JOURNAL OF VIROLOGY, Feb. 1986, p. 547–551
0022-538X/86/020547-05$02.00/0

Vol. 57, No. 2

# Cleavage of a Viral Polyprotein by a Cellular Proteolytic Activity

YING C. TIAN AND DING S. SHIH*

*Department of Biochemistry, Louisiana State University, and Louisiana Agricultural Experiment Station, Baton Rouge, Louisiana 70803*

Received 28 May 1985/Accepted 6 August 1985

**The 200,000-dalton polyprotein encoded by the bottom component RNA of cowpea mosaic virus was synthesized in rabbit reticulocyte lysates, and this in vitro-synthesized protein was isolated from the lysate reaction mixture by sucrose density gradient centrifugation. Incubation of the isolated polyprotein with buffer caused no change in the protein, but incubation with reticulocyte lysates or with fractionated lysate proteins resulted in cleavage of the protein into the expected cleavage products (32,000- and 170,000-dalton proteins). This finding indicated that reticulocytes contain a proteolytic activity that is needed for the primary cleavage reaction. A cleavage assay in which we used partially purified preparations showed that cleavage was an ATP-dependent reaction.**

The synthesis of the proteins of animal picornaviruses and plant comoviruses involves extensive series of proteolytic reactions. Translation products are first cleaved at the nascent chain stage to produce primary cleavage products. These initial cleavages are referred to as the primary cleavages. Many of the primary cleavage products are intermediate proteins which must undergo additional cleavages, the secondary cleavages, to form stable viral structural and nonstructural proteins. In the case of the picornaviruses, a final maturation cleavage occurs after encapsidation of the virion RNA. It is known that virus-encoded proteases are responsible for at least some of the secondary cleavage reactions. For example, proteins p22 and P3-7c of encephalomyocarditis virus and poliovirus, respectively, catalyze cleavage of viral capsid protein precursors into capsid proteins (9, 14). The cleavage sites and the mechanism used for generating these viral enzymes are known (5, 13, 15, 20).

Very little is known about the primary cleavage reactions and about the enzymes involved in these reactions. The results of inhibitor studies have suggested that primary cleavage reactions are catalyzed by cellular enzymes (2, 11, 23). However, no direct evidence for this has been reported thus far.

In this paper we show that the large 200,000-dalton (200K) polyprotein synthesized from the bottom component RNA (B-RNA) of cowpea mosaic virus (CPMV) could be specifically cleaved to form two smaller proteins by a proteolytic activity present in rabbit reticulocyte lysates. The same cleavage could also be obtained with fractionated reticulocyte proteins.

CPMV, which is similar to the picornaviruses in many aspects, is the type member of the plant comovirus group. The viral genome consists of two single-stranded RNA molecules which are encapsidated in separate isometric particles (middle and bottom components) (1, 25). The two genomic RNAs, middle component RNA and B-RNA, are 3,481 and 5,889 nucleotides long, respectively (12, 26). Both RNAs are polyadenylated and have a genome-linked protein at their 5′ termini (3, 4). The B-RNA can replicate independently in cowpea mesophyll protoplasts, causing formation of eight virus-specific proteins (7, 19). In rabbit reticulocyte lysates B-RNA can be translated into a 200K polyprotein which is cleaved during synthesis to produce a 32K protein and a 170K protein (17). The 170K protein, in turn, is cleaved to form either of two pairs of proteins, a 110K-60K pair and a 87K-84K pair (18). An important characteristic of the cleavage of the 200K polyprotein in the reticulocyte lysate is that the reaction requires the reducing agent dithiothreitol (DTT) (17). In the absence of DTT, the cleavage reaction does not take place, and the protein accumulates in the lysate reaction mixture. However, the polyprotein is readily cleaved once DTT is added.

## MATERIALS AND METHODS

**Virus and viral RNA.** The conditions used for growing CPMV and for preparing the B-RNA have been described previously (18).

