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Foot-and-Mouth Disease Virus Protease 3C Induces Specific Proteolytic Cleavage of Host Cell Histone H3

MATTHIAS M. FALK,[1]* PABLO R. GRIGERA,[2] INGRID E. BERGMANN,[3] ANDREE ZIBERT,[1] GERD MULTHAUP,[1] AND EWALD BECK[1]

Zentrum für Molekulare Biologie Heidelberg, Universität Heidelberg, D-6900 Heidelberg, Federal Republic of Germany[1]; Centro de Virología Animal, Serrano 661 (1414), Buenos Aires, Argentina[2]; and Pan American Foot-and-Mouth Disease Center, CEP 20001, Rio de Janeiro, Brazil[3]

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In foot-and-mouth disease virus (FMDV)-infected cells, the disappearance of nuclear protein histone H3 and the simultaneous appearance of a new chromatin-associated protein termed Pi can be observed (P. R. Grigera and S. G. Tisminetzky, Virology 136:10–19, 1984). We sequenced the amino terminus of protein Pi and showed that Pi derives from histone H3 by proteolytic cleavage. The 20 N-terminal amino acid residues of histone H3 are specifically cleaved off early during infection. Truncated histone H3 remains chromatin associated. In addition, we showed that the histone H3-Pi transition is catalyzed by the FMDV 3C protease. The only known function of the viral 3C protease was, until now, the processing of the viral polyprotein. The viral 3C protease is the only FMDV protein required to induce the histone H3-Pi transition, as well as being the only viral protein capable of cleaving histone H3. No viral precursor fusion protein is needed for this specific cleavage as was reported for the processing of the poliovirus P1 precursor polyprotein by 3C/D protease. As the deleted part of the histone H3 corresponds to the presumed regulatory domain involved in the regulation of transcriptionally active chromatin in eucaryotes, it seems possible that this specific cleavage of histone H3 is related to the host cell transcription shutoff reported for several picornaviruses.

The propagation of picornaviruses, involving the replication of the genome, RNA and protein syntheses, processing of the primary translation products, and encapsidation, occurs entirely in the cytoplasm of the infected cell. Although apparently no functions of the host cell nucleus are involved in the picornaviral life cycle, it is possible to isolate viral proteins from poliovirus and foot-and-mouth disease virus (FMDV) from nuclear fractions (4, 17, 22). Therefore, it was postulated that in poliovirus-infected HeLa cells some virus-specific gene products interact with chromatin (17). Little is known about picornavirus-induced changes in the structure or function or both of host cell chromatin. Changes in the microheterogeneity of histone H1 after mengovirus infection of Ehrlich ascites tumor cells were reported by Traub and Traub (57).

After infection with picornaviruses, host cell RNA, DNA, and protein syntheses are abolished and the cellular machinery is subverted to the production of viral protein and RNA (35, 51), an event often referred to as host cell shutoff. The specific inhibition of host cell protein synthesis is thought to result in part from the inactivation of the translational initiation factor eIF-4F or cap-binding protein complex (16, 53) through cleavage of the largest subunit of this complex, termed p220, by the viral 2A protease as shown for enteroviruses and rhinoviruses (34) or the L/L′ protease of FMDV (14). Therefore, cellular cap-dependent mRNA translation would decrease, whereas the translation of uncapped viral mRNA is favored by ribosome binding at an internal ribosome entry site within the 5′ untranslated region of the picornaviral mRNA (for a review, see reference 25). However, at least one other event in addition seems to be required for complete inhibition of host cell protein synthesis (8). Recently, Black et al. (5) reported an increased eIF-2α phosphorylation followed by degradation of cellular 68,000-M_r protein kinase in poliovirus-infected cells. Whereas mechanisms inhibiting host cell protein synthesis are well documented, little is known about the picornavirus-induced inhibition of host cell RNA synthesis that is reported for mengovirus, poliovirus, encephalomyocarditis (EMC) virus (reviewed in reference 13), and FMDV (10; M. Tesar and O. Marquardt, Virology, in press; I. Bergmann, unpublished data). Infection of BHK-21 cells and IB-RS2 cells (a swine-derived cell line) with FMDV causes the disappearance of histone H3 and the simultaneous appearance of a new chromatin-associated polypeptide termed Pi, which migrates between H2A and H4 on sodium dodecyl sulfate (SDS)-polyacrylamide gels (21). On the basis of the observations obtained for poliovirus (4, 17) and for mengovirus (57) mentioned above, one could argue that FMDV-specific gene products may interact with the chromatin of the infected cell, resulting in histone modification. Therefore, we hypothesized that protein Pi might be a specific degradation product of histone H3 produced by a viral protease or an endogeneous proteolytic activity induced during infection.

In this report, we show unambiguously that protein Pi corresponds to truncated histone H3 missing the first 20 N-terminal amino acid residues by determining the amino-terminal amino acid sequence of protein Pi. In addition, by the incubation of isolated nuclei with in vitro-synthesized proteins derived from different regions of the FMDV genome, we demonstrated that this specific cleavage of histone H3 is performed by the FMDV 3C protease. No other viral protein is capable of cleaving histone H3 in a similar way under native conditions. Until now, the only reported function of the 3C protease is the proteolytic processing of the viral precursor polyprotein into the mature gene products.

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* Corresponding author.

MATERIALS AND METHODS

Cells and viruses. BHK-21 clone 13 cells and HeLa cells were grown in monolayer culture in Dulbecco minimum essential medium supplemented with 10% newborn calf serum or 10% fetal calf serum, respectively, and 100 U of penicillin and streptomycin per ml.

BHK-21 cells were infected with FMDV type C3 strain Resende at a multiplicity of infection of approximately 10 PFU by the method of Grigera and Tisminetzky (23).

HeLa cells were infected in a similar way with attenuated Sabin I poliovirus.

Labeling of cells. Cells were seeded 1 day before labeling into tissue culture plates of 10-cm diameter. Exponentially growing monolayers were labeled for 14 h with 20 μCi of [^{35}S]methionine (>1,000 Ci/mmol Amersham Corp., Arlington Heights, Ill.) per ml in Dulbecco minimum essential medium lacking methionine (GIBCO Laboratories, Grand Island, N.Y.).

