, electrophoresis, and autoradiography of the samples were as described previously (18). The marker lane (M) represents a standard *in vitro* translation sample programmed with EMC virion RNA. The designations P3', 3A'BC, and 3A'B denote peptides containing the truncated form of EMC protein 3A, as described in the text.

from the P3 region of the EMC genome *in vitro*, including an active 3C protease (Fig. 3).

As a result of the sequencing work (17), bacterial DNA plasmids containing portions of the EMC genome became available. Construction pE5T10, containing the large fragment of EMC, was used as the source of 5' noncoding and plasmid vector sequences. Clone pEM3 was used to supply the P3 region segment because the viral sequences contained within this clone were specifically determined (17). Plasmid DNA from pEM3 was digested with restriction enzymes *NruI* and *MluI*. The gel-purified fragment (encompassing the P3 region) was ligated into pE5T10 which had been cut with *BalI* and *MluI*. Linkage of these two fragments coupled the normal EMC polyprotein initiation codon into the same translational reading frame that produces the P3 protein. The key elements of the resulting clone, pE5P3 (EMC, 5' noncoding, P3), are illustrated in Fig. 4.

RNA transcribed *in vitro* from pE5P3 was used to direct translation reactions in reticulocyte extracts. A typical time course of protein synthesis is shown in Fig. 5. After 10 min of incubation, a large peptide (P3') was evident, with an electrophoretic mobility slightly faster than the authentic P3 marker peptide. The smaller size of P3' relative to P3 was expected, as cleavage with *NruI* during the cloning procedure resulted in a DNA fragment missing 33 codons from the 5' end of the P3 region (amino end of the peptide; refer to Fig. 4).

With increasing time of incubation, other bands (3CD, 3D, 3A'BC, 3C, and 3A'B) also appeared in the translation

FIG. 6. Construction of plasmid pE5P3c. The procedures used to insert 12 bases into the 3C region of clone pE5P3 are outlined. The *Bgl*/II restriction sequence and the sequence of the inserted *Cla*I linker are underlined. Cys-159 is denoted with an asterisk (*). Amino acid residues unique to clone pE5P3c (PSMD) are in boldface type.

samples. The overall peptide profile is characteristic of normal P3 region processing catalyzed by viral protease 3C (12, 18, 19). This result indicates that the 3C sequences contained within P3' were active when expressed in vitro. Peptides 3A'BC and 3A'B were like P3' in that they represented slightly shorter versions of their normal processing counterparts. Tryptic profiles (not shown) of clone-derived 3D and 3D isolated from infected cells were indistinguishable, confirming that correct processing had indeed occurred and that the normal viral translational reading frame was expressed in vitro. These results are the first demonstration that the P3 peptide of EMC is capable of autocatalytic processing in the absence of peptides from the L, P1, and P2 regions of the viral genome.

Inactivation of cloned 3C protease by an engineered insertion. EMC 3C protease belongs to the class of thiol enzymes (8, 20). Sequence homology with other picornaviral proteases suggests that the catalytically active residues within 3C include Cys-159 and His-177 (EMC 3C has a total of 205 residues) (1). To test the effects of genetic manipulation on this region, 12 nucleotides were inserted into a unique *Bgl*/II restriction site within the 3C region of pE5P3 to form pE5P3c. As a result of this insertion, plasmid pE5P3c contained four extra codons (Pro, Ser, Met, and Asp) between the sequences encoding Cys-159 and His-177 (Fig. 6).

In vitro transcription and translation of pE5P3c DNA produced a peptide (P3'c) which was similar in size to that expressed by the parental clone (Fig. 7, lane 1). However, unlike P3', the P3'c peptide showed no evidence of spontaneous processing even after prolonged incubation (lane 2).

Addition of active 3C protease from exogenous sources resulted in the normal appearance of 3CD, 3D, 3C, 3A'BC, and 3A'B. The processing occurred after the addition of enzyme isolated from infected cells (F4 fraction, lane 5) or of protease from a cell-free translation of virion RNA (lane 3) or pE5P3-derived RNA (lane 4). The data show that the four-codon insertion inactivated the proteolytic ability of the endogenous 3C sequences within P3'c, but did not affect the capacity of P3'c to serve as substrate in bimolecular reactions with added 3C enzyme.