**Preparation of the 200K polyprotein.** The B-RNA-encoded 200K polyprotein was synthesized in rabbit reticulocyte lysates which were obtained from Green Hectares, Oregon, Wis. The conditions used for protein synthesis have been described previously (18, 21), except that DTT was omitted from the reaction mixture. Translation was at 30°C for 45 min. After translation, the reaction mixture (100 μl) was diluted with 2 volumes of buffer A (20 mM Tris acetate, pH 7.6, 80 mM potassium acetate, 0.6 mM magnesium acetate, 0.3 mM DTT) and applied to a 10 to 40% sucrose density gradient prepared in the same buffer. The gradient was centrifuged in a Beckman type SW28 rotor at 27,000 rpm for 36 h and was then divided into 1-ml fractions. Fractions containing the polyprotein were identified by scintillation counting and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The 200K protein-containing fractions were stored at −80°C.

**Partial purification of the cleavage enzyme.** A 35-ml portion of rabbit reticulocyte lysate was diluted with an equal volume of buffer B (100 mM Tris acetate, pH 7.6, 50 mM potassium acetate, 10 mM DTT, 20% glycerol), and the diluted lysate solution was centrifuged in a Beckman type Ti70 rotor at 45,000 rpm for 2 h. The resulting supernatant was fractionated by ammonium sulfate precipitation. Proteins precipitated between 50 and 60% saturation were collected, and the protein precipitate was dissolved in 1.5 ml of buffer C (20 mM Tris hydrochloride, pH 7.6, 50 mM KCl,

* Corresponding author.