Extraction of cell nuclei and chromatin. Cell nuclei were prepared under physiological conditions by the method of Zentgraf and Franke (62). Cell monolayers were extensively washed with ice-cold Dulbecco minimum essential medium and treated with 1 ml of cell lysis buffer A (100 mM NaCl, 50 mM Tris hydrochloride [pH 7.4], 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10% sucrose, 0.05% Nonidet P-40, 0.05% sodium deoxycholate) per dish at 0°C.

After lysis, cells were scraped from the dishes with a rubber policeman and transferred to Eppendorf tubes (1.5-ml volume). A total cell homogenate was obtained by pipetting the solution up and down a few times. Samples were centrifuged at 1,000 × *g* for 5 min at 4°C to pellet the nuclei. The nuclei were washed once in buffer A and used directly in the in vitro assay or were suspended in 0.5 ml buffer B (as buffer A, but containing 1% Nonidet P-40 and 0.5% sodium deoxycholate). The nuclear material was fractionated by being pelleted for 5 min at 1,000 × *g* through a 1-ml cushion of 20% sucrose in buffer B. Purity of resuspended nuclear material was verified by microscopic examination. Nuclear material obtained by both methods gave identical results in the in vitro assay described below.

Protein sequencing. Chromatin from FMDV-infected BHK-21 cells was prepared and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) as described elsewhere (56). To visualize protein bands, we soaked the gels in 1 M KCl–10% acetic acid (39). The bands representing protein Pi were excised from the gel, and the protein was electroeluted by the method of Hunkapillar et al. (27). SDS was removed from protein Pi by ion-pair extraction with anhydrous acetone-triethylamine-acetic acid-water (85:5:5:5, by volume) (31). The lyophilized protein was redissolved in formic acid and sequenced automatically by gas-phase sequencing with on-line high-pressure liquid chromatographic analysis as described by Hope et al. (26).

Construction of FMDV cDNA clones. FMDV cDNA clones were constructed by standard procedures (36). The cDNA of FMDV strain O_1K (18) was cloned from position 743 to the 3′ end including the poly(A) sequence (position 8000) into the transcription vector pSP64 (38) (see Fig. 4). In vitro transcription of the *Hpa*I-linearized plasmid (pSP0.7-8.0) with SP6 polymerase resulted in an RNA coding for the whole FMDV O_1K polyprotein. Linearization of this plasmid with *Xba*I (position 5413 of the viral genome) resulted in a deletion of the 3BCD-coding region and the C-terminal part of 3A on the transcript.

Plasmid pSP5.1-8.0 contains the FMDV polyprotein-coding sequence 3′ to the *Eco*RI site at position 5149 and leads to an RNA encoding the whole P3 precursor protein except for the 24 N-terminal amino acid residues of 3A.

Plasmid pSP5.1-6.4 was constructed by the insertion of a 1.3-kilobase *Eco*RI-*Hin*dIII fragment (FMDV positions 5149 to 6448) into pSP65. The derived RNA started as described for pSP5.1-8.0 and stopped after 30 amino acid residues of the 3D-coding region.

A Ser-163 mutant of 3C protease (pSP5.1-6.4M6) was derived from plasmid pSP5.1-6.4 by oligonucleotide site-directed mutagenesis, resulting in a Cys-163 to Ser-163 amino acid exchange as described elsewhere (A. Zibert and E. Beck, manuscript in preparation).

Plasmid pSP5.4-6.4 was derived from pSP5.1-6.4 by deletion of the *Eco*RI-*Xba*I fragment (FMDV positions 5149 to 5413). In vitro transcription resulted in an RNA coding for the 3C protease, the VPgs ($3B_{123}$), and 20 N-terminal amino acid residues of 3D. In vitro translation could initiate at position 5825 in the $3B_2$ coding region.

Linearization of pSP5.4-6.4 with *Pst*I (FMDV position 6296) led to an RNA coding for an incomplete 3C protease lacking the 30 C-terminal amino acid residues.

In vitro transcription-translation and histone H3 processing. Transcription reactions were essentially performed by standard procedures (38). ^{7}mG(5′)ppp(5′)G (Boehringer GmbH, Mannheim, Federal Republic of Germany) was added to obtain capped mRNA as described by Mayer et al. (37). Synthetic RNAs (1 μg/μl) were translated in rabbit reticulocyte lysate (Amersham) (1 μg of RNA per 10 μl of lysate). To check in vitro translation efficiency, we supplemented small samples of the translation mixtures with [^{35}S]methionine. After incubation for 1 h at 30°C, isolated labeled nuclei corresponding to a 10- to 15-cm^2 dish of culture cells were mixed with 30 to 50 μl of translation mixture and incubated at 30°C. To avoid degradation of core histones during incubation by a chromatin-associated protease (12, 33) reported to cleave native histone H3 between Lys-23 and Ala-24 (9), we added 2 mM diisopropylfluorophosphate (DFP) to the incubation mixture. After different times of incubation with the reticulocyte lysate, nuclear material was pelleted at 10,000 × *g* for 5 min and suspended in sample buffer. SDS-PAGE was done by the method of Thomas and Kornberg (56). Gels were soaked in 1 M sodium salicylate for fluorography before drying.

