Inducible Expression of Encephalomyocarditis Virus 3C Protease Activity in Stably Transformed Mouse Cell Lines

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An inducible expression vector system has been developed to facilitate the study of the effects of individual virus gene products on cell function. The vector utilizes the mouse metallothionein promoter carried on the bovine papillomavirus genome. Conditions which optimize the induced expression of open reading frames inserted downstream from the mouse metallothionein promoter have recently been described. In this communication we describe the use of this system to clone and express the encephalomyocarditis virus 3C protease in cultured mouse cells. Stably transformed cell lines could be induced to produce levels of 3C protease activity comparable to those observed during normal virus infection. In spite of this, no effects on cellular protein synthesis rate or membrane permeability were observed. It was also discovered that 3C protease as well as 3C protease-containing polyproteins are turned over. This was true not only in the induced cell clones, but also during the normal course of encephalomyocarditis virus infection, as well as in translation systems *in vitro*. This phenomenon was highly specific for this family of polypeptides, perhaps explaining their apparent lack of cytotoxic effects.

One of the least understood subjects in virology is the mechanism of viral cytopathogenesis (2). The identification and characterization of specific viral gene products responsible for cytotoxic effects, and the mode of action of these products during an infectious cycle, remain fundamental problems. A major difficulty facing researchers interested in this question has been the lack of a suitable experimental system. Clearly, transient transfection of cells with viral genes is usually not a viable approach, due to the variable fraction of cells that take up and express DNA and to cytopathic effects caused by the procedure itself. To avoid these difficulties, a system is required which enables individual viral genes to be stably inserted into growing cells in an unexpressed mode and then to be induced at some later time under well-defined conditions. This necessitates that a mechanism must be available to tightly regulate the expression of the inserted viral genes without damaging the cells artifactually.

The development of a method for the induction of high levels of expression of genes inserted behind the mouse metallothionein (mMT-I) promoter has been recently reported (30). This procedure employed a modified version of the expression vector system first described by Pavlakis and Hamer (23). The original vector contained the bovine papillomavirus genome into which the mMT-I gene and a modified pBR322 segment had been inserted. This vector was subsequently improved by deleting the mMT-I 5' untranslated region and by inserting a polylinker and a chloramphenicol acetyltransferase (CAT) gene downstream of the mMT-I transcription initiation site. Transfection of cultured mouse cells with these plasmids leads to the formation of stably transformed cells (23, 25, 28, 30). Activation of the promoter by treatment of the cells with zinc in the presence of cycloheximide, followed by maintenance of the cells in dactinomycin or cordycepin (*i.e.*, superinduction), has led to

as much as a 50-fold increase in production of proteins coded for by sequences downstream from the mMT-I promoter (30). We report here our first attempt to use this expression system for the identification and study of viral genes coding for cytotoxic proteins.

As a candidate cytotoxic gene we selected the encephalomyocarditis (EMC) virus genomic region which codes for the EMC virus 3C protease (17, 18). We reasoned that production of a foreign protease in a cell might be expected to have detrimental effects upon cell structure and function. EMC virus 3C protease is a 22-kilodalton (kDa) protein which plays a major role during the virus replication cycle (10, 15, 16). Like all picornaviruses, EMC virus contains a single positive-stranded RNA which codes for a single large polyprotein. Current nomenclature divides this polyprotein into a leader (L) segment and three polyprotein segments, P1, P2, and P3 (reading from the amino to the carboxy terminus). These polyprotein segments, as well as the individual virus proteins subsequently derived from them, are generated by a proteolytic cascade in which the polyprotein precursor is cleaved into smaller precursors and then into the functional proteins (10, 15). Most of the cleavages in this cascade are carried out by the 3C protease, which resides in the P3 segment of the polyprotein precursor (17, 18). 3C protease functions intramolecularly to cleave itself out of the precursor (6, 19), and it also acts to cleave out other EMC virus proteins at certain glutamine-glycine and glutamine-serine junctions (17, 22).

Here we describe the successful cloning and inducible expression of the EMC virus 3C protease (and most likely the neighboring 3B genomic protein) in cultured mouse cells. No evidence of cytotoxic effects attributable to these proteins was detected. However, novel information has been obtained concerning the surprising lability and the extremely high specificity of the protease. In addition, the data demonstrate the potential usefulness of this inducible vector system for producing significant quantities of specific viral proteins in stable cell lines. This should facilitate efforts to

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FIG. 1. Inducible expression vector and construction of plasmids carrying EMC virus sequences. (A) The parental expression vector. pBMC3 and pBMC4 are identical except for the orientation of the inserted polylinker (indicated by the dotted line). Solid lines represent bovine papillomavirus sequences (bacterial pML DNA, which carries an ampicillin resistance gene and a bacterial origin of replication, is not shown [30]). Open boxes represent the mMT-I gene, from which the 5' untranslated region has been deleted, and flanking sequences. Stippled areas represent introns. The hatched box represents sequences coding for CAT. (B) Sequence of the polylinker region of pBMC3. Unique restriction sites are indicated. The mMT-I TATA box is marked by the double underline. The transcription start site is indicated by the arrow. Potential translation initiation codons are boxed, and termination codons are underlined. (C) Features of the expression vector pBMCE1. The sequences coding for individual EMC virus proteins and CAT are labeled. Incomplete sequences are marked by a prime mark ('). Restriction sites are marked: BII, *Bss*HII; SI, *Sal*I; XI, *Xho*I; EI, *Eco*RI. (D) Features of the in vitro transcription vector pE3A'BCD'. To prepare mRNA in vitro, pE3A'BCD' was linearized with *Eco*RI and employed as a template for SP6 RNA polymerase.

understand virulence and virus gene product action in infected cells.