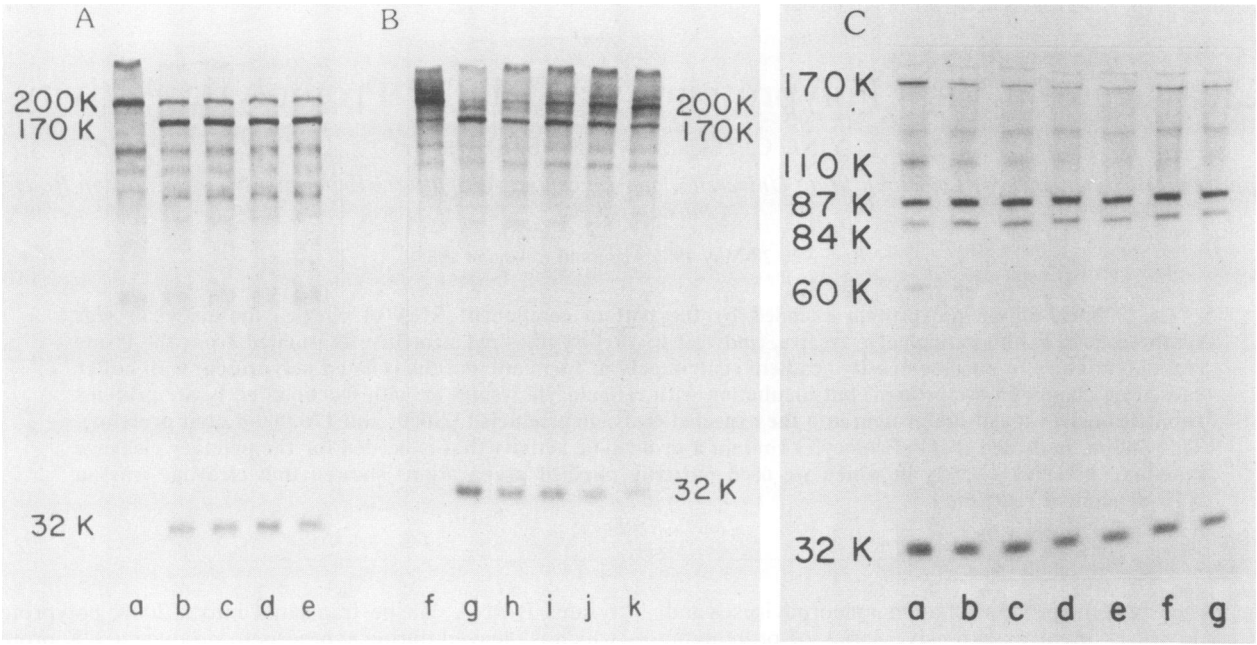


FIG. 1. Effects of dilution on the cleavage of CPMV proteins. (A) The conditions used for protein synthesis have been described previously (18, 21), except that no DTT was added to the reaction mixture and translation was for 45 min. After translation the reaction mixture was adjusted to 5 mM DTT and 1.6 mM ATP, divided into 5-μl portions, and diluted 0- to 80-fold with a blank lysate translation solution. The diluted solutions were overlaid with mineral oil (to prevent evaporation) and were incubated at 30°C for 2 h. At the end of incubation, a sufficient amount of blank lysate was added to each solution to make all solutions equal in volume. Lane a, Sample withdrawn after 45 min of translation; lanes b through e, samples diluted 0-, 10-, 40-, and 80-fold, respectively. (B) The conditions used for translation and for sample treatment were the same as those described above for panel A, except that a dilution buffer containing 20 mM Tris acetate, 80 mM potassium acetate, 0.6 mM magnesium acetate, 5 mM DTT, and 1.6 mM ATP was used to dilute the translation mixture. Lane f, Sample withdrawn after 45 min of translation; lanes g through k, samples diluted 0-, 5-, 10-, 20-, and 40-fold, respectively. (C) Translation carried out in the presence of 5 mM DTT for 60 min. The same dilution buffer that was used in the experiment shown in panel B was used to dilute the reaction mixture. Diluted samples were incubated for 5 h. Lanes a through g, Samples diluted 0-, 10-, 20-, 40-, 80-, 150-, and 200-fold, respectively.

1 mM EDTA, 10 mM mercaptoethanol, 10% glycerol). The solution was applied to a Sephadex G-150 column (2.5 by 90 cm) which was equilibrated in the same buffer. The column was eluted at a flow rate of approximately 20 ml/h, and 3-ml fractions were collected. Fractions containing cleavage activity were pooled, and ammonium sulfate was added to 70% saturation. The suspension was centrifuged, and the protein precipitate was dissolved in 1.5 ml of buffer C. The solution was dialyzed against buffer C and applied to a DEAE-cellulose column (1.5 by 45 cm) which was equilibrated with the same buffer. The column was washed with buffer C (at a flow rate of 3 ml/h) until the absorbance reached a basal level. A 0.05 to 0.5 M NaCl gradient having a total volume of 200 ml was applied, and fractions (approximately 2.5 ml) were collected. After gradient elution, the column was further eluted with about 30 ml of 1 M NaCl. Fractions from each UV-absorbing peak were pooled, and proteins were precipitated with ammonium sulfate. The protein precipitates were dissolved in buffer A, and the solutions were dialyzed against buffer A. The dialyzed solutions were stored at −80°C.

**Cleavage assay.** A typical cleavage assay mixture (25 μl) contained 10 μl of the 200K polyprotein substrate isolated from sucrose density gradients, 10 μl of enzyme solution (or 10 μl of buffer for the control samples), 5 mM DTT, 1.6 mM ATP, and 0.01 mg of bovine serum albumin. (The gradient fractions used in the assay contained almost no reticulocyte proteins; therefore, bovine serum albumin was added to the assay solutions as a carrier protein to keep the 200K protein in solution.) The assay solutions were incubated at 30°C for 5 h.

**Electrophoresis.** Protein samples were analyzed on 7.5% sodium dodecyl sulfate-polyacrylamide gels. The conditions used for gel electrophoresis have been described previously (18, 21). Viral protein bands were detected by fluorography.

## RESULTS

**Effect of dilution on cleavage of the 200K polyprotein.** Pelham reported that diluting 200K protein-containing reticulocyte lysate reaction mixtures 10-fold with a blank lysate solution caused no change in the rate of cleavage of the polyprotein, but that similar dilution with buffer inhibited cleavage (17). This suggests that the primary cleavage reaction is catalyzed by a reticulocyte enzyme.

