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Identification of Putative Polymerase Gene Product in Cells Infected with Murine Coronavirus A59

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The virion RNA of mouse hepatitis virus, strain A59 (MHV-A59) is believed to be the mRNA for the viral RNA-dependent RNA polymerase. The cell-free translation of virion RNA results in the synthesis of two predominant products p220 and p28 (M. R. Denison and S. Perlman, 1986, *J. Virol.* 60, 12–18). p28 is a basic protein and is readily detected by two-dimensional gel electrophoresis. When infected cells and isolated virions were assayed for this protein by two-dimensional gel electrophoresis, p28 could be detected in infected cells labeled at late times after infection, but not at early times or in purified virions. p28 represents the first protein product of the putative coronavirus polymerase gene to be identified in infected cells. © 1987 Academic Press, Inc.

The genome of mouse hepatitis virus (MHV), a member of the coronavirus family, is a polyadenylated positive-strand RNA (*2, 3*). The MHV virion does not contain its own RNA-dependent RNA polymerase, and the first event in the replication of this virus, after adsorption, penetration, and uncoating must be the synthesis of a polymerase. Virus-specific polymerase activity has been detected at both early and late times in the infectious cycle (*4, 5*). The polymerase detected at early times p.i. synthesizes RNA complementary to virion RNA, whereas the late appearing polymerase synthesizes subgenomic and genomic RNA of the same polarity as virion RNA (positive-strand RNA) (*6*).

The viral RNA polymerase is postulated to be a translation product of virion RNA (*2, 7*). The study of the viral polymerase in intact cells is hampered by the small amount of polymerase present, and no proteins with polymerase activity have yet been isolated. To study the synthesis of the putative viral polymerase under more favorable conditions, we translated virion RNA in cell-free lysates from rabbit reticulocytes (*1*). We showed that the virion RNA is translated *in vitro* into several large peptides of molecular weight greater than 200,000, with a predominant product of 220,000 (p220), in agreement with other workers (*7*), and also identified a 28,000-Da protein which was the N-terminal portion of the larger precursor proteins. In the presence of protease inhibitors such as leupeptin, the synthesis of p220 and p28 was diminished, and a new protein with mol wt 250,000 became evident. As the first step in determining if such proteolytic processing occurs in infected cells, we assayed infected cells for the presence of p28. Our results showed that this protein was present at late times p.i. and was not present in isolated virions.

MHV-A59, originally obtained from Dr. Susan Weiss, was grown in BALB/c 17CL-1 cells and titered in L-2 cells. Both were grown in monolayer cultures in Dulbecco's minimum essential medium (DMEM) with 10% fetal calf serum.

When MHV virion RNA is translated in a cell-free rabbit reticulocyte lysate, two major products p220 and p28 are apparent after 60 min of incubation (*1*). Neither product appears if translation is performed in the presence of either $ZnCl_2$ or leupeptin, both inhibitors of serine and thiol proteases. To determine if either p220 or p28 was present in infected tissue culture cells, we labeled cells with [^{35}S]methionine and analyzed the products at different times after infection. MHV does not efficiently shut off host cell protein synthesis until very late times after infection, making the analysis of virus-specific proteins difficult. Consequently, we analyzed infected cell lysates by two-dimensional gel electrophoresis in order to maximize our ability to detect either p220 or p28.

In preliminary experiments, we determined that p220 synthesized in the cell-free lysate did not enter the first dimension of two-dimensional gels whether isoelectric focusing (IEF) or nonequilibrium pH gradient electrophoresis (NEPHGE) was used, probably because of its large size. On the other hand, we found that p28 could readily be detected using NEPHGE gels in the first dimension, but could not be detected if IEF gels were used (Fig. 1). This suggested that p28 was a basic