MATERIALS AND METHODS

Plasmids. The parental expression vector pBMC3 was constructed through a complex series of manipulations. (Complete details of the construction of the expression vector pBMC3 and pBMC4 are too involved to present in this communication. Individuals requesting the vectors will be provided with such a description as well as restriction maps.) Briefly, the 741-base-pair *Hind*III-to-*Ban*I fragment from pKK232-8 (Pharmacia), which contains the sequence coding for CAT, was rendered blunt by treatment with *Escherichia coli* DNA polymerase Klenow fragment. This DNA was inserted into a unique *Sma*I site located downstream from the mMT-I promoter in the mMT-I gene, carried by a modified version of pJYMMT(E) (5, 25). The resulting plasmid was digested with *Hind*III to obtain a 3,940-base-pair fragment which includes the mMT-I gene with the inserted CAT sequence. This DNA fragment was then inserted into the pBPV2308 shuttle vector (23, 25), which had been digested with *Hind*III. The resulting plasmid, pBMC1, was treated with *Sal*I, *E. coli* DNA polymerase

Klenow fragment, and T4 DNA ligase to remove the *Sal*I site present in the bovine papillomavirus sequence. This results in pBMC2. A 39-base-pair DNA sequence with *Xho*I-compatible termini and carrying restriction endonuclease sites not present in pBMC2 (i.e., a polylinker) was synthesized. pBMC2 was digested at a unique *Xho*I site located between the mMT-I transcription start site and the beginning of the CAT coding sequence. The synthetic DNA was inserted into this site, which gave rise to two plasmids, pBMC3 (Fig. 1A and 1B) and pBMC4, which are identical except for the orientation of the polylinker.

The expression plasmid carrying EMC virus 3C protease coding sequences was prepared as follows. pBMC3 was digested at the *Eco*RV and *Apa*I sites present in the polylinker (Fig. 1B). The 864-base-pair *Nru*I-to-*Apa*I fragment, which includes sequence coding for part of 3A (3A'), all of 3B and 3C, and a small portion of 3D (3D'), was prepared from pEM3 (17). (The primes are used here to indicate incomplete sequences.) Ligation of this DNA fragment into the treated pBMC3 resulted in pBMCE1 (Fig. 1C). An in vitro transcription vector carrying the identical EMC virus coding segment was constructed by digesting pBMCE1 with *Sal*I and *Eco*RI (located in the CAT gene) and inserting this

fragment into pGEM-3Z (Promega Biotec) digested with the same endonucleases. This plasmid was designated pE3A'BCD' (Fig. 1D). Another transcription vector, pE5LVPO, which carries sequences coding for the EMC virus leader (17) protein and proteins 1A and 1B' (the carboxy-terminal amino acid is missing from the 1B protein coded for by this sequence), has been previously described (21).

Isolation of cell lines, superinduction, and preparation of lysates. pBMCE1 and pBMC3 were introduced into mouse C127 cells by cotransfection with pSV2neo (29). Clones resistant to G418 were selected (29), and cell lysates were assayed for CAT activity. Those lines constitutively expressing high levels of CAT were maintained for study. For some experiments an additional cell line, designated 400 (prepared by S. Daniels-McQueen), was employed. The 400 line was derived by transforming C127 cells with pBMCF1, which is the pBMC3 parental vector containing the coding sequence for rabbit ferritin inserted into the polylinker. These cells were useful because they constitutively produce very high levels of CAT activity.

To induce the activation of the mMT-I promoter, the superinduction protocol was executed (30), with modifications prescribed by the specific experiment. Typically, cells cultured in Costar six-well dishes (9 cm² per well) for 48 h in Earle minimal essential medium containing 2% fetal calf serum were treated with 30 μ M ZnSO₄ plus 1 μ g of cycloheximide per ml for 6 h (30). This medium was removed, the cells were washed three times with Earle balanced salt solution, and then medium containing either 2 μ g of dactinomycin per ml or 50 μ g of cordycepin per ml was added. At appropriate times, cells were washed three times with physiologically buffered saline (PBS) and scraped into the same solution. Cells were pelleted by centrifugation and then lysed in buffer D (10 mM HEPES [N-2-hydroxyethyl]piperazine-N'-2-ethanesulfonic acid; pH 7.5], 10 mM KCl, 1.5 mM MgCl₂, and 2 mM dithiothreitol), 0.25 M Tris hydrochloride (pH 7.5), or immune buffer (30), depending upon the particular assays to be performed.

Infections with EMC virus. The EMC virus preparation was the generous gift of A. Burness and associates. Virus infection of transformed C127 cells was carried out with attached cells cultured in either 100-mm dishes or Costar 6-2311 dishes as previously described (26). The infections employed 10 or 20 PFU of virus per cell. At various times postinfection, the cells were washed three times with PBS and then lysed in either immune buffer or buffer D.