As a first step in investigating the primary cleavage reaction, we expanded the dilution experiment of Pelham. The 200K polyprotein was synthesized in the reticulocyte lysate in the absence of DTT, and then the reaction mixtures were serially diluted either with a blank lysate translation solution or with a dilution buffer. (Both the blank lysate solution and the dilution buffer contained 20 mM Tris acetate [pH 7.6], 80 mM potassium acetate, 0.6 mM magnesium acetate, 5 mM DTT, and 1.6 mM ATP.) The diluted reaction mixtures were incubated for 1 or 2 h to allow cleavage to occur, and then the samples were analyzed on a slab gel. Figure 1A shows that diluting the protein synthesis reaction

mixtures 10- to 80-fold with the lysate solution had no effect on cleavage of the polyprotein (Fig. 1A, lanes b through e). On the other hand, as shown by the gel patterns in Fig. 1B, a gradual decrease in the cleavage of the polyprotein was observed when the reaction mixtures were diluted with buffer (Fig. 1B, lanes g through k). The protein profiles show that in the undiluted control sample (lane g) the 200K polyprotein was almost completely cleaved to the 32K and 170K products after incubation with 5 mM DTT. However, in the diluted samples, increasing amounts of uncleaved 200K protein and decreasing amounts of the cleavage products were observed as dilution was increased from 5- to 40-fold (lanes h through k).

We have shown previously that a secondary cleavage reaction (cleavage of the 170K protein to form the 87K-84K protein pair) is not affected by buffer dilution (18). In the present study we performed a similar dilution experiment as a positive control. Protein synthesis reaction mixtures were incubated for 1 h in the presence of DTT. Under such conditions the predominant products were the 170K and 32K proteins. The reaction mixtures were then diluted with buffer, and the diluted solutions were incubated for 5 h to allow cleavages to occur. The gel patterns in Fig. 1C show that formation of the 87K-84K protein pair was not affected at all by dilution of the reaction mixture with buffer, even when the mixture was diluted 200-fold. (This insensitivity of the cleavage reaction to buffer dilution suggests that the reaction is an autocatalytic reaction. On the other hand, formation of the 110K-60K pair was sensitive to buffer dilution. Thus, either a cellular enzyme or a virus-encoded enzyme must be responsible for the latter cleavage reaction.)

The results described above indicated that cleavage of the 200K protein is not a process mediated by a virus-encoded protein or a process that occurs autocatalytically, but is a process which is catalyzed by a reticulocyte factor.

**Cleavage of the 200K protein by a reticulocyte factor.** In order to directly demonstrate that a reticulocyte factor is involved in the primary cleavage reaction, we synthesized the 200K protein and isolated the protein by sucrose density gradient centrifugation. The isolated protein was incubated with either dilution buffer or with the blank lysate translation solution (Fig. 2). The protein profiles in Fig. 2 show that the 200K polyprotein remained uncleaved when it was incubated with buffer (Fig. 2, lanes a and c), but was cleaved to form the 32K and 170K proteins after it was incubated with the lysate solution (lanes b and d). This result directly demonstrated that a reticulocyte factor, presumably a protease, is needed for the primary cleavage reaction.

**Partial purification of the cleavage activity.** Cleavage of the isolated 200K protein could also be demonstrated with fractionated lysate components. Assays performed with different ammonium sulfate-precipitated fractions indicated that the cleavage activity existed predominantly in the 50 to 60% fraction (Fig. 3A, lane b). Some activity also existed in the 40 to 50% fraction (lane a), but no activity was found in the 60 to 70% fraction (lane c) or in the fractions containing higher concentrations. Separation of the proteins present in the 50 to 60% saturation fraction on a Sephadex G-150 column resulted in resolution of the precipitated proteins into two major peaks. The first peak eluted at the void volume, and it contained the cleavage activity. The second peak contained the hemoglobin. Further fractionation of the materials recovered from the Sephadex column on a DEAE-cellulose column resulted in additional separation of the isolated proteins. The elution profile is shown in Fig. 3B. A cleavage assay of the pooled fractions from each peak