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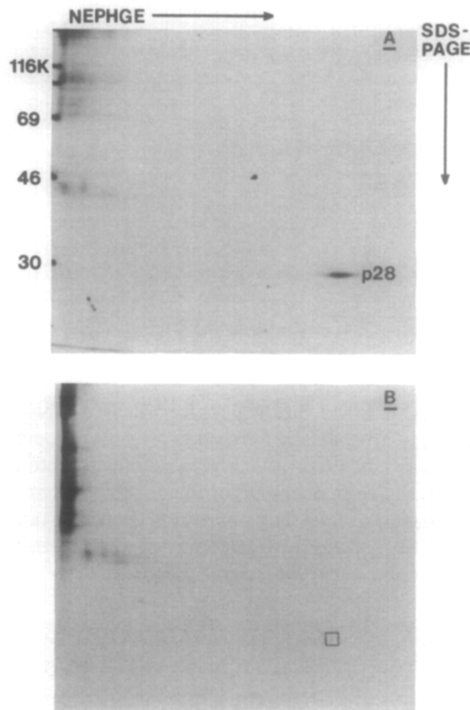

FIG. 1. Two-dimensional gel electrophoresis of cell-free translation products of MHV-A59 virion RNA. [^{35}S]Methionine cell-free products were prepared (*1*) and analyzed by two-dimensional gel electrophoresis by the method of O'Farrell and O'Farrell (*14*) with modifications for resolution of basic proteins in the first dimension as described by O'Farrell *et al.* (*15*). Samples were solubilized in buffer containing 9.5 *M* urea, 2% (w/v) Nonidet-P40, 2% ampholines, pH 3.5–10 (LKB), and 5% 2-mercaptoethanol (*16*). Separation in the first dimension (horizontal) was by nonequilibrium pH gradient electrophoresis (NEPHGE) with 5% pH 7–9 ampholines (LKB) and in the second dimension (vertical), by discontinuous SDS–polyacrylamide gel electrophoresis (*17, 18*). Approximately 1.5×10^5–5×10^5 cpm of [^{35}S]met-labeled protein were loaded on each 1.5-mm tube gel. The top reservoir was filled with 0.01 *M* phosphoric acid and the lower reservoir with 0.02 *M* NaOH, and the gels were run for 1600–2000 V hr without preequilibration. Gels were then removed, soaked in two changes of SDS sample buffer (*19*) for 45 min each, and laid on top of the stacking portion of 10% SDS–polyacryalmide gels. The gels were overlaid with hot agar, cooled, and electrophoresed at 20 mA for approximately 4 hr. These gels were then fixed, stained, and processed for fluorography using 1 *M* sodium salicylate (*20*). For determination of molecular weights, ^{14}C-labeled markers (Amersham Radiochemical Centre) were polymerized into tube gels lacking ampholines, equilibrated in SDS sample buffer, and placed on the sides of the second dimension gels prior to overlaying with hot agar. (A) Contains the control cell-free translation with molecular weight standards and p28 indicated. (B) Shows the cell-free products in the presence of the protease inhibitor leupeptin. The position of p28 from (A) is indicated by the square. The anode for the first dimension is at the right.

protein and presumably did not enter the IEF gel for this reason. p28 is not synthesized in the presence of leupeptin when analyzed by one-dimensional SDS–polyacrylamide gel electrophoresis and, as expected, the p28 which we have identified by two-dimensional gel electrophoresis was similarly not synthesized in the presence of this inhibitor.

In the next set of experiments, uninfected and infected cell lysates were analyzed for p28 by two-dimensional gel electrophoresis. p28 was detected in L-2 cells labeled between 5 and 8 hr p.i., the time of maximal viral RNA and protein synthesis, but could not be detected in either uninfected cells or infected cells labeled prior to 5 hr p.i. The results of representative experiments are shown in Figs. 2 and 3. p28 was not detected in uninfected cells (Figs. 2C and 3C) or in infected cells labeled for the period 1–5 hr p.i. (Figs. 2A and 3A). When p28 labeled in cell-free lysates was added to infected cell lysates labeled 1–5 hr p.i. prior to electrophoresis, it was easily detected (Figs. 2B and 3B), confirming its apparent absence in cells labeled at early times after infection. On the other hand, when cells were labeled 7–7.5 hr p.i., a protein migrating with the identical mobility as the cell-free product was readily detected in infected cells (Figs. 2D and 3D). The nucleocapsid protein N and the transmembrane protein E1 were also apparent in infected cells labeled at this time (Fig. 2D).