Assays for 3C protease and CAT activity. 3C protease activity was measured by the ability of in vitro-translated protease or mouse cell lysates to cleave the EMC virus leader (L) protein from the polyprotein L-1AB'. RNA transcripts (14) were prepared from pE5LVPO linearized with *Xba*I. These transcripts (0.03 μ g/ μ l) were translated in reticulocyte lysate (13) in the presence of [³⁵S]methionine to produce labeled L-1AB' polypeptide. Translation reactions were terminated by treatment with cycloheximide and RNase A (12, 21). Transcripts were also prepared from pE3A'BCD' linearized with *Eco*RI and were translated in reticulocyte lysate in the absence of label. Superinduced or uninduced cells were lysed by freeze-thawing three times in buffer D. These preparations were centrifuged for 10 min at 16,000 $\times$ g, and the supernatants were recovered. Assays of in vitro-translated protease were carried out by combining 2 μ l of labeled L-1AB' translation reaction mixture with 4 μ l of unlabeled 3A'BCD' translation mixture. The reactions were incubated at 30°C for 3 to 18 h. Assays for protease activity

in cell lysates were carried out by combining 0 to 4 μ g of cellular protein in 4 μ l of buffer D with 3 μ l of the labeled L-1AB' translation mixture. These reactions were incubated at 30°C for 3 h. Reaction products were analyzed by electrophoresis through sodium dodecyl sulfate (SDS)-polyacrylamide gels (11) containing 12% acrylamide, followed by fluorography. Quantitation of released 1AB' diprotein was accomplished using a Joyce-Loebl densitometer to scan the fluorograms.

Cell lysates for assays of CAT activity were prepared by freeze-thawing as described above, except 0.25 M Tris hydrochloride (pH 7.5) was used as the lysing buffer. Reaction conditions were those previously described (4), and acylated and unacylated [¹⁴C]chloramphenicol products were separated by silica gel thin-layer chromatography.

In vivo labeling with [³⁵S]methionine and immune precipitation. Uninduced, superinduced, or virus-infected cells were labeled with [³⁵S]methionine (30) at the indicated times during the course of certain experiments. Typically, cells cultured in Costar six-well dishes were washed three times with Earle balanced salt solution, and minimal essential medium (minus unlabeled methionine) containing 50 to 100 μ Ci of [³⁵S]methionine (Amersham) per ml was added. After an incubation of 1 h, the medium was removed. For those experiments requiring chases, the cells were washed twice with unlabeled complete medium and then incubated for the indicated time with this same medium. To prepare cell lysates for trichloroacetic acid precipitations or for immune precipitations, the cells were washed three times with PBS and then lysed in immune buffer (30). Incorporation of label into the cells was determined by precipitation with trichloroacetic acid and liquid scintillation counting. Immune precipitations were carried out using the previously described procedures (30). The EMC virus 3C protease antisera were prepared by G. D. Parks. These antisera were prepared using synthetic peptides described in the Results section. Immune-precipitated proteins were detected by electrophoresis through SDS-polyacrylamide gels (11) containing 12% acrylamide, followed by fluorography. In some cases, total labeled cell lysates were analyzed by electrophoresis through SDS-polyacrylamide gels having a gradient of 7.5 to 15% acrylamide or through gels of 20% acrylamide (3).

RESULTS

Construction of plasmids and isolation of cell lines. The parental expression vector pBMC3 and its features are described in Fig. 1A and 1B. The gene coding for CAT is included downstream from the polylinker to provide a secondary marker for the selection of clones, as well as for a means to quantitate expression from the plasmids carried in different cell lines (4). Although our principal interest was in the gene coding for 3C protease, a larger fragment of the EMC virus genome was inserted into the polylinker of pBMC3. This fragment was obtained from the plasmid pEM3 (17) and includes a portion of the sequences coding for protein 3A (3A'), all of the sequences coding for 3B and 3C, and a small portion of the sequences coding for 3D (3D'). This DNA insert was chosen because it facilitated the plasmid construction, particularly in regard to aligning the reading frame with the first AUG initiation codon in the polylinker (Fig. 1B). In addition, since 3C protease acts autocatalytically, it was desirable to provide flanking amino acid sequences so that proper processing to the 22-kDa protein would be facilitated (10, 22). However, it should be noted that the minimum numbers of amino acids flanking the

FIG. 2. Assays for 3C protease activity. All reactions were analyzed by electrophoresis through SDS-polyacrylamide gels, followed by fluorography. (A) Assays for cleavage of L-1AB' by *in vitro*-translated protease. Labeled L-1AB' translation reaction mixture was combined with either unlabeled translation mixture lacking mRNA (lanes 1 and 3) or unlabeled 3A'BCD' translation mixture (lanes 2 and 4) as described in Materials and Methods. These mixtures were incubated at 30°C for 3 h (lanes 1 and 2) or 18 h (lanes 3 and 4). (B) Assays for cleavage of L-1AB' by lysates from uninduced or superinduced cells. Line 120 cells were superinduced with zinc plus cycloheximide as described in Materials and Methods. At the indicated times after switching to medium containing dactinomycin, the cells were washed with PBS, scraped, and then lysed by freeze-thawing in buffer D. A 2.5- μ g sample of cellular protein was then incubated with labeled L-1AB' translation reaction mixture at 30°C for 3 h. Lane 1 shows the analysis of a control reaction minus cell lysate. Other lanes show analyses of lysates prepared from cells after treatment with zinc plus cycloheximide followed by incubation in dactinomycin for the indicated times: lane 2, 0 h; lane 3, 1 h; lane 4, 2 h; lane 5, 4 h; and lane 6, 8 h.

cleavage junctions that are required for processing have not yet been precisely determined. (It will be demonstrated below that the processing of the polyprotein encoded by the genomic region employed was slow, especially at the 3C/3D junction.) The important features of the final expression plasmid, pBMCE1, are depicted in Fig. 1C. mRNAs transcribed from the mMT-I promoter would be chimeric, containing an open reading frame encoding the EMC virus polyprotein, another encoding CAT, and a third encoding mMT-I. To provide mRNA for *in vitro* translation experiments, an *in vitro* transcription vector was constructed which carries the cloned EMC virus sequences behind the SP6 RNA polymerase promoter (14; Fig. 1D). This plasmid is called pE3A'BCD'.

pBMCE1 was introduced into mouse C127 cells by co-transfection with pSV2neo (29). Clones resistant to G418 were selected (29), and cell lysates were assayed for CAT activity (4). Three cell lines constitutively expressing the highest levels of CAT were maintained for study (lines 111, 119, and 120). A control cell line transformed with the parental pBMC3 plasmid was also isolated (line C22).