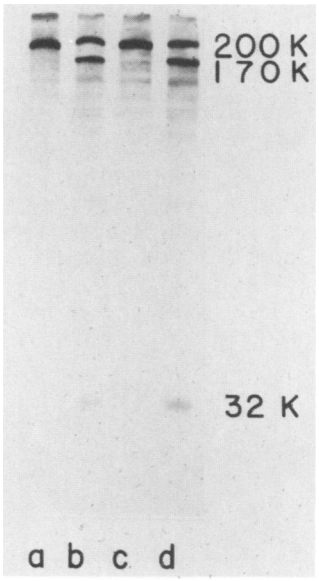


FIG. 2. Cleavage of isolated 200K polyprotein with unfractionated reticulocyte lysates. The conditions used for isolating the polyprotein and for cleavage assays are described in Materials and Methods. Lanes a and c, Polyprotein from two different gradient fractions incubated with the dilution buffer; lanes b and d, the same two fractions incubated with the blank lysate translation solution.

revealed that fraction II (Fig. 3A, lane e), but not any other fraction (lanes d, f, g, and h), possessed the cleavage activity. The minor protein bands in lane f were probably nonspecific cleavage products caused by other reticulocyte proteases that were concentrated in the eluted fraction.

**Requirement of ATP for the cleavage reaction.** Pelham reported that addition of glucose 6-phosphate to the reticulocyte lysate reaction mixture prevented cleavage of the 200K protein (17). This suggests that the cleavage reaction is an ATP-dependent reaction. It is known that rabbit reticulocytes contain a nonlysosomal, ATP-dependent protein degradation system (for a review, see reference 10). This proteolytic system, which can be stimulated by ubiquitin, appears to be responsible for selective degradation of abnormal polypeptides. In this degradation system, ATP seems to serve at least the following two distinct functions: (i) it is required for the formation of covalent linkage between ubiquitin and substrate proteins in order to enhance the susceptibility of the substrate proteins to reticulocyte proteases (10), and (ii) it is involved in an unidentified reaction step which is independent of ubiquitin (24).

By using the preparations obtained from the DEAE-cellulose column as enzyme sources, we determined that cleavage of the 200K protein is an ATP-dependent reaction. Furthermore, we found that under the assay conditions used in this study cleavage was not stimulated by ubiquitin (Fig. 4). Figure 4, lanes a through d, contained samples which contained, respectively, 0, 20, 100, and 200 μg of ubiquitin per ml with no ATP added; lanes e through h contained the same samples but with ATP. The protein profiles indicate that cleavage could occur only in the presence of ATP and that no increase in cleavage was observed with increasing ubiquitin concentrations.

