*Acta virol.* **36**: 245-252, 1992

# COMPARISON OF TWO DEFECTIVE HEPATITIS A VIRUS STRAINS ADAPTED TO CELL CULTURES

P. REINER, M. REINEROVÁ*, Z. VESELOVSKÁ*

Institute of Preventive and Clinical Medicine, 833 01 Bratislava; *Cancer Research Institute, Slovak Academy of Sciences, 812 32 Bratislava, Czechoslovakia

*Received December 6, 1990; revised May 17, 1991*

*Summary.* - The replication of two defective hepatitis A virus strains in cell culture was examined. The w.t. HAS-15 strain growing in FRhK-4 cells produced infectious icosahedral virions 27 nm in size as well as round shaped particles with lipids attached to their surface. The morphogenesis of HAV was membrane-dependent and the detected particles were in various degree of maturation. The MBB 11/5 strain growing in PLC/PRF/5 cells produced mainly noninfectious empty procapsids without RNA genome. The translation of viral proteins was uninhibited in both strains. The reason for restricted replication competence of both strains seemed to be different. In HAS-15, highly efficient encapsidation of the progeny RNA positive-strand lowered the formation of replicative intermediate forms. In MBB 11/5, nearly exclusive empty procapsid production gave evidence for the failure of the VPg primer protein attachment to viral RNA. Changes in the efficacy of viral genome replication were a result of the adaptation of HAV to propagation *in vitro*.

*Key words: hepatitis A virus propagation; restricted genome replication; procapsid formation*

## *Introduction*

Hepatitis A virus (HAV) is an enterovirus classified as member of the family *Picornaviridae* (Gust *et al.*, 1983). The virion is a naked icosahedron containing positive-stranded RNA consisting of 7478 nucleotides with a poly(A)tail (Cohen *et al.*, 1987; Paul *et al.*, 1987*a*). Three major capsid polypeptides have been identified with $M_r$ of 33,000 (VP1), 27,000 (VP2) and 29,000 (VP3) of which VP1 appears to be the dominant surface protein (Wheeler *et al.*, 1986). The net electrophoretic profiles of HAV-infected cells regularly reveal 17 virus - specified proteins. The viral RNA, unlike to most eukaryotic mRNAs, is not capped at its 5'end. Instead, a small polypeptide termed VPg ($M_r$ - 2,000) is

covalently attached to the noncoding 5'region (5'NCR) of the virus genome (Wimmer, 1982; Weitz *et al.*, 1986). VPg is not necessary for the function of viral mRNA. Unlike most picornaviruses, HAV neither inhibits host-cell proteosynthesis nor produces obvious cytopathic effect. Wild-type strains of HAV grow poorly in cell cultures and in a majority of attempts the replication terminates in persistent rather than in acute lytic infection (Provost, 1984 and references therein). The molecular basis for cell culture adaptation is largely unknown. In the present study, we analysed two *in vitro* propagated HAV strains in order to get insight into changes that occur during adataption to cell culture.

## *Materials and Methods*

*Virus strains.* Two *in vitro* adapted strains of HAV were utilized. The wild strain of HAV, named HAS-15 was recovered by Hollinger and attenuated in Prof. Maynard's laboratory (Bradley *et al.*, 1984). HAS-15 was propagated in foetal rhesus monkey kidney (FRhK-4) cell culture. The strain MBB 11/5 originated from North Africa (Frosner *et al.*, 1979). The virus was maintained and attenuated in Prof. Deinhardt's laboratory (Paul *et al.*, 1987*a*). The latter strain was propagated in human hepatocellular carcinoma cells PLC/PRF/5. Both viruses were concentrated after ultracentrifugation by freezing-thawing, disintegrated and clarified by centrifugation in Beckman SW 60 Ti rotor at 485 000 x g for 4 hr. The purified sedimented HAV was quantified as described for EMC virus characterization (Reuckert and Pallansch, 1981) by measuring the absorbance at 260 nm (1 mg/ml virus is 7.7 $A_{260}$ units).