To determine if p28 was a minor protein actually present in virions, infected 17CL-1 cells were labeled with [^{35}S]methionine and virus was purified as described in Fig. 4. A portion of the labeled virus preparation was analyzed directly by two-dimensional gel electrophoresis whereas a second part was mixed with the [^{35}S]methionine-labeled cell-free products prior to analysis (Fig. 4). No p28 could be detected associated with purified virions.

Although proteolytic processing is an important component of the replication of many viruses (*8*), the glycoprotein E2 is the only structural protein known to undergo cleavage in coronavirus-infected cells (*2, 3*). Virion RNA is translated in a rabbit reticulocyte cell-free system into a series of related large proteins which are processed into smaller products, including a 28,000-Da basic protein (*1, 7*). The presence of p28 in infected cells suggests that the same proteolytic processing occurs in infected cells as occurred in the cell-free system and that the viral polymerase is translated initially as a large precursor protein which is processed into several smaller proteins.

The functions of p28 and the other translation products of virion RNA are not known at present. The fact that p28 is a basic protein which is most abundant in infected cells at late times after infection suggests that it may be involved in the processing of either genomic or subgenomic RNA. Coronaviruses replicate by a unique mechanism in which each positive-strand RNA

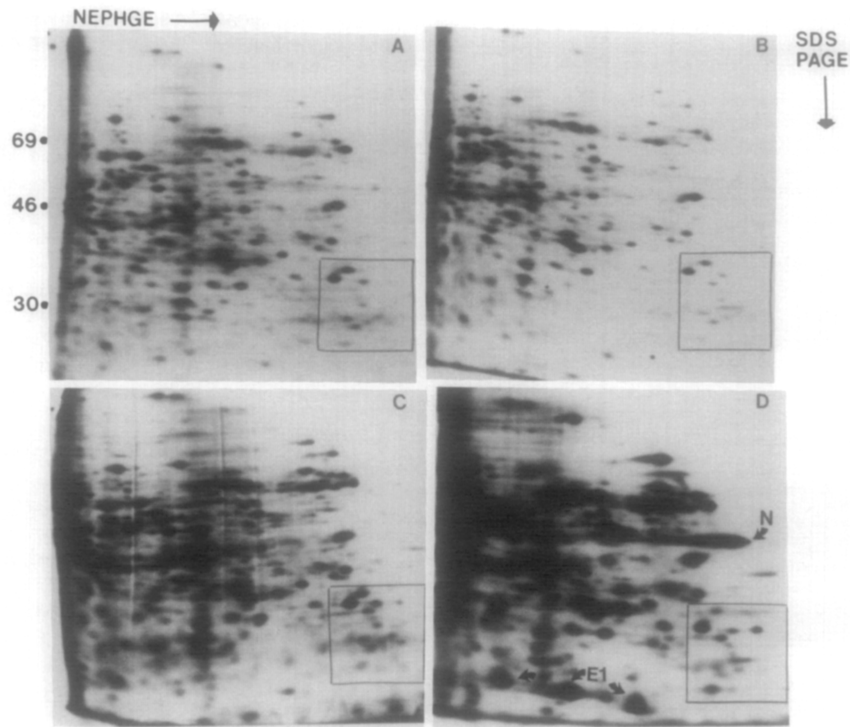

Fig. 2. Two-dimensional gel electrophoresis of intracellular proteins. L-2 cells were infected with MHV-A59 at a m.o.i. of 10. At the times indicated below, the cells were resuspended in DMEM lacking methionine supplemented with 2% dialyzed fetal calf serum and labeled with [^{35}S]methionine (100 μCi/ml) for the lengths of time shown. Cells were washed with ice-cold phosphate-buffered saline, and the cell pellet was prepared for analysis by two-dimensional gel electrophoresis as described in Fig. 1. (A) Infected cells labeled from 1 to 5 hr p.i. (B) Mixture of a portion of the sample shown in (A) and [^{35}S]methionine-labeled cell-free products. (C) Uninfected cells labeled for 7 hr. (D) Infected cells labeled from 7 to 7.5 hr p.i. The positions of the nucleocapsid N protein and the transmembrane E1 protein are indicated. In each panel, the portion of the gel containing p28 was magnified (the area marked by a box is shown in Fig. 3).

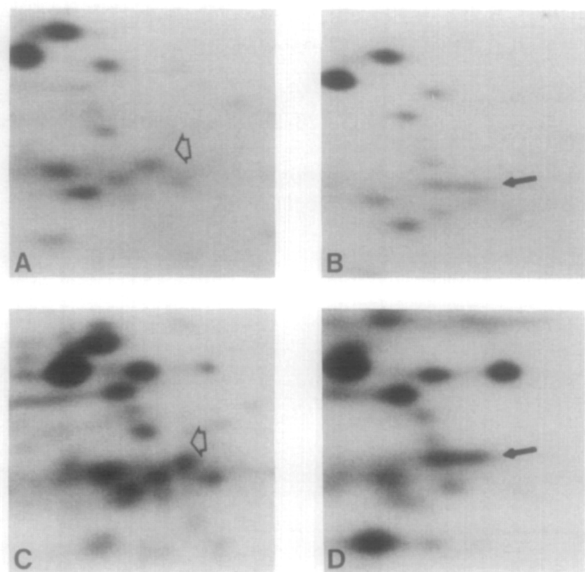

Fig. 3. Expanded view of boxed area shown in Fig. 2. The portion of the gels shown in Fig. 2, which included p28, was magnified. The solid arrows shown in (B) and (D) indicate the position of p28, whereas the open arrows in (A) and (C) indicate the corresponding position where p28 would be located if it were present in these samples.

molecule is joined to a leader RNA by a mechanism that does not involve splicing (*9, 10*). p28 may be involved in some aspect of the synthesis or processing of leader RNA, such as the decision as to whether genomic RNA will function as mRNA or previrion RNA.

The cell-free translation and processing of MHV virion RNA resembles that of the B component of cowpea mosaic virus (CPMV) (*11, 12*). In both cases, a primary translation product with molecular weight greater than 200,000 Da is observed, with rapid processing to proteins with approximate molecular weight of 170,000 and 32,000 (CPMV) or 200,000 and 28,000 (MHV). Both the 170K and 32K CPMV proteins are present in infected cells, and the 32K protein, which has protease activity (*13*), is believed to be involved in the processing of capsid proteins. By analogy with CPMV, p28 may be a viral protease, although the precursor to the viral polymerase would be the only substrate for such an enzyme in MHV-infected cells.

p28 is the first translation product of MHV virion RNA to be identified in infected cells and its study should prove useful in the study of the MHV polymerase structure and function.

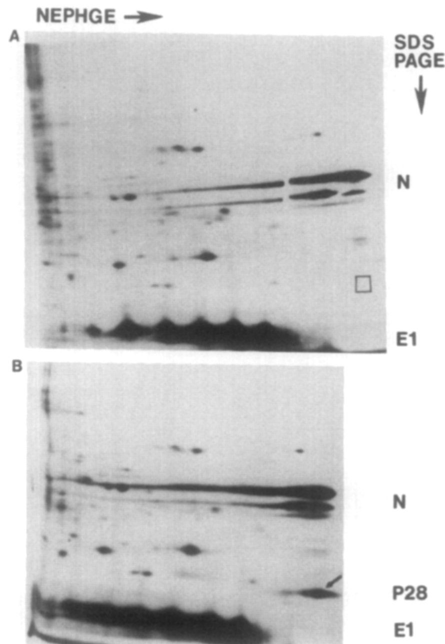

FIG. 4. Two-dimensional gel electrophoresis of virion proteins. 17CL-1 cells were infected with MHV-A59 at a m.o.i. of 0.04. At 4 hr p.i., the cells were placed in DMEM lacking methionine with 2% dialyzed fetal calf serum, and 30 min later, labeled with [^{35}S]methionine (100 μCi/ml) (New England Nuclear Corp.). At 16 hr p.i., the cells were all in syncytia and virus was prepared as previously described by differential centrifugation and sucrose gradient velocity sedimentation (*1*). Virus was concentrated from the final sucrose gradient by centrifugation for 3 hr at 30,000 rpm in the Beckman SW56 rotor. The pellet was prepared for analysis by two-dimensional gel electrophoresis as described in Fig. 1. (A) Virion proteins. (B) Mixture of [^{35}S]methionine-labeled virion proteins and [^{35}S]methionine-labeled cell-free products. N and E1 proteins are indicated.

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