## DISCUSSION

**Identification of the primary cleavage enzyme.** Our results demonstrate that cleavage of the CPMV 200K polyprotein

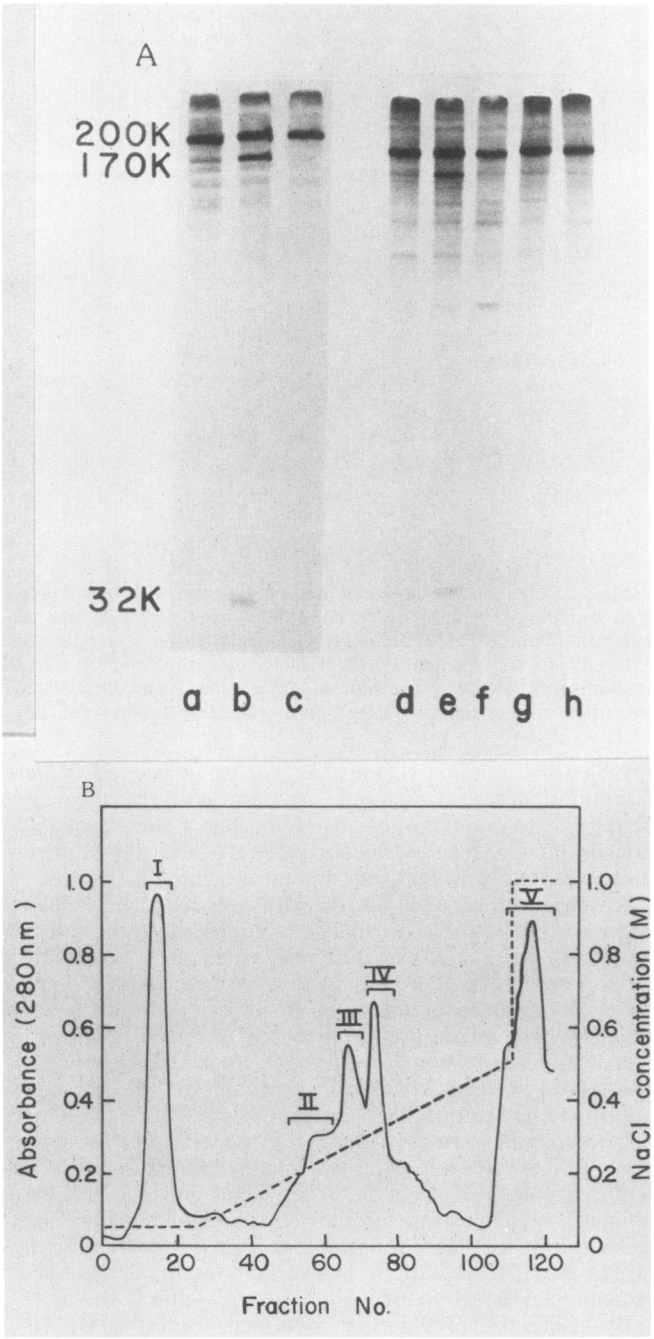


FIG. 3. Cleavage of isolated 200K polyprotein with fractionated lysate components. (A) Lanes a through c, Polyprotein incubated with reticulocyte proteins derived from 40 to 50% (lane a), 50 to 60% (lane b), and 60 to 70% (lane c) ammonium sulfate-saturated fractions; lanes d through h, polyprotein incubated separately with fractions I through V eluted from a DEAE-cellulose column. (B) DEAE-cellulose elution profile. The chromatography conditions used are described in Materials and Methods.

requires a cellular factor. These results represent the first direct evidence for involvement of a cellular protein in a primary cleavage reaction. At the present time, the identity of the primary cleavage enzyme has not been determined for any viral system. Therefore, identification of the 200K cleavage enzyme should be an important and interesting pursuit. What is this enzyme that causes cleavage of only one peptide bond on a large 200K protein molecule? Is this enzyme involved in cleaving any of the picornavirus precursor proteins which have been shown to be actively processed in the reticulocyte lysate (16, 21, 22)? What is the relationship of this enzyme, if any, to the reticulocyte ATP-dependent protein degradation system? Purification and further characterization of the cleavage activity will be necessary to answer these and other related questions.

It should be pointed out that since the viral proteins synthesized in CPMV-infected cowpea protoplasts and in reticulocyte lysates are similar (6, 8), it is reasonable to assume that a corresponding cleavage enzyme exists in plant cells. Identification of this plant enzyme should also be a meaningful problem.

**Requirement of DTT for isolating reactive 200K molecules.** An important factor that contributed to the demonstration of reticulocyte cleavage activity is the DTT dependence of the cleavage reaction. By omitting DTT from the lysate protein synthesis reaction mixture we were able to synthesize uncleaved 200K protein molecules. However, we found that in order to isolate reactive 200K protein molecules it was necessary to include a low concentration (0.3 mM) of DTT in the sucrose density gradient solution, because otherwise the isolated polyprotein was not cleavable. This DTT requirement may be explained as follows. In a non-DTT-supplemented translation reaction mixture some of the synthesized 200K protein molecules are maintained in a native or near-native conformation by low levels of reducing agents present in the reticulocyte lysate. However, in a DTT-free gradient the endogenous reducing agents are separated from the polyprotein, and this causes irreversible denaturation of the polyprotein.