RESULTS

Identification of protein Pi as part of histone H3. To investigate the hypothesis that protein Pi represents a proteolytic degradation product of histone H3 derived by specific cleavage at an exposed site of this histone in native chromatin, we sequenced the amino-terminal end of protein Pi. For this purpose, FMDV-infected BHK-21 cells were lysed 4 h postinfection (p.i.) and chromatin was prepared as described in Materials and Methods. Histones were analyzed by SDS-PAGE. The band representing protein Pi was excised from the gel and electroeluted. The 20 N-terminal amino acid residues of the polypeptide were sequenced by automatic Edman degradation combined with on-line high-pressure liquid chromatographic analysis. The determined amino acid sequence could be perfectly aligned with amino acids Ala-21 to Arg-40 of calf thymus histone H3 (Fig. 1). The high-pressure liquid chromatograms derived from the sequencing reactions of the 20 amino-terminal amino acid residues of protein Pi are shown in Fig. 2. Not only the amino acid sequence but also the methylation of Lys-7 and

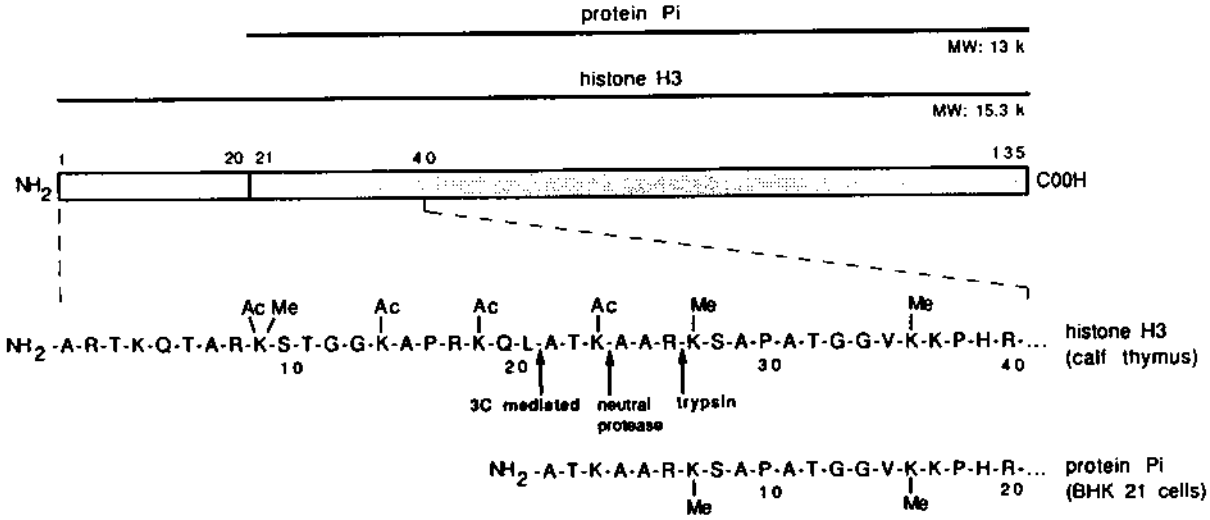

FIG. 1. Identification of protein Pi as part of histone H3. The sequences shown correspond to the hydrophilic N-terminal domain of calf thymus histone H3 carrying the posttranslational modifications acetylation (Ac) and methylation (Me) and to the N-terminal part of protein Pi as derived from BHK-21 cells after infection with FMDV. As shown by protein sequencing and in vitro transcription-translation assays, protein Pi originates from histone H3 by viral protease 3C-mediated cleavage between Leu-20 and Ala-21. Cleavage by the tightly chromatin-associated neutral protease (9) and cleavage of isolated chromatin or core particles with trypsin (6) yields similar degradation products, indicating that the amino-terminal portion of histone H3 is exposed and accessible in the core particle. Schematic drawings of histone H3 and protein Pi are shown above. MW, Molecular weight; K, $\times 10^3$.

Lys-16 can be clearly seen in the chromatograms. On the other hand, Lys-17 was not methylated. All these observations are completely in agreement with histone H3 from calf thymus (6), unequivocally identifying protein Pi as amino-terminal-truncated histone H3.

Examination of chromatin-associated proteins isolated at different times after infection showed that degradation of histone H3 started at 1.5 h p.i. and that it was converted completely to protein Pi after 6 h. The life cycle of FMDV was completed within the same time. Virus particles were released by host cell lysis 4 to 6 h after infection. The other histones of the infected cells showed no visible change on SDS gels (Fig. 3A) (23). Therefore, the cleavage between Leu-20 and Ala-21 of histone H3 seems to be the only proteolytic event, although this amino acid pair exists at additional positions in the other histone types.

Virus-induced processing of histone H3 in vitro. To characterize the virus-induced proteolysis of histone H3 in more detail, we incubated [^{35}S]methionine-labeled nuclei derived from uninfected BHK-21 cells with a cytoplasmic extract of infected cells. Cytoplasmic extract was obtained by lysing the cells at 4 h p.i. Crude cellular materials such as nuclei and membrane fragments were pelleted by 1,000 $\times$ *g* centrifugation. The clear supernatant was used in this assay (see Materials and Methods). Figure 3B shows that in vitro processing of histone H3 to protein Pi was possible under these conditions. Histone H3 was already partially converted to protein Pi 1 h after incubation of nuclei with cytoplasmic extract from infected cells (Fig. 3B, lane 2) but remained stable in nuclei incubated with cytoplasmic extract derived from mock-infected cells (Fig. 3B, lane 1). These findings clearly showed that a proteolytic activity is present in the cytoplasm of the FMDV-infected cell, directly or indirectly cleaving histone H3. (Previous published failure to cleave histone H3 in vitro in a comparable experiment [23] is most likely due to different experimental conditions used.)

Addition of 2 mM *N*-ethylmaleimide (NEM) (Fig. 3B, lane 3) or 2 mM iodoacetamide (Fig. 3B, lane 4) to the incubation mixture before incubation completely inhibited the histone H3 processing. The same was found in presence of 2 mM L-1-chloro-3-(4-tosylamido)-4-phenyl-2-butanone (TPCK) or 2 mM $ZnCl_2$ (data not shown).

Peptide chloromethylketones such as TPCK irreversibly inhibit both serine and cysteine proteinases by covalent binding to the hydroxyl group of serine residues or to the sulfhydryl group of cysteine residues, respectively (46). On the other hand, iodoacetamide and NEM are known as cysteine protease-specific inhibitors which inactivate only cysteine proteases by alkylation of the cysteine sulfhydryl group at the active site (50).