Cleavage of the leader peptide is catalyzed by protease 3C. The protease responsible for the first cleavage event within the EMC capsid region (L-P1-2A to L and P1-2A) has never been specifically identified (3, 27). Using a cloned construction, pE5LVP0 (EMC, 5' noncoding, Leader, VP0), which expressed a peptide containing this site, we tested the ability of various 3C enzyme preparations to catalyze scission of the leader protein from its precursor.

Clone pE5LVP0 contains a 5' noncoding segment of EMC and extends 3' from the polyprotein initiation site through the leader and VP0 coding regions, stopping just short (two amino acids) of the VP0-VP3 cleavage site (Fig. 4). Cell-free transcription and translation of plasmid-derived RNA produced an L-VP0 peptide (Fig. 8, lane 1) which was stable after incubation in reticulocyte extracts (lane 2). However, addition of either F1 or F4 protease from infected cells (lanes 7 and 8) or 3C expressed from pE5P3 (lane 5) resulted in cleavage of L-VP0 into a leader-sized peptide (L) and VP0 (1AB and ε1). The L-VP0 peptide was not affected by incubation with extracts containing brome mosaic virus translation products (lane 3), EMC capsid precursor protein

(lane 4), or the P3'c peptide (lane 6). The VP0 derived from the cloned peptide (L-VP0) contained the same methionine-labeled tryptic fragments as authentic VP0 produced in vivo (not shown).

These experiments show that L-VP0 cleavage is catalyzed by viral protease 3C and not, as often speculated, by a "host" or other reticulocyte enzyme (17, 27, 28). Furthermore, this result is evidence that clone-derived 3C protease (pE5P3) can catalyze a capsid region processing step in a manner similar to that of the enzyme isolated from infected HeLa cells.

DISCUSSION

Among picornavirus RNAs, the genome of EMC is unusual in its ability to direct high levels of viral protein synthesis in reticulocyte extracts (28). Past experiments have suggested this property might reside primarily within the 5' noncoding region of the genome (833 bases), between the poly(C) tract (bases 149 through 291) and the polyprotein initiation codon (base 834) (5; unpublished observations). Our engineered constructions support this idea. Plasmids pE5P3 and pE5P3c contained only 480 5' noncoding viral

FIG. 7. Expression and cleavage of P3'c protein. Plasmid DNA from pE5P3c was transcribed in vitro, and the products were used to program [³⁵S]methionine-labeled protein synthesis in reticulocyte lysate. After 40 min of incubation at 30°C, the reaction was stopped by addition of cycloheximide and RNase, and a sample (2 µl) was saved (lane 1). Similar samples were mixed with buffer (2 µl of phosphate-buffered saline, lane 2), fraction F4 protease (5 µl, lane 5), a lysate sample which had translated EMC virion RNA (1 µl, lane 3), or a lysate sample which had translated pE5P3-derived RNA (1 µl, lane 4). After further incubation at 30°C (12 to 15 h), the samples were fractionated by gel electrophoresis and visualized by autoradiography. The marker lane (M) represents a standard in vitro translation sample programmed with EMC virion RNA.

FIG. 8. Expression and cleavage of L-VP0 protein. Plasmid pE5LVP0 was transcribed and translated as described for plasmid pE5P3c in the legend to Fig. 7. A portion (2 µl) of the [³⁵S]methionine-labeled sample was saved (lane 1). Similar samples were mixed with 1 to 3 µl of untranscribed lysate (lane 2), lysate which had translated brome mosaic virus RNA (lane 3), lysate containing EMC capsid precursor proteins (lane 4), lysate which had translated pE5P3-derived RNA (lane 5), lysate which had translated pE5P3c-derived RNA (lane 6), fraction F1 protease (lane 7), or fraction F4 protease (lane 8). Samples were incubated (30°C, 3 h) and then analyzed by gel electrophoresis and autoradiography. Lane M represents peptides produced during cell-free translation of EMC virion RNA.

bases, derived from the genomic segment immediately 5' to the polyprotein initiation codon. Sequences from the small fragment, poly(C), L, P1, and P2 regions were absent. Yet, like virion RNA, the clone-derived transcripts were efficiently translated in vitro. While our experiments were not intended to precisely define the particular viral sequences required for efficient protein synthesis, it is clear that the clones contain all the elements necessary for cell-free translation.