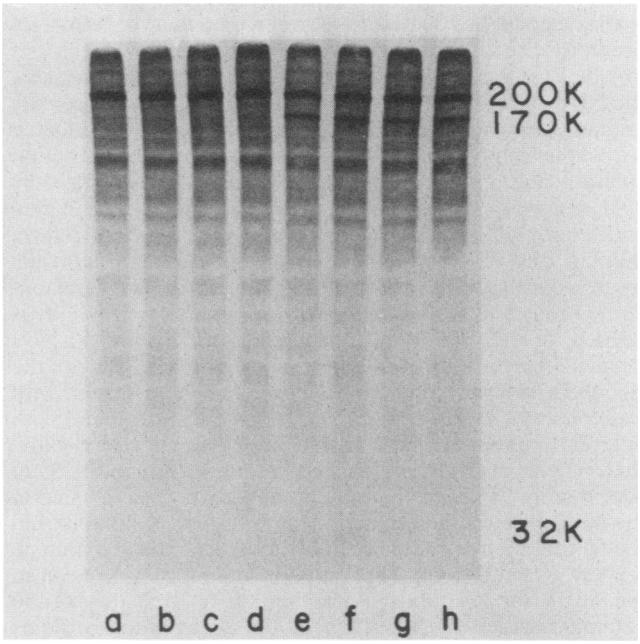


FIG. 4. Requirement of ATP for the cleavage reaction. DEAE-cellulose fraction II was used as the enzyme source. Lanes a through d, Samples incubated with, respectively, 0, 20, 100, and 200 μg of ubiquitin per ml (ubiquitin was a gift from Hans-Christian Thogersen of the laboratory of Aaron Klug; lanes e through h, same as lanes a through d, respectively, except that 1.6 mM ATP was included in the samples.

Furthermore, it should be pointed out that in some severe cases omission of DTT from the gradients resulted in complete precipitation of the polyprotein during centrifugation.

## ACKNOWLEDGMENTS

We thank Hans-Christian Thogersen for providing a sample of ubiquitin.

This work was supported by Public Health Service grant AI17764 from the National Institute of Allergy and Infectious Diseases and by funds provided by the Louisiana Agricultural Experiment Station.