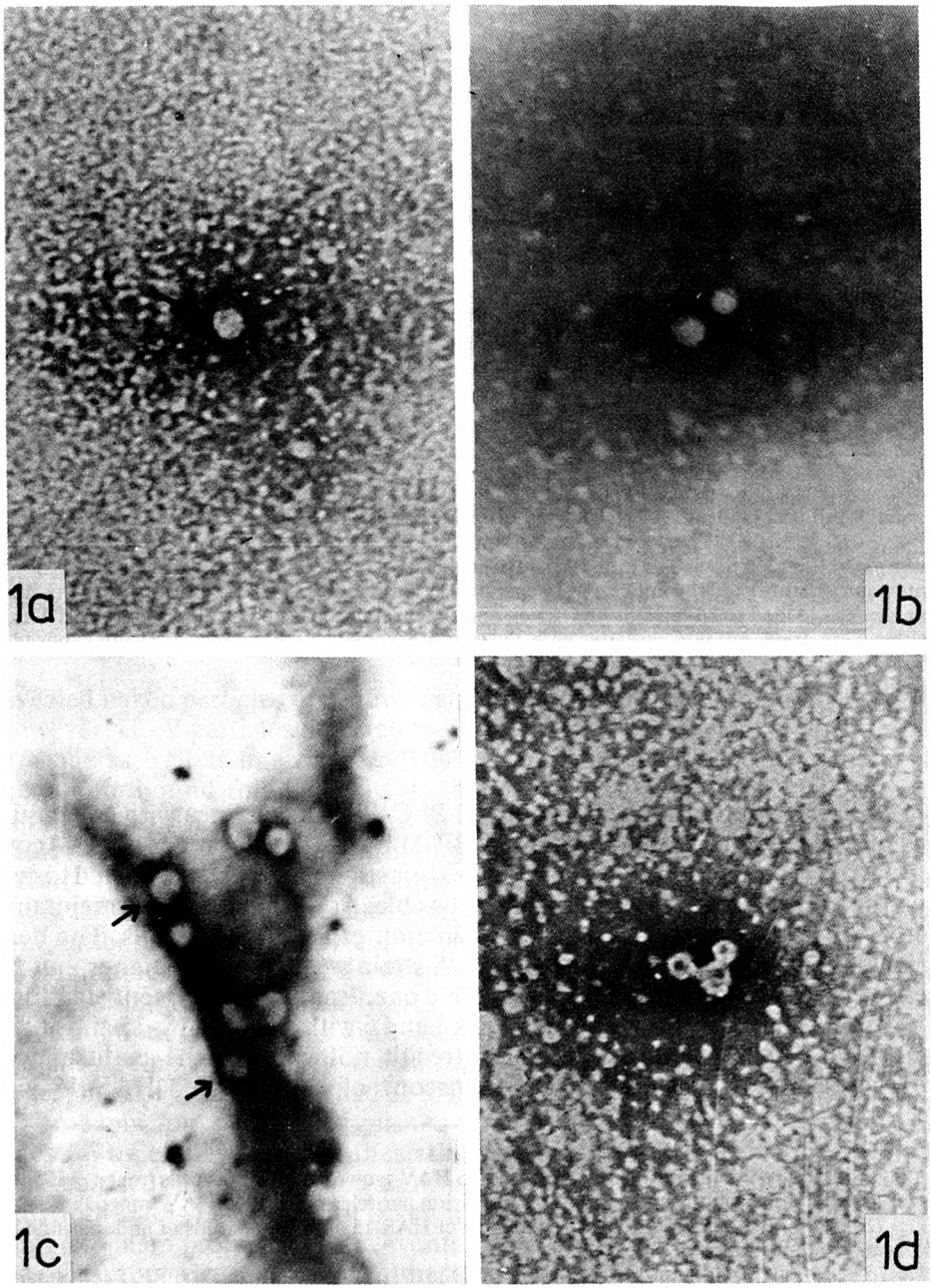
*Indirect immunofluorescence (IF) test.* The formation of hepatitis A antigen (HAAg) was checked using convalescent sera diluted 1:20 in PBS (starting for one hour at 37 °C, washing 3 times with PBS, and then stained for another hour with antihuman IgG swine globulins labelled with fluorescent isothiocyanate purchased at Sevac, CSFR). The coverslips were washed with PBS and mounted into 90 % glycerol in PBS.