Inducible expression of EMC virus 3C protease. For initial studies on the expression of EMC virus 3C protease, a functional assay was employed to detect specific protease activity in the cells (21, 22). For this purpose, mRNA was prepared by *in vitro* transcription from the plasmid pE5LVPO (21), which carries sequences encoding the EMC L-1AB' polyprotein (the carboxy-terminal amino acid is missing from B) downstream from the T7 RNA polymerase promoter (14). Translation of this mRNA *in vitro* provides a good substrate for 3C protease, since this enzyme cleaves the leader polypeptide (L) from the polyprotein (1AB') rapidly and with high specificity (12, 21). This activity is demonstrated in Fig. 2A by the ability of translation lysates programmed with mRNA transcribed from pE3A'BCD' to cleave [³⁵S]methionine-labeled L-1AB' polyprotein. The 36.4-kDa 1AB' diprotein product is visible in the autoradiogram (Fig. 2A, lanes 2 and 4). The presence of two bands corresponding to 1AB' (lane 2) has been previously observed

(21, 22). The released leader protein could also be resolved when the concentration of reticulocyte lysate was minimized. (The prolonged exposure of the autoradiograms shown in Fig. 2 reveals trace amounts of foreshortened "early quitter" polypeptides. Not unexpectedly, these are also cleaved by high levels of protease, as seen in lanes 2 and 4 of Fig. 2A.)

Using the cleavage of the L-1AB' polyprotein as an assay, lysates prepared from cell lines transformed with pBMCE1 were tested for 3C protease activity. Lysates prepared from 120 cells in the absence of induction did not contain measurable protease activity (Fig. 2B, lane 2). In contrast, prior superinduction of 120 cells led to the production of readily measured levels of 3C activity (lanes 3 to 6). Interestingly, the level of activity peaked 2 h after the shift from zinc plus cycloheximide to dactinomycin (lane 4). The activity then diminished to about one-sixth of this maximum level by 8 h after the shift. No protease activity was detected 12 h after the shift. The 119 cells behaved similarly. In contrast, no protease activity was detected at any time in superinduced C22 cells.

To confirm these results, we next assayed the expression of 3C protease by immune precipitation with anti-3C antibodies. Antibodies were prepared (by G. D. Parks at the University of Wisconsin, Madison) in rabbits against the following synthetic polypeptides: a sequence of 15 amino acids (LDIQGNPVMDFEKY) corresponding to the 3B/3C junction; a sequence of 14 amino acids (RDNTSKFVKAB DVC) corresponding to a region near the center of 3C; and a region of 10 amino acids (FEPQGALERL) corresponding to the 3C/3D junction. The affinity of these antibodies for antigens produced *in vivo* did not appear to be high, since long autoradiography times accompanied by high backgrounds were usually necessary to detect antibody-specific proteins. Figure 3A shows the results of immune precipitations from lysates prepared from uninduced and superinduced C22, 119, and 120 cells. The immune-precipitated proteins can be compared with immune-precipitated (Fig. 3A, lane 7) and nonprecipitated (lane 8) proteins synthesized by *in vitro* translation of transcripts prepared from pE3A'BCD'. It is not possible to unequivocally assign identities to all of the immune-precipitated proteins, but their molecular weights strongly suggest the presence of 3A'BCD', 3CD', and 3C, in addition to other processing intermediates (Fig. 3A, lanes 7 and 8). Although the immune precipitates shown in Fig. 3A (lanes 4 and 6) do not indicate the presence of all the proteins produced *in vitro*, it is evident that both the 119 and 120 cells produced proteins recognized by anti-3C antibodies, the largest of which corresponds in size to the unprocessed precursor, 3A'BCD'. The presence of other bands produced *in vitro* could often be detected by longer fluorographic exposure (for example, see lane 1 in Fig. 3B). As expected, the expression of these proteins in the cells is dependent upon activation of the mMT-I promoter by the superinduction protocol, which increased their rate of synthesis by approximately 50-fold.

In light of the observed loss in protease activity with time during superinduction (Fig. 2B), it was important to look for a corresponding loss in immune-precipitable 3C-containing proteins during the incubation of the cells with dactinomycin (Fig. 3B). Line 120 cells were pulsed with [³⁵S]methionine during superinduction. The cells were then chased with medium lacking label. Immune precipitation of lysates from these cells indicated that all these proteins were rapidly lost (Fig. 3B). The lack of accumulation of 22-kDa protein in the cells suggests either that the higher-molecular-weight pre-

FIG. 3. Immune precipitation of 3C-containing proteins produced *in vivo*. (A) Assay for the production of immune-precipitable proteins in uninduced and superinduced cells. Cells were superinduced by treatment with zinc plus cycloheximide, followed by incubation with dactinomycin along with [35 S]methionine for 1 h, as described in Materials and Methods. Lysates were then prepared and immune precipitated with a mixture of the 3C antisera described in the text. The immune-precipitated proteins were then analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. *In vitro* markers were prepared by the translation of transcripts from pE3A'BCD' (Fig. 1D) in reticulocyte lysate in the presence of [35 S]methionine. The analyses of immune precipitates from uninduced and superinduced pairs of cultures are shown: C22 cells (lanes 1 and 2), 119 cells (lanes 3 and 4), and 120 cells (lanes 5 and 6). Lane 7 shows the analysis of the immune-precipitated *in vitro* translation reaction, and lane 8 shows the analysis of the same reaction mixture prior to immune precipitation. (B) Analysis of immune-precipitated proteins in superinduced cells during pulse-chase. Line 120 cells were superinduced as described above. After a 1-h pulse with [35 S]methionine in the presence of dactinomycin, the medium was removed, the cells were washed, and medium lacking label but containing dactinomycin was added for the indicated times. Lysates were then prepared, immune precipitated, and analyzed. In the experiment shown in panel B, three times as much [35 S]methionine was used as for panel A. The precipitated products are shown following chase times of 0 h (lanes 1 and 2), 1 h (lanes 3 and 4), or 2 h (lanes 5 and 6).