However, 2 mM DFP, a serine protease inhibitor, did not inhibit the specific histone H3 cleavage reaction. DFP was used in all in vitro incubation reactions to prevent histone degradation by the so-called neutral protease, a serine-type enzyme, as described by Kurecki et al. (33).

Butterworth and Korant (11) reported the observation of large picornaviral peptides in the presence of zinc ions, and Pelham (44) and Gorbalenya and Svitkin (21) reported a specific inhibition of 3C protease from EMC virus by NEM and iodoacetamide. On the basis of these data, and since picornaviral and plant comovirus 3C proteases are related cysteine proteases (3, 19, 61; for a review, see reference 32) with homology to cellular cysteine and serine proteases (20), we suggest that the FMDV 3C protease is involved in this specific histone H3-Pi transition.

Assay for histone H3 cleavage by different FMDV gene products. To prove that the FMDV 3C protease participates in the histone H3-Pi transition, we constructed a set of transcription vectors containing cDNAs of different parts of the viral genome inserted adjacent to an SP6 promoter (Materials and Methods) (Fig. 4). RNA transcripts were synthesized in vitro and translated in rabbit reticulocyte lysates.

Samples of each reaction were supplemented with

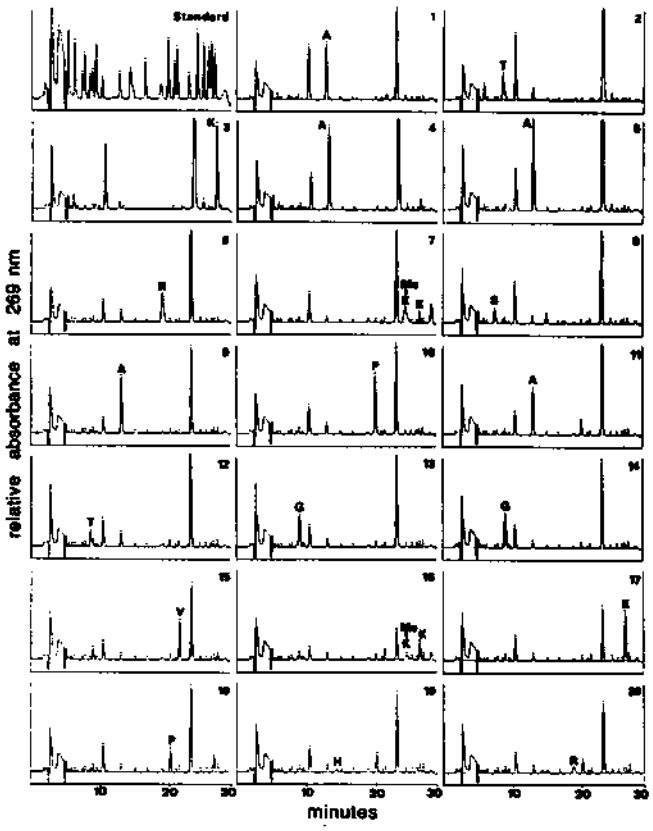

FIG. 2. High-pressure liquid chromatograms of the 20 amino-terminal amino acid residues derived from amino acid sequencing of protein Pi. Protein Pi was isolated from FMDV-infected BHK-21 cells at 4 h p.i. and purified as described in the text. Peaks representing an amino acid residue are marked with the corresponding letter. While Lys-17 is not methylated, the methylation (Me) of Lys-7 and Lys-16 can be seen on the chromatograms. These results are in agreement with the methylation pattern of histone H3 of calf thymus (6).

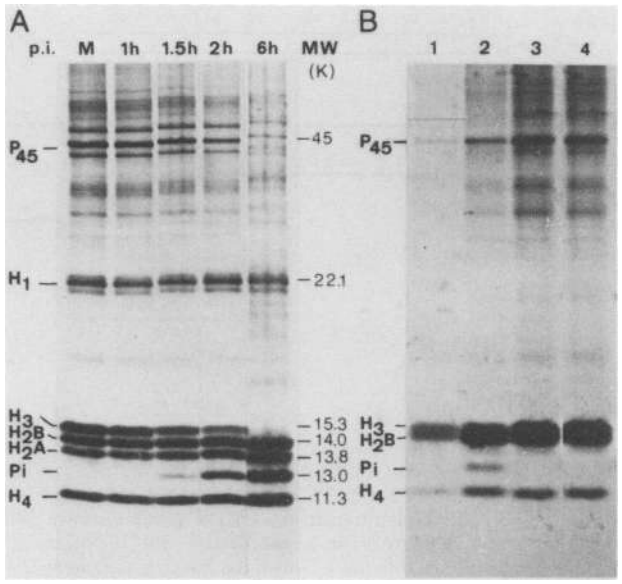

FIG. 3. Histone H3 cleavage in vivo and in vitro. (A) Chromatin of BHK-21 cells was prepared at 1, 1.5, 2, and 6 h p.i. with FMDV (lane M, mock-infected cells as control). Proteins were analyzed by SDS-PAGE and stained with Coomassie blue. A histone standard (Sigma) and marker proteins (Amersham) were used as references. At 1.5 h p.i., the degradation of histone H3 becomes visible by the appearance of the histone H3 cleavage product Pi. By 6 h p.i., histone H3 of the infected cell is completely converted into protein Pi. (B) [^{35}S]methionine-labeled nuclei of uninfected BHK-21 cells were prepared and incubated with a cytoplasmic fraction from infected cells obtained at 3 h p.i. The histone H3-Pi transition occurs in the presence of viral proteins (lane 2) but not in the corresponding extract from mock-infected cells (lane 1). Histone H3 cleavage is blocked by adding a cysteine protease inhibitor such as NEM (lane 3) or iodoacetamide (lane 4) to the incubation mixture. Histones H1 and H2A are not labeled because they contain no methionine residues. MW, Molecular weight; K, $\times 10^3$.