Unlike most eucaryotic mRNAs, translational activity directed by picornavirus RNAs does not require a 5' capping group (7). It is interesting that our plasmid-derived transcripts likewise retained this viral property and expressed protein effectively in vitro without treatment in specific capping reactions.

Based on our results, we propose that the 5'-linked viral constructions represent examples of a general method for cell-free expression of any coding segment from the EMC genome. By taking advantage of the *BalI* restriction sequence at the EMC polyprotein initiation codon, it should also be possible to construct 5'-linked hybrid plasmids which actively express coding sequences from different viruses (e.g., polio- or rhinovirus) or from other exogenous sources.

The EMC P3 region sequences within clones pE5P3 and pE5P3c were correctly expressed in vitro into peptides P3' and P3'c. The P3' protein contained an active 3C protease, as evidenced by the typical P3 region processing profile

during translation reactions (12, 18, 19). The coding segment within pE5P3 was derived from pEM3, a clone which was isolated and examined as part of the EMC sequencing project (17). Thus, the *in vitro* activity shows that the published version of the 3C sequence is that of a competent viral protease.

Expression and processing of P3' demonstrated that peptides from other regions of the EMC genome (L, P1, or P2) are not required in any way for proper cleavage of the P3 region. The spontaneous nature of the P3' cascade supports previous contentions that the endogenous 3C protease is capable of monomolecular autocatalysis (11, 19). That is, 3C sequences within the precursor molecule (P3, 3ABC, or 3CD) release themselves by catalyzing self-cleavage reactions. Our experiments with P3'c, which contains an insertion within the putative active site of 3C, strengthen this argument, as inactivation of the endogenous enzyme seemed to preclude self-cleavage. The P3'c peptide, however, was still functional as a substrate in bimolecular reactions catalyzed by added 3C.

The results with pE5P3 and pE5P3c provide further evidence that the P3 region processing cascade can be carried out by two independent, autocatalytic pathways, with either monomolecular or bimolecular reaction mechanisms. Previously, the two pathways were distinguishable only by dilution processing experiments (19). We can now use P3'c to study exclusively the bimolecular P3 region reactions in the absence of any monomolecular cleavage. Furthermore, we can use pE5P3 as a convenient *in vitro* source of active 3C protease free of viral peptides from the L, P1, and P2 regions.

We have also developed a rapid purification scheme for the isolation of 3C from EMC-infected HeLa cells. In the past, enzymatic activity was partially purified from cell-free translation samples programmed with virion RNA (18). Alternatively, 3C was isolated from virus-infected Krebs-2 cells by lengthy chromatographic steps after denaturation with urea (8). Our method is based on cation exchange and takes advantage of the high positive charge on the EMC 3C peptide (6). The procedure results in stable preparations of highly active protease free of other detectable viral proteins.

The purified 3C and pE5P3-derived enzymes were equally effective in processing reactions with substrate prepared from clone pE5LVP0. This result demonstrates that 3C protease can catalyze the scission of the leader protein from its precursor, the first step within the EMC capsid region cascade (3, 27). This event had previously been attributed to an unidentified host protease (17, 27, 28). The L-VP0 cleavage probably occurs between the glutamine-glycine amino acid pair at position 67-68 of the polyprotein (17). This is the only EMC 3C site thus far identified which is not flanked by a proline residue. Future mutagenesis experiments with pE5LVP0 and other clones will be directed toward determining the effects of flanking sequences on protease recognition of the cleavage sites.

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