cursors were being degraded directly, or that they were being processed to 3C protease which was then very rapidly degraded. It is highly likely that at least some of these precursor polyproteins can also act as a functional protease (6, 8, 20, 31) and thus contribute to the activity levels observed in the experiments described in Fig. 2.

It is worthy of note that the net decline in protease activity after 4 h of superinduction (Fig. 2) indicates that the mRNA encoding this enzyme is also rapidly inactivated. The same inference is drawn from the fact that superinduction of 119 and 120 cells leads to only a surprisingly small net increase in intracellular CAT concentration (see Fig. 7A and accompanying discussion below). Pulse-chase experiments indicate that the mRNA half-life in 120 cells is less than 2 h. This is in marked contrast to results obtained using rabbit ferritin as a reporter protein, in which no decrease in the rate of translation was observed for at least 6 h of superinduction, suggesting that in this construct the mRNA is quite stable (30; unpublished observations). The reason for these large apparent differences in mRNA stability is not clear.

3C protease and 3C-containing polyproteins are unstable in EMC virus-infected cells as well as *in vitro*. The observations that superinduced 3C protease activity (Fig. 2B) and immune-precipitable 3C-containing translation products (Fig. 3B) disappeared with time raise interesting questions concerning EMC virus protein stability. However, it was first important to determine whether this turnover occurs only in transfected cells. To address this concern, the expression of 3C protease in EMC virus-infected cells was studied. To facilitate these experiments, the 400 cell line was chosen. These cells, which were derived by transforming C127 cells with pBMCF1, were selected because they constitutively produce high levels of CAT activity, which is useful for quantitating cell lysis, membrane permeability, or both.

Lysates were prepared from EMC virus-infected 400 cells and assayed for protease activity capable of cleaving the L protein from the L-1AB' polyprotein (Fig. 4A). Measurable 3C protease activity was detected by 4 h postinfection (Fig. 4A, lane 4), and the activity level peaked at 6 h (lane 5). After this, the activity level declined. The extent of this activity loss between 6 and 8 h postinfection was found to be 37%, 44%, and 55% in three separate experiments. (400 cells infected with EMC virus lysed at approximately 12 h post-infection; see Fig. 7B for a similar result.) This result

suggests that a "pulse" of protease activity lasting 4 to 6 h occurs in EMC virus-infected cells, which is comparable to the pulse observed in superinduced transfected cells.

The 3C protein produced in EMC virus-infected cells could also be detected by immune precipitation with the anti-3C antibodies. Line 400 cells were labeled with [35 S]

FIG. 4. Assays for 3C protease in EMC virus-infected cells. Attached 400 cells cultured in 100-mm dishes were infected with 20 PFU of EMC virus per cell. At various times postinfection, the cells were washed with PBS and lysates were prepared for protease activity assays as described or for immune precipitation as described in Materials and Methods. (A) Assays for protease activity. Lysates from EMC virus-infected cells (4 μ g of protein) were assayed for the ability to cleave the L protein from L-1AB'. Lane 1 shows the result of the assay of mock-infected cell lysate. The remaining lanes show the analyses of lysates prepared at the indicated times postinfection: lane 2, 0 h; lane 3, 2 h; lane 4, 4 h; lane 5, 6 h; lane 6, 8 h. (B) Assay for immune-precipitable proteins. The medium from cells infected for 5 h was replaced with medium containing [35 S]methionine, followed by 1 h of incubation. After washing, this medium was replaced with unlabeled medium. After the indicated lengths of time, cell lysates were prepared and assayed for proteins precipitable by the 3C antisera mixture: lanes 1 and 2, no chase; lane 3, 1-h chase; lanes 4 and 5, 2-h chase. [35 S]methionine-labeled *in vitro* translation products produced by the pE3A'BCD' transcript (Fig. 3) are shown in lane 6.

FIG. 5. Stability of EMC virus proteins produced by in vitro translation. (A) Transcript (0.02 $\mu\text{g}/\mu\text{l}$) prepared from pE3A'BCD' (see Fig. 1D) was translated in reticulocyte lysate in the presence of [^{35}S]methionine. Samples were removed and analyzed by SDS-polyacrylamide gel electrophoresis at the indicated times: lane 2, 20 min; lane 3, 40 min; lane 4, 120 min. Lane 1 is a minus-RNA control. (B) Comparison of the stability of EMC virus 3C-containing proteins and BMV proteins synthesized in vitro. Equal quantities (0.02 $\mu\text{g}/\mu\text{l}$) of pE3A'BCD' transcript and BMV RNA were translated in reticulocyte lysate in the presence of [^{35}S]methionine. Samples were removed at various times, and trichloroacetic acid-precipitable ^{35}S was determined. Symbols: ●, pE3A'BCD' transcript; ○, BMV RNA.