[^{35}S]methionine to check in vitro translation efficiency (Fig. 5). Viral RNA isolated from strain O Israel was translated in parallel to show processing of FMDV polyprotein in vitro (59) (Fig. 5, lanes 1a and 1b). Translation of the viral RNA and RNAs derived from pSP0.7-8.0, pSP5.1-8.0, pSP5.1-6.4, and pSp5.4-6.4 yielded different, smaller protein bands representing viral proteins derived from the large precursor proteins by proteolytic cleavage, indicating synthesis of active 3C protease (Fig. 5, lanes 2, 4, 5, and 6) (34). Proteins obtained by in vitro translation were characterized by immunoprecipitation with specific antisera raised against viral proteins (55) (data not shown).

After 1 h of incubation, the different cold translation reactions were mixed with freshly isolated [^{35}S]methionine-labeled nuclei of BHK-21 cells and incubated for an additional hour. To avoid degradation of core histones during incubation by the chromatin-associated neutral protease (9, 12, 33), we added 2 mM DFP.

Protein Pi was produced in extracts after preincubation with either viral RNA or plasmid-derived RNA coding for the whole polyprotein (Fig. 6A, lanes 3 and 4). In contrast, protein Pi was not present either in isolated nuclei alone (lane 1) or in isolated nuclei incubated with reticulocyte lysate not primed with RNA (lane 2).

To investigate whether the FMDV L/L′ protease (54) or another protein of the P1/P2 precursor protein could be responsible for the histone H3-Pi transition, we cleaved plasmid pSP0.7-8.0 with *Xba*I (position 5413 of FMDV genome). The resulting synthetic RNA coded for the whole FMDV polyprotein up to the *Xba*I site in the 3A-coding region (Fig. 4). Histone H3 was not cleaved under these conditions (Fig. 6A, lane 5), demonstrating that neither active L/L′ protease nor another protein of the P1/P2 region of the FMDV genome is responsible for the specific histone H3 cleavage.

The proteolytic activity was localized to the C-terminal part of the viral polyprotein by the following experiments. The translation products of transcripts coding for the whole P3 region of FMDV genome or parts of it (pSP5.1-8.0, pSP5.1-6.4, and pSP5.4-6.4; Fig. 4) always induced histone H3 cleavage (Fig. 6A, lanes 6 to 8). Protein translated from an RNA coding exclusively for the viral 3C protease and one VPg (pSP5.4-6.4, lane 8) was also able to cleave histone H3 in this assay.

Inactivation of the viral protease 3C by deletion of the 30 C-terminal amino acid residues (pSP5.4-6.4xP; Fig. 6, lane 9) or by adding specific cysteine protease inhibitors such as NEM (Fig. 6, lane 10) or iodoacetamide (Fig. 6, lane 11) before incubation with isolated nuclei blocked the histone H3-Pi transition completely. Also, histone H3 cleavage was

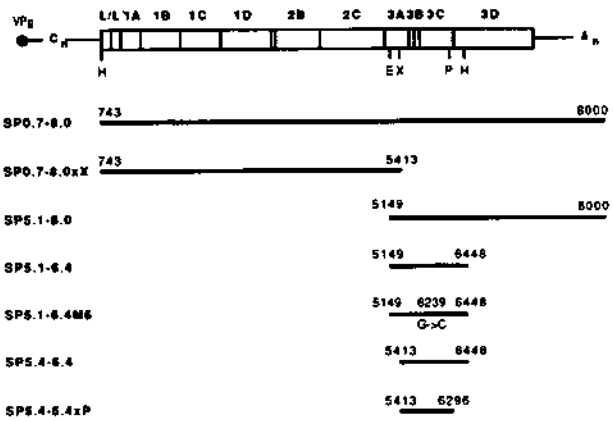

FIG. 4. FMDV RNAs transcribed in vitro. The diagram shows the RNA transcripts derived from the linearized plasmids pSP0.7-8.0, pSP5.1-8.0, pSP5.1-6.4, pSP5.1-6.4M6, pSP5.4-6.4, and pSP5.4-6.4xP in their relative positions to the FMDV genome shown in the upper part of the figure. The gene of the viral 3C protease is highlighted. pSP5.1-6.4M6 has a G-to-C exchange at position 6239 of the viral genome that leads to a Cys-to-Ser amino acid change at position 163 of the 3C protease. The numbers refer to the cleavage positions of restriction endonucleases in the viral genome used for construction of FMDV cDNA clones and for linearization of the plasmids. H, *Hin*dIII; E, *Eco*RI; X, *Xba*I; P, *Pst*I.

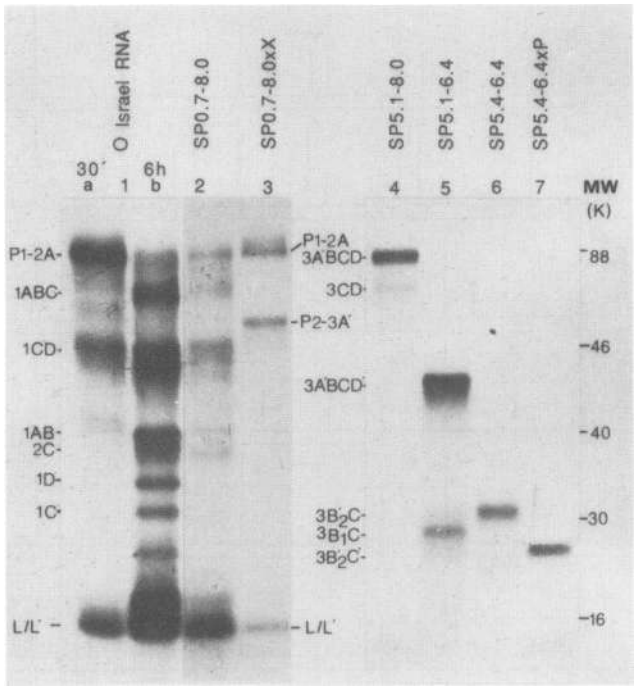

FIG. 5. In vitro translation of transcribed FMDV RNAs in the presence of [^{35}S]methionine. The products of the in vitro translation reactions synthesized by FMDV RNAs described in the legend to Fig. 4 (translation products of pSP5.1-6.4M6 are not shown) were analyzed by SDS-PAGE and autoradiography (lanes 2 to 7). Proteins obtained were characterized by their size and by immunoprecipitation with specific antisera (55). The appearance of smaller protein bands indicates in vitro processing of the viral precursor proteins by the active 3C protease. Lanes 1a and 1b show translation products derived from viral RNA after 30 min and 6 h of incubation, respectively. Truncated proteins are indicated ('). MW, Molecular weight; K, $\times 10^3$.