## LITERATURE CITED

1. **Bruening, G.** 1977. Plant covirus systems: two-component systems, p. 55–141. *In* H. Fraenkel-Conrat and R. R. Wagner (ed), Comprehensive virology, vol. 11. Plenum Publishing Corp., New York.
2. **Burroughs, J. N., D. V. Sangar, B. E. Clarke, D. J. Rowlands, A. Billiau, and D. Collen.** 1984. Multiple proteases in foot-and-mouth disease virus replication. J. Virol. **50**:878–883.
3. **Daubert, S. D., G. Bruening, and R. C. Najarian.** 1978. Protein bound to the genome RNAs of cowpea mosaic virus. Eur. J. Biochem. **92**:45–51.
4. **El Manna, M. M., and G. Bruening.** 1973. Polyadenylate sequences in the ribonucleic acids of cowpea mosaic virus. Virology **56**:198–206.
5. **Emini, E. A., M. Elzinga, and E. Wimmer.** 1982. Carboxy-terminal analysis of poliovirus proteins: termination of poliovirus RNA translation and location of unique poliovirus polyprotein cleavage sites. J. Virol. **42**:194–199.
6. **Franssen, H., R. Goldbach, and A. van Kammen.** 1984. Translation of bottom component RNA of cowpea mosaic virus in reticulocyte lysate: faithful proteolytic processing of the primary translation product. Virus Res. **1**:39–49.
7. **Goldbach, R., G. Rezelman, and A. van Kammen.** 1980. Independent replication and expression of B-component RNA of cowpea mosaic virus. Nature (London) **286**:297–300.
8. **Goldbach, R., G. Rezelman, P. Zabel, and A. van Kammen.** 1982. Expression of the bottom component RNA of cowpea mosaic virus: evidence that the 60-kilodalton VPg precursor is cleaved into single VPg and a 58-kilodalton polypeptide. J. Virol. **42**:630–635.
9. **Hanecak, R., B. L. Semler, C. W. Anderson, and E. Wimmer.** 1982. Proteolytic processing of poliovirus polypeptides: antibodies to polypeptide P3-7c inhibit cleavage at glutamine-glycine pairs. Proc. Natl. Acad. Sci. USA **79**:3973–3977.
10. **Hershko, A., and A. Ciechanover.** 1982. Mechanisms of intercellular protein breakdown. Annu. Rev. Biochem. **51**:335–364.
11. **Korant, B. D.** 1972. Cleavage of viral precursor proteins in vivo and in vitro. J. Virol. **10**:751–759.
12. **Lomonossof, G., and M. Shanks.** 1983. The nucleotide sequence of cowpea mosaic virus B-RNA. EMBO J. **2**:2253–2258.
13. **Palmenberg, A. C., E. M. Kirby, M. R. Janda, N. L. Drake, G. M. Duke, K. F. Potratz, and M. J. Collet.** 1984. The nucleotide and deduced amino acid sequences of the encephalomyocarditis viral polyprotein coding region. Nucleic Acids Res. **12**:2969–2985.
14. **Palmenberg, A. C., M. Pallansch, and R. R. Rueckert.** 1979. Protease required for processing picornaviral coat protein resides in the viral replicase gene. J. Virol. **32**:770–778.
15. **Palmenberg, A. C., and R. R. Ruckert.** 1982. Evidence for intramolecular self-cleavage of picornaviral replicase precursors. J. Virol. **41**:244–249.
16. **Pelham, H. R. B.** 1978. Translation of encephalomyocarditis virus RNA *in vitro* yields an active proteolytic processing enzyme. Eur. J. Biochem. **85**:457–482.
17. **Pelham, H. R. B.** 1979. Synthesis and proteclytic processing of cowpea mosaic virus proteins in reticulocyte lysates. Virology **96**:463–477.
18. **Peng, X. X., and D. S. Shih.** 1984. Proteolytic processing of the proteins translated from the bottom component RNA of cowpea mosaic virus. The primary and secondary cleavage reactions. J. Biol. Chem. **259**:3197–3201.
19. **Rezelman, G., R. Goldbach, and A. van Kammen.** 1980. Expression of bottom component RNA of cowpea mosaic virus in cowpea protoplasts. J. Virol. **36**:366–373.
20. **Semler, B. L., R. Hanecak, C. W. Anderson, and E. Wimmer.** 1981. Cleavage sites in the polypeptide precursors of poliovirus protein P2-X. Virology **114**:589–594.
21. **Shih, D. S., C. T. Shih, O. Kew, M. Pallansch, R. Rueckert, and P. Kaesberg.** 1978. Cell-free synthesis and processing of the proteins of poliovirus. Proc. Natl. Acad. Sci. USA **75**: 5807–5811.
22. **Shih, D. S., C. T. Shih, D. Zimmern, R. R. Rueckert, and P. Kaesberg.** 1979. Translation of encephalomyocarditis virus RNA in reticulocyte lysates: kinetic analysis of the formation of virion proteins and a protein required for processing. J. Virol. **30**:472–480.
23. **Summers, D. F., E. N. Shaw, M. L. Stewart, and J. V. Maizel.** 1972. Inhibition of cleavage of large poliovirus-infected precursor proteins in infected HeLa cells by inhibitors of proteolytic enzymes. J. Virol. **10**:880–884.
24. **Tanaka, K., L. Waxman, and A. L. Goldberg.** 1984. Vanadate inhibits the ATP-dependent degradation of proteins in reticulocytes without affecting ubiquitin conjugation. J. Biol. Chem. **259**:2803–2809.
25. **van Kammen, A.** 1967. Purification and properties of the components of cowpea mosaic virus. Virology **31**:633–642.
26. **Van Wezenbeek, P., J. Verver, J. Harmsen, V. Vos, and A. van Kammen.** 1983. Primary structure and gene organization of the middle component RNA of cowpea mosaic virus. EMBO J. **2**:941–946.