methionine for 1 h at 5 h postinfection, and the medium was then replaced with chase medium lacking label. Lysates were prepared from pulse-labeled and chased cells and assayed for immune-precipitable proteins. A 22-kDa protein was readily detected in all lysates (Fig. 4B). Although it is not shown in Fig. 4B, P3 polyprotein (86 kDa) and another high-molecular-size 3C protease-containing precursor (69 kDa) were also immune precipitated, but neither of these precursors was detected after a 1-h chase. Few if any of the smaller precursor polyproteins that predominate in transfected cells or in vitro were evident. Nevertheless, the 22-kDa protein in infected cells comigrated with the in vitro-translated protein tentatively identified as 3C protease (Fig. 4B). The 22-kDa protein in the infected cells was degraded during the chase, with only a small fraction of the 6-h level (Fig. 4B, lanes 1 and 2) present 2 h later (lanes 4 and 5). Thus it is clear that the stability of 3C protease in virus-infected cells is similar to that of the polyprotein precursors seen in transfected cells.

Interestingly, 3C-containing polyproteins synthesized in vitro were also found to be rapidly degraded. This was observed when a saturating amount of the SP6 transcript of pE3A'BCD' was translated in reticulocyte lysate in the

presence of [^{35}S]methionine. Continued incubation of the translation reaction led to a decrease in most of the translated proteins, including the 22-kDa 3C protease (Fig. 5A). The loss of the higher-molecular-weight proteins may, at least in part, represent processing to p22. To determine whether this degradation is specific for EMC virus proteins, an identical experiment was carried out with a saturating amount of brome mosaic virus (BMV) mRNA (Fig. 5B). Here, trichloroacetic acid-precipitable [^{35}S]methionine was quantitated during the incubation of the BMV mRNA and the pE3A'BCD' transcripts in reticulocyte lysates. In contrast to the EMC virus products, it is clear that the BMV translation products were relatively stable.

Effects of the expression of 3C protease on cell viability and function. Having demonstrated the inducible expression of EMC virus 3C protease activity, our efforts turned toward examining the effects of 3C-containing polyproteins on the host cell. Cells were frequently examined during the course of each superinduction experiment, but no alterations in morphology that might indicate cytopathic effects were evident. Moreover, no signs of cell lysis were observed. Before drawing any conclusions concerning cytotoxicity, however, it was necessary to determine whether the peak levels of protease produced in the cells were similar to those produced during infection with EMC virus. Measurements of protease activity in cell lysates revealed that the highest levels occurred in the 120 cells 2 to 4 h after superinduction (Fig. 2B). The 119 cells produced about two-thirds of this level, and the 111 cells produced one-half or less. Comparison of this protease level with that which occurred in the EMC virus-infected cells at 6 h postinfection demonstrated that the peak activities in superinduced 120 cells and virus-infected cells were quite similar (Fig. 6). As noted above, these peak activity levels are maintained for only a few hours in both types of cells. We therefore conclude that the 120 cells can produce levels of protease activity that are comparable in magnitude and duration to those that occur during EMC virus infection.

The effects of superinduction on cell function and integrity were then examined. As a gauge of metabolic activity, the ability of cells to incorporate amino acids into protein following the superinduction of 3C protease expression was quantitated. At 16 h after the shift from zinc plus cycloheximide to dactinomycin, superinduced (and control) cells were labeled with [^{35}S]methionine for 1 h, and the incorporation of label into protein was then measured. While this particular superinduction protocol reduced incorporation in general (probably due to the presence of dactinomycin for 16 h), cells transformed with pBMC3 and pBMCE1 were affected to approximately the same extent (Table 1). Comparable results were obtained when cells were pulse-labeled 4 h after the shift to dactinomycin, i.e., shortly after the maximum 3C protease concentration had been attained. Substitution of cordycepin for dactinomycin (24, 27) also led to a quantitatively similar superinduction of 3C protease expression (data not shown). However, there was less overall inhibition of protein synthesis with cordycepin (Table 2). Here, cells were again pulse-labeled with [^{35}S]methionine for 1 h, beginning either 4 h after the shift to medium containing (or lacking) cordycepin or 12 h after the removal of cordycepin. We conclude from the data in Tables 1 and 2 that the expression of the protease does not appear to have a significant adverse effect on cellular protein synthesis, either during its period of maximum expression or subsequently.

The integrity of cell membranes during superinduction

FIG. 6. Quantitation of 3C protease levels in superinduced transfected cells and in EMC virus-infected cells. Line 120 cells were superinduced with zinc plus cycloheximide, followed by dactinomycin for 2 h. Line 400 cells were infected with 10 or 20 PFU of EMC virus per cell, and cells were harvested 6 h postinfection. Lysates were prepared from both the superinduced and the infected cells and assayed for the ability to cleave L-1AB', as described in Materials and Methods. The resulting fluorograms were then scanned using a Joyce-Loebl densitometer, and the amount of released 1AB' diprotein per microgram of infected cellular protein was calculated, taking into account the efficiency of the initial infections. These were $\geq 80\%$ as determined by counting surviving cells 18 h postinfection. Symbols: $\blacktriangle$, lysate from superinduced 120 cells; $\circ$, lysate from cells infected with 10 PFU of virus per cell; $\bullet$, lysate from cells infected with 20 PFU of virus per cell.

was also studied. Leakage of cellular proteins from infected cells is an effect commonly produced by a wide variety of viruses (2), as is ultimate cell lysis. Leakage and lysis were measured by making use of the CAT enzyme encoded by the pBMC3 and pBMCE1 plasmids. Assays of CAT activity in media in which uninduced and superinduced C22, 119, and 120 cells were grown revealed that no increase in the leakage of CAT was detected as a result of superinduction, even 12 h after the protease activity peak has been attained (Fig. 7A). (As noted above, superinduction of 119 and 120 cells results in a surprisingly small net increase in intracellular CAT activity. This is a result of the high stability of the CAT protein in combination with the extreme lability of the encoding mRNA. In contrast, a sizeable increase in the CAT

TABLE 1. Uptake and incorporation of [35 S]methionine into uninduced and superinduced cells in the presence of dactinomycin

Cell line	35 S incorporated (10^3 cpm/ μ g of protein)		
	Uninduced	Superinduced ^a	Superinduced/uninduced
C22	5.80	2.56	0.44
C22	7.28	3.34	0.46
119	5.94	2.48	0.42
120	7.46	3.01	0.40
111	9.58	3.36	0.35

^a Zinc plus cycloheximide for 6 h and then dactinomycin for 16 h.