not observed in the presence of the 3C-M6 protease mutant (pSP5.1-6.4M6; Fig. 6, lane 12). In this mutant, amino acid residue Cys-163 was changed to a serine residue by site-directed mutagenesis (Zibert and Beck, in preparation). By analogy to related cysteine proteases from other picornaviruses and comoviruses (3, 19, 61), Ser-163 is located in the active site of FMDV 3C protease. This protease mutant is also incapable of processing the viral polyprotein in vitro (data not shown). Similar results have been reported by Ivanoff et al. (28) with an analogous amino acid exchange in the active site of poliovirus 3C protease.

The results obtained from these in vitro experiments clearly demonstrated that 3C protease of FMDV is the only viral gene product involved in the specific histone H3 cleavage reaction. From our in vitro incubation assay we can conclude that truncated histone H3 still remains chromatin associated. Isolated chromatin was pelleted after incubation with rabbit reticulocyte lysate to separate the chromatin from contaminating globin of the reticulocyte lysate before analysis by SDS-PAGE (see Materials and Methods). Protein Pi was always bound to the pelleted material (Fig. 6).

To demonstrate specific histone H3 cleavage in the presence of 3C protease alone, we studied the kinetics of appearance of protein Pi after incubation of labeled nuclei in a reticulocyte extract preincubated with SP5.4-6.4 RNA as described above. After different times of incubation, samples were removed and chromatin was analyzed by SDS-PAGE. The fast appearance of protein Pi (5 to 15 min) demonstrated the high specificity of this cleavage reaction, although histone H3 was only partially converted to protein Pi and thus the decrease of histone H3 was not visible on the gel. This observation could be explained first by the quality of the isolated chromatin used in the assay, which remained only partly in native form during incubation, and second by the fact that globin from reticulocyte lysates has almost the same electrophoretic mobility as histone H3 on Kornberg SDS gels (56), therefore interfering with histone H3, H2A, and H2B resolution.

The histone proteins (including protein Pi) and the other labeled nuclear proteins were mostly stable against proteolytic degradation even during very long incubation periods (8 h in Fig. 7, lane 7), demonstrating a low level of contaminating proteases other than 3C in this system. Weaker protein bands in lane 7 were most likely caused by lower amounts of labeled proteins analyzed in this lane.

In an attempt to demonstrate direct cleavage of histone H3 by FMDV 3C protease, we incubated purified histones isolated from calf thymus (Sigma Chemical Co., St. Louis, Mo.) in the presence of 3C protease immunoprecipitated by a polyclonal antibody against 3C (55). No cleavage reaction occurred under these incubation conditions free of other proteases derived from reticulocyte lysate or isolated nuclei (data not shown).

Chromatin isolated in native form from HeLa cells was also used in the in vitro cleavage assay. In the presence of FMDV 3C protease derived from pSP5.4-6.4, HeLa cell histone H3 was also cleaved (Fig. 6B, lane 2), demonstrating that this specific histone H3 cleavage is not restricted to chromatin derived from cells that can be infected with FMDV. Cleavage of histone H3 was not observed, however, in chromatin from poliovirus Sabin I-infected HeLa cells isolated analogously as described for BHK-21 cells at 10 h p.i. (data not shown).