*Electron microscopy (EM) and immune electron microscopy (IEM).* The free as well as membrane-bound virus particles were used directly for EM. For IEM, 100 $\mu$l of resuspended virus was reacted with 900 $\mu$l of 1:10 diluted human anti-HAV convalescent serum and incubated at 37 °C for one hour. The immunocomplexes were ultracentrifugated at 180 000 x g for 30 min and the resulting pellets were resuspended in 50 $\mu$l of distilled water. Then they were transferred onto electron microscopy grids coated by formvar, negatively stained with 1 % phosphotungstic acid, acid pH 7.0 and viewed in a Tesla T 500 electron microscope.

*Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).* Hepatitis A virus particles, labelled with $^{35}$S-methionine were pelleted as described. The pellets were submitted to SDS-PAGE in the Multiphor II electrophoretic system (Pharmacia-LKB, Sweden) at 190 mA and 216 V, according to LKB Application note No. 306.

*Preparation of $^{35}$S-methionine-labelled virus.* Cultures of FRhK-4 and PLC/PRF/5 cells infected with either HAS-15 or MBB 11/5 strains of HAV were kept at 33 °C in medium containing 2 % dialyzed foetal calf serum and metabolically labelled with L-$^{35}$S methionine (Amersham, UK) at concentration 3.7 MBq.ml$^{-1}$.

*Immunoprecipitation and analysis of antigens.* Virus pellets were resuspended in the lysis buffer and a radioactivity of suspension was measured. For immunoprecipitation, 10 $\mu$l of undiluted serum was added to virus lysates with activity $4x10^5$ cpm and incubated 1 hr at 4 °C. Formed immune complexes were precipitated with 30 $\mu$l of protein A-Sepharose CL-4B 50 % vol/vol (Pharmacia-LKB) and incubated again 1 hr at 4 °C, followed by centrifugation at 12,000 x g for 1 min in a microcentrifuge. The precipitated immune complexes were washed three times in 400 $\mu$l




For legend see next page.

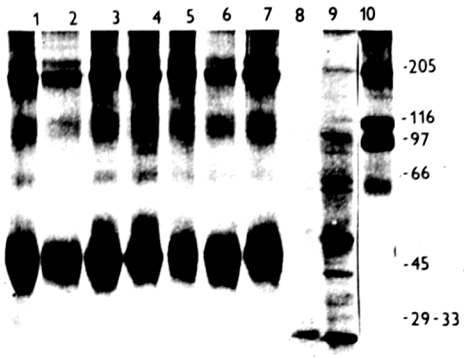



**Fig. 2**
SDS-PAGE of purified HAV
The pellets of both virus strains were analysed in 10 % polyacrylamide gel and stained with Coomassie brilliant blue. Lanes 2, 3, 4 and 5 - different isolates of HAS-15 strain of HAV; lanes 1, 6 and 7 - isolates of MBB 11/5 strain of HAV; lane 8 - analysis of cell lysate of contactly inhibited PLC//PRF/5 cells, infected with MBB 11/5 strain of HAV; lane 9 - analysis of cell lysate contactly inhibited FRhK-4 cells, infected with HAS-15 strain of HAV; lane 10 - molecular weight standards. Protein bands in lanes 1 to 7 between 45 and 55 kD correspond to the uncleaved 1D2A precursor protein. Bands in lanes 8 and 9 with electrophoretic mobility between 29 and 33 kD correspond to VP1 - VP3 proteins.

of NET-N buffer (150 mmol/l NaCl, 5 mmol/l EDTA, 50 mmol/l Tris-HCl pH 8.0, 0.05 % Nonidet P-40, 0.01 % $NaN_3$). The washed immunoprecipitates were suspended in the electrophoresis sample buffer (2 % SDS, 0.05 mol/l Tris-HCl pH 6.8, 8 % glycerol, 0.01 % bromophenol blue, 5 % mercaptoethanol) and the antigen was separated by 3 min boiling. After centrifugation the supernatant was loaded to 10 % SDS-PAGE gel, and the protein separated by electrophoresis. The gel slices were dried, put into cassettes with röntgen film screen (Perlux, Germany) and exposed for 21 days at -20 °C.