TABLE 2. Uptake and incorporation of [35 S]methionine into uninduced and superinduced cells in the presence of cordycepin

Zinc plus cycloheximide incubation for 6 h	35 S incorporated (10^2 cpm/ μ g of protein) after incubation:			
	Minus cordycepin		Plus cordycepin	
	For 4 h	For 16 h	For 4 h	For 4 h, then 12 h minus cordycepin
—	15.7	11.9	10.5	13.3
+	11.8	11.8	12.4	13.4

level was produced in C22 cells by superinduction [not shown]. Evidence of this increase can be seen in the amounts of unreacted chloramphenicol in Fig. 7A, lanes 3 and 5.) In control experiments, the deliberate lysis of cells in medium (Fig. 7A) or infection of 119 cells with EMC virus (Fig. 7B) released large quantities of CAT. In the latter case, cells lysed only 6 h after the protease activity peak was attained. These results indicate that the protease does not alter membrane permeability to macromolecules such as CAT.

It was of interest to determine whether the superinduced 3C protease activity cleaved host proteins in transfected cells. A partial answer to this question was obtained by analyzing pulse-labeled and chased polypeptides in superinduced cells by SDS-polyacrylamide gel electrophoresis and fluorography. The results (Fig. 8A) indicate that most of the major host polypeptides are not cleaved by 3C protease. (The absence of pronounced 3C polypeptide bands in Fig. 8A, lane 4, was not surprising. The normal course of EMC virus infection in transformed C127 cells is slow, being very similar to that seen in Krebs 2 ascites cells [7]. In both cell lines, even major viral proteins [e.g., γ and E, which are the products of the 1C and 3D genes, respectively] are not easily detectable by labeling until about 4 h postinfection and do not represent a high percentage of all proteins synthesized until very late in the infectious cycle. Minor proteins, such as 3C protease, are often difficult to detect at any time. Cytopathic effects [e.g., cellular "rounding up"] begin at 8 h, and cell lysis begins at about 12 h postinfection in both cell lines.) Similar to the results obtained *in vivo*, the much higher levels of protease activity synthesized *in vitro* (see Fig. 2A) did not visibly alter proteins present in reticulocyte lysate (Fig. 8B).

DISCUSSION

The results of the experiments reported here have demonstrated that stable cell lines capable of expressing cloned virus genes in a controlled manner can be readily prepared. The expression vector system which has been developed, when coupled with the procedure for superinduction (30), has made it possible to selectively induce the expression of EMC virus 3C protease. This in turn has allowed studies to be carried out on the behavior of this protein *in vivo* and the effects of its expression on cell function.

The data obtained reveal that EMC virus 3C protease and its precursors are rapidly turned over in stably transfected cells, in normal infected cells, and *in vitro*. Thus it is clear that the turnover of 3C-related proteins is a natural phenomenon. It is difficult at this time to say what proteolytic mechanism may be responsible for this turnover. The activity is clearly not encoded by a region of the EMC virus genome outside of the segment present in pBMCE1. This

FIG. 7. Assays for the release of CAT into medium following superinduction or EMC virus infection. (A) Release of CAT from superinduced cells. C22, 119, and 120 cells in six-well dishes were superinduced with zinc plus cycloheximide followed by dactinomycin for 18 h. A set of control cells remained untreated for the same period of time. A sample of medium (400 μ l) was removed from each culture. The cells were then lysed in the remaining medium (1.1 ml) by freeze-thawing three times. Cell debris was pelleted by centrifugation for 10 min at $16,000 \times g$, and 400- μ l volumes of each supernatant were removed. A 3- μ l portion of each medium sample from the C22 cells and 5 μ l of each medium sample from the 119 and 120 cells were assayed for CAT activity as described in Materials and Methods. Acylated and unacylated chloramphenicol were detected by autoradiography of the silica gel thin layers. Samples: Lane 1, minus medium control; lanes 2 through 5, medium from C22 cells; lanes 6 through 9, medium from 119 cells; lanes 10 through 13, medium from 120 cells; lane 14, 0.1 U CAT standard; lane 15, 0.01 U CAT standard. Media from lysed or unlysed control or superinduced cells are indicated in the figure. (The C22 cell cultures were all considerably more dense than the 119 or 120 cultures in this experiment, accounting for a difference in the absolute amounts of CAT activity present.) (B) Release of CAT from EMC virus-infected cells. Uninduced 119 cells in 100-mm dishes were infected with 20 PFU of EMC virus per ml. At various times postinfection, 1/10 of the medium was removed and replaced with the same volume of fresh medium. The removed medium was then assayed for CAT activity as described above, except that 40 μ l of medium was used for each assay. Lane 1 shows a minus-medium control, and lane 8 shows a 0.01 U CAT standard. Other lanes show media from infected cell cultures at the indicated times postinfection: lane 2, 0 h; lane 3, 4 h; lane 4, 6 h; lane 5, 8 h; lane 6, 12 h; and lane 7, 24 h.

leaves either a host proteolytic pathway or the 3C protease itself. It also must be emphasized that this proteolysis does not occur with all EMC virus proteins, since during a very long incubation (18 h) the 1AB' dipeptide (Fig. 2A) was not degraded in reticulocyte lysate. Similar results and conclusions were obtained by immune precipitation of capsid proteins synthesized in virus-infected cells (data not shown).