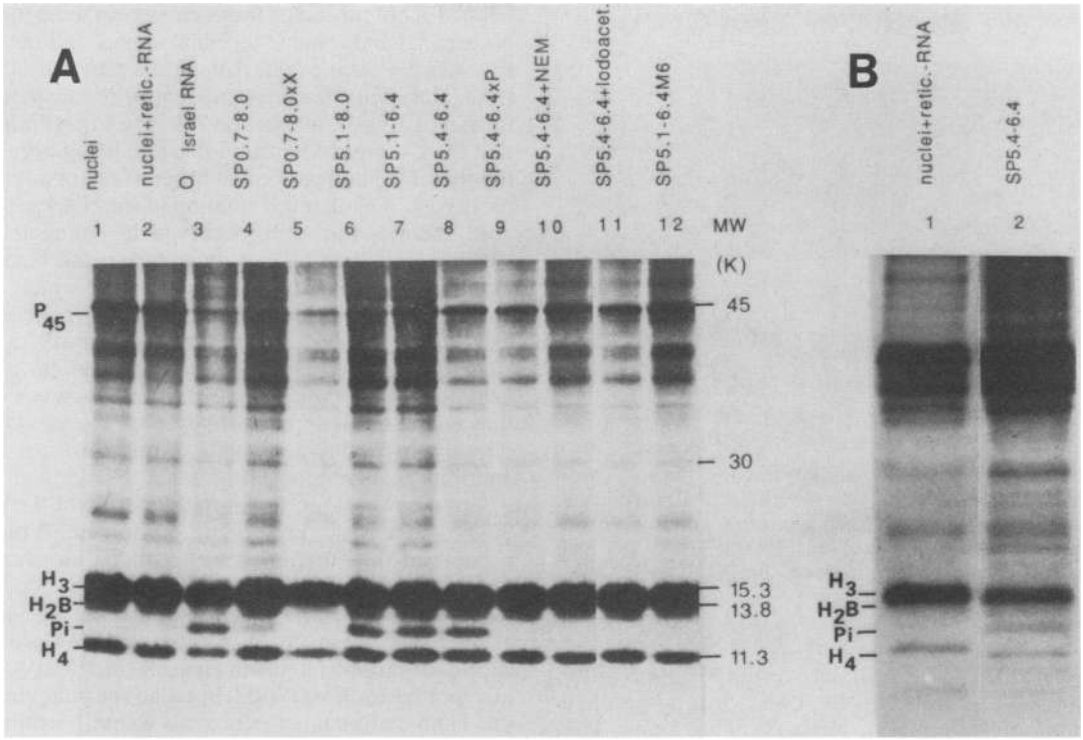

FIG. 6. In vitro histone H3 cleavage assay. (A) [^{35}S]methionine-labeled nuclei were isolated from BHK-21 cells and incubated with rabbit reticulocyte lysate and the FMDV RNAs described in the legend to Fig. 4. Proteins were analyzed by SDS-PAGE and autoradiography. Only in the reactions in which an RNA coding for the whole protease 3C is translated (Fig. 5) is histone H3 converted to protein Pi (lanes 3, 4, 6, 7, and 8). Lanes 5 and 8 show that the 3C protease is the only viral protein required for the cleavage. The histone H3-Pi transition is blocked by termination of RNA transcription at the *PstI* site within the coding region of 3C (lane 9), by adding cysteine protease inhibitors such as NEM (lane 10) or iodoacetamide (lane 11) to the incubation mixture, or by using a Cys-163 to Ser-163 mutant of 3C protease (lane 12). As controls, labeled nuclei alone (lane 1) and nuclei incubated with rabbit reticulocyte lysate without RNA (lane 2) are shown. (B) HeLa cell histone H3 is also cleaved by FMDV 3C protease in vitro. [^{35}S]methionine-labeled nuclei isolated from HeLa cells were incubated with rabbit reticulocyte lysate by using FMDV RNA SP5.4-6.4 (lane 2). As a control, lane 1 shows labeled nuclei incubated with rabbit reticulocyte lysate not suplemented with viral RNA. MW, Molecular weight; K, $\times 10^3$.

DISCUSSION

In this work, we demonstrated that during FMDV infection, 20 N-terminal amino acid residues of the nuclear protein histone H3 are specifically cleaved off and that the viral 3C protease is responsible for this proteolytic cleavage. Deacetylation (2) or another modification of histone H3 is not the reason for the visible H3-Pi transition. The cleavage reaction occurred very early within the first 2 h after infection and was mediated only by the viral 3C protease. Neither the viral L/L′ protease (54) nor another viral protein could perform this cleavage. No viral precursor fusion protein was needed for this specific histone H3 cleavage reaction, as was reported for the correct processing of the poliovirus P1 precursor polyprotein by 3C/D protease (40).

From these data, we cannot determine whether histone H3 is cleaved directly by viral 3C protease or indirectly by activating another unknown cellular protease, as in case of the p220 cleavage of the cap-binding protein complex by the viral L/L′ protease (14). Since our in vitro experiments were always done with chromatin isolated in native form containing further nuclear components, the activation of a ubiquitous unknown nuclear protease present in IB-RS2 cells, BHK-21 cells, and HeLa cells cannot be excluded. This postulated unknown protease was not inhibited by 2 mM DFP, which was added to all in vitro incubation reaction mixtures to prevent histone degradation by the tightly chromatin-associated neutral protease, a serine-type enzyme described by Kurecki et al. (33). Organophosphorous compounds such as DFP are irreversible inhibitors of serine proteases, acting by phosphorylation of the active-site serine (46). Therefore, any cellular protease that may be involved is certainly not a serine-type enzyme. Attempts to cleave purified histone H3 directly by 3C protease isolated from the in vitro translation system as an immunoprecipitate were unsuccessful. However, this does not prove the indirect cleavage of histone H3 by a second protease, because (i) histone H3 was possibly not in a native conformation and (ii) the 3C protease could have been inactivated by steric hindrance by the antibodies.

To look for common structural features, we compared the observed histone H3 cleavage site with the known 3C protease cleavage sites in the FMDV polyprotein (Table 1), although the amino acid sequences surrounding the natural 3C cleavage sites in FMDV (59), poliovirus (42), and EMC virus (43) do not share significant primary structure similarity. However, a common 3C protease substrate specificity exists, as shown by using chimeric picornavirus polyproteins (15). There is no Leu-Ala pair in the P1/P1′ position of the viral polyprotein as at the histone H3 cleavage site. However, Leu exists at position P1 at the uncommon 1D/2A 3C protease processing site (Table 1), and Ala in the P1′ position belongs to the group of small uncharged amino acid residues

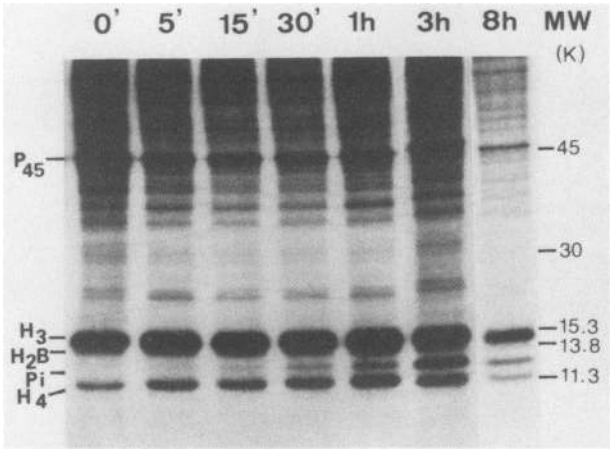

FIG. 7. Kinetics of histone H3 cleavage in vitro. Labeled chromatin derived from BHK-21 cells was incubated in a reticulocyte lysate expressing the plasmid pSP5.4-6.4-encoded 3C protease. At different times after incubation started (0, 5, 15, and 30 min; 1, 3, and 8 h), samples were removed and analyzed by SDS-PAGE. MW, Molecular weight; K, $\times 10^3$.