## *Results*

After infection of both FRhK-4 and PLC/PRF/5 cell lines with cell culture adapted HAV strains HAS-15 and MBB 11/5, the development of HAAg was tested by indirect immunofluorescence. Infection of cells has never led to cytopathic effect and no plaque assay was possible. As all cultures were maintained at 33 °C, there was a gradual 10 % extinction of PLC/PRF/5 cells. The death rate of cells infected with the MBB 11/5 strain was two times higher, but this effect was rather a toxic than a cytopathic one. Immunofluorescent staining of HAAg reached optimum between days 3 and 6 with strain HAS-15 and at days 6 and 9 with the strain MBB 11/5 (result not shown); the cultures were observed for 21 days. Electron microscopy of concentrated HAS-15 virus

**Fig. 1**
*Fig. 1a:* Infectious icosahedral virions of HAS-15 HAV growing in FRhK-4 cells (magn. x 120 000).
*Fig. 1b:* Infectious round shaped, lipid-bound virion particles of HAS-15 HAV (magn. x 120 000).
*Fig. 1c:* Membrane-dependent virus replication of HAS-15 among the folds of endoplasmic reticulum (magn. x 120 000).
*Fig. 1d:* Non-infectious empty procapsids of MBB 11/5 HAV growing in PLC/PRF/5 cells (magn. x 120 000).

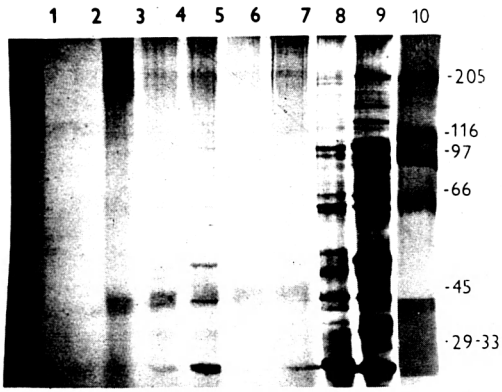



**Fig. 3**
Autoradiogram of $^{35}$S-methionine labelled viral proteins after immunoprecipitation
Lane 1 - purified, $^{35}$S-methionine labelled MBB 11/5 precipitated with negative anti-VHA serum; lane 2 - purified non-labelled HAS-15 precipitated with positive anti-VHA serum; lane 3 - $^{35}$S-labelled HAS-15 with negative anti-VHA serum; lane 4 - $^{35}$S-labelled HAS-15 precipitated with convalescent serum; lane 5 - $^{35}$S-labelled HAS-15 precipitated with highly positive human anti-HAV IgG; lane 6 - $^{35}$S-labelled MBB 11/5 precipitated with convalescent serum; lane 7 - $^{35}$S-labelled MBB 11/5 precipitated with highly positive human anti-HAV IgG; lane 8 - net electrophoretic profile of cellular and virus-specific $^{35}$S-methionine labelled proteins synthesized by PLC/PRF/5 cells infected with MBB 11/5 HAV; lane 9 - net electrophoretic profile of cellular and virus-specific $^{35}$S-labelled proteins synthetized by FRhK-4 cells infected with HAS-15 HAV; lane 10 - molecular weight standards.

revealed naked particles of 27 nm in size and icosahedral symmetry (Fig. 1-1). However, HAV particles detected by EM consisted of distinct types, differing in shape as well as in size (Fig. 1-2); the majority of particles was found in membrane bound foldings (Fig. 1-3). The smooth surface of some virions suggested that lipids were bound to them. This view was strengthened by immune electron microscopic findings using hyperimmune rabit anti-HAV serum which has never aggregated more than five virions (unpublished findings). The MBB 11/5 strain virion particles assembled by human covalescent antibodies were mainly empty capsids lacking of core RNAs (Fig. 1-4). The immunocomplexes of HAV particles were submitted to SDS-PAGE analysis using two human anti-HAV hyperimmune globulins (Fig. 2). Whole virions of both HAV strains were neutralized by polyclonal human antibodies and showed no differencies in electrophoretic profiles of their proteins. The largest bulk of viral proteins had an electrophoretic mobility between 45 and 55 kD probably due to not accomplished processing of precursor proteins (Fig. 2, lanes 1-7). Cellular proteosynthesis decreased in non-transformed cells which were contactly inhibited when grown in monolayers. After infection of cells with both HAS-15 and MBB 11/5 strains, the synthesis of viral proteins represents the main proteosynthetic event. There was a considerable difference in the amount of virus proteins synthesized by cells infected by either HAS-15 strain or MBB 11/5. The cell lysate of FRhK - 4 cells infected by HAS-1 strain

contained more virus-specific proteins than PLC/PRF/5 cells infected by the MBB 11/5 strain (Fig. 2, lanes 8 and 9). On the background of ongoing cellular proteosynthesis, capsid