The striking differences existing between the kinetics of the processing of precursors to 22-kDa 3C protease in the infected cells and in the stable cell lines containing pBMCE1 are of interest. In the 119 and 120 cells, as well as during *in vitro* translation of the pE3A'BCD' transcript, the relative proportion of 3C-containing polyprotein precursors was very high, with no obvious accumulation of the 22-kDa protein as a function of time. In the infected cells, on the other hand, a very much smaller proportion of precursors was observed. These data may indicate that a larger segment of the EMC virus genome, including most or all of the 3D region, is required to generate the efficient physiological processing to 22-kDa 3C protease (6, 8, 32). Other explanations for this observation are possible, however. In any case, the accumulation of the 3CD' precursor, which is particularly striking (Fig. 3), has also been observed to occur when poliovirus precursors lacking the complete poliovirus 3D region are expressed in *E. coli* (6).

Our results also indicate that the expression of 3C protease actively produces few or no obvious cytopathic effects. These data certainly do not rule out the possibility that EMC virus 3C protease might be cytotoxic under other circumstances or at higher concentrations. In particular, the possibility still exists that 3C protease in combination with other EMC virus proteins may be detrimental to cells. Nevertheless, we feel it is significant that no adverse effects of 3C protease expression on the transfected cell lines studied here

were ever observed. Although no direct evidence was obtained to demonstrate the expression of the 3B genomic protein, it is difficult to imagine that it (or at least its 3A'B precursor) is not present in the cells as well. Thus, 3B is probably not cytotoxic either.

As to why 3C protease by itself is not acutely cytotoxic, the most likely explanation is its reported substrate specificity (1, 10, 22). 3C protease cleaves only certain amino acid junctions in EMC virus polyprotein (22). Even poliovirus polyproteins, which are also processed by a similar poliovirus 3C protease, do not serve as substrates for the EMC virus enzyme (10). Moreover, no cellular protein has yet been identified as a substrate for any picornaviral protease (reviewed in reference 10). Consistent with these results, examination of total labeled proteins in lysates prepared from superinduced 120 cells showed that of those polypeptides resolvable in a one-dimensional analysis, none are affected by EMC virus protease activity. Of course, potential cleavage of host cell proteins may be minimized because of the rapid turnover of 3C protease activity.

Most cell lines transformed with the plasmids described here were found to be quite stable (23, 25, 28). The 119 and 120 lines continued to produce nearly constant levels of CAT and 3C protease activity for at least 6 months. The 111 line, however, showed a drastic decrease in both CAT and protease activity during month 3 after transfection. We observed a similar occurrence with one of the C22 cell lines. The reason for these occasional sudden declines in CAT production is not known.

This plasmid system shows promise for the study of the individual function of virtually any viral gene. Efforts to identify EMC virus cytotoxic genes or gene combinations are in progress. Of critical importance for the success of this undertaking will be whether the level of gene expression in

FIG. 8. Effect of 3C protease on host proteins. (A) Analysis of total cellular proteins from uninduced and superinduced cells. Line 120 cells were superinduced with zinc plus cycloheximide as described in Materials and Methods. The cells were washed, and medium containing [35 S]methionine in the presence or absence of dactinomycin was added. After 1 h these media were replaced with identical media lacking label. After incubation for various times, lysates were prepared and equal amounts of protein were analyzed by 7.5 to 15% gradient polyacrylamide gel electrophoresis. Lane 1, Uninduced cells, 0-h chase; lane 2, uninduced cells, 1-h chase; lane 3, uninduced cells, 2-h chase; lane 4, superinduced cells, 0-h chase; lane 5, superinduced cells, 1-h chase; lane 6, superinduced cells, 2-h chase. The asterisk marks the location of heme oxygenase, the expression of which has been shown to be stimulated by a variety of alterations in environmental conditions (9). (B) Analysis of reticulocyte lysate proteins following translation of transcript from pE3A'BCD'. A transcript encoding EMC virus 3A', 3B, 3C, and 3D' genes was prepared from pE3A'BCD' (see Fig. 1D) and was translated in reticulocyte lysate. At various times, 2- μ l samples were removed for analysis by 20% polyacrylamide gel electrophoresis. After fixing in 10% acetic acid, the gel was stained with Coomassie blue. Lane 1 shows lysate incubated for 120 min in the absence of mRNA. The remaining lanes show lysates analyzed after translation for the indicated times: lane 2, 20 min; lane 3, 40 min; lane 4, 120 min; lane 5, 200 min. Lane 6 shows the analysis of lysate after translation of BMV RNA for 200 min.

the uninduced state is low enough to allow cell growth, yet in the induced state is comparable to that attained in the course of normal virus infection. The ratio of the induced to uninduced synthesis rates for the EMC virus 3C protease precursors described here is approximately 50-fold, similar to what was previously observed using rabbit ferritin as a reporter protein (30). However, this high induction ratio for the synthesis rate may or may not be reflected in the steady-state concentrations of protein ultimately attained, since other factors, such as protein and mRNA stability, can

greatly influence the result. We are currently addressing some of these issues in an attempt to improve this expression vector for the purpose at hand.

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