(Gly, Ala, Ser) which are also allowed at the P1′ position for correct processing, as shown for the EMC virus polyprotein by Parks and Palmenberg (43). Helix-breaking amino acid residues located at positions P5 (Pro) and P2′ (Thr) around the cleavage site and a small unpolar amino acid (Ala) at position P4′ agree with a hypothetical model of a 3C processing site. The only exception to this model is Arg in position P4. Until now, the only known large polar amino acid residue in position P4 is also reported for the unusual 1D/2A 3C protease processing site of the FMDV polyprotein. The information obtained from these data indicate that histone H3 cleavage, if it is indeed directly catalyzed by 3C protease, would occur at an unusual 3C protease processing site.

Cleavage between Leu-20 and Ala-21 of histone H3 is the only observed FMDV-induced alteration of histones, although additional Leu-Ala amino acid pairs exist at other positions in the histone proteins. This could be explained by the conformation and the arrangement of the core histones in core particles and is in agreement with earlier experiments on the proteolytic degradation of chromatin. After digestion of isolated chromatin or core particles with trypsin, a well-defined set of protected fragments is obtained (6, 7). Only the N-terminal and some C-terminal amino acid residues of core histones are cleaved off, despite the fact that other potential trypsin cleavage sites are present in the histone chains (for example, Lys-37 in histone H3, Fig. 1). It was concluded that the C-terminal hydrophobic regions of core histones are tightly folded and packed and therefore are not accessible to proteases. A similar degradation of the N-terminal portion of core histones has been observed in uninfected cells from various organisms and is attributed to the neutral protease closely associated with chromatin (33), which seems to be involved in transcriptional control (12). This cleavage, however, does not occur at the same position as the trypsin cleavage in histone H3 but between amino acid residues Lys-23 and Ala-24 (9) (Fig. 1). These findings demonstrate that unlike the C-terminal hydrophobic regions, the amino-terminal hydrophilic domains of core histones are exposed and thus accessible to proteases.

Up to now, the only reported function of the picornavirus 3C protease is the proteolytic processing of the viral polyprotein into the mature gene products, an event that takes place in the cytoplasm of the infected cell. The cleavage of histone H3 by FMDV 3C protease during infection may explain the presence of picornaviral 3C protease or 3C precursor proteins in the nucleus of the FMDV-infected cell, as reported for FMDV (22) but also for poliovirus (4, 17). In our hands, attenuated poliovirus Sabin I strain, which carries 56 nucleotide substitutions dispersed all over the genome excluding the 3C protease-coding region (41), did not cleave histone H3 in infected HeLa cells. The same result was obtained after infection of mouse L cells with EMC virus (S. Sankar, personal communication). These observations may indicate that this histone H3 cleavage is an FMDV-specific event.

Although a histone modification is not observable in poliovirus-infected cells, host cell transcription shutoff occurs. Published mechanisms describe the inhibition of at least one factor that is required for specific transcription by polymerase II (13) or the direct modification of RNA polymerase II (48) during poliovirus infection.

Brown et al. (10) supposed that the inhibition of cellular RNA synthesis after viral infection could be the result of the general blocking of DNA expression by histones. Thus, the observed histone H3 cleavage early during FMDV infection could contribute to the host cell transcription shutoff caused by this virus. Histone H3 has been localized to the surface of

TABLE 1. FMDV 3C protease cleavage sites of the viral polyprotein strain O_1K (18) compared with the observed histone H3 cleavage position[a]

Cleavage site	P5	P4	P3	P2	P1/P1′	P2′	P3′	P4′	P5′
P1B/1C	Phe	Pro	Ser	Lys	Glu/Gly	Ile	Phe	Pro	Val
P1C/1D	Asp	Ala	Arg	Ala	Glu/Thr	Thr	Ser	Ala	Gly
P1D/2A	Val	Lys	Gln	Thr	Leu/Asn	Phe	Asp	Leu	Leu
P2B/2C	Arg	Ala	Glu	Lys	Gln/Leu	Lys	Ala	Arg	Asp
P2C/3A	Pro	Ile	Phe	Lys	Gln/Ile	Ser	Ile	Pro	Ser
$P3A/3B_1$	Gln	Pro	Gln	Ala	Glu/Gly	Pro	Tyr	Ala	Gly
$P3B_1/3B_2$	Leu	Pro	Gln	Gln	Glu/Gly	Pro	Tyr	Ala	Gly
$P3B_2/3B_3$	Pro	Val	Val	Lys	Glu/Gly	Pro	Tyr	Glu	Gly
$P3B_3/3C$	Leu	Ile	Val	Thr	Glu/Ser	Gly	Ala	Pro	Pro
P3C/3D	Glu	Pro	His	His	Glu/Gly	Leu	Ile	Val	Asp
Histone H3	Pro	Arg	Lys	Gln	Leu/Ala	Thr	Lys	Ala	Ala

[a] As discussed in the text, the histone H3 cleavage site fits quite well with the authentic 3C cleavage sites in the viral polyprotein, except for arginine in position P4.

the core particle (30) and appears to be the first component of the particle to be digested by trypsin in vitro (6). The nucleosomal structure in transcriptionally active chromatin is not disintegrated, only conformationally altered (52, 58), as demonstrated, for example, by the accessibility of sulfhydryl groups in the center of histone H3 to SH reagents in active chromatin (1, 47). Acetylation of lysine residues of core histones H3 and H4 correlates with this nucleosome alteration. Johnson et al. (29) postulated that acetylation of lysine amino groups on H3 amino termini would alter their positive charge and thus their interaction with negatively charged DNA phosphates. Although a direct link between transcriptionally active genes and core histone acetylation is still missing, there is much indirect evidence supporting this view (24, 29, 45; for reviews, see references 49 and 60).

Our in vitro cleavage assay showed that truncated histone H3 remains chromatin associated. This would be a prerequisite for host cell transcription shutoff by the mechanism postulated above. Thus, if core histone acetylation is the basis for transcriptional activity of eucaryotic genes, cleavage of the N-terminal domain of histone H3 early during infection could be an efficient way for FMDV to switch off host cell transcription. In combination with the reported mechanism involved in protein synthesis shutoff owing to cleavage of the cap-binding protein complex by the FMDV leader protease (14), this specific histone H3 cleavage could contribute to the almost complete breakdown of host cell functions during viral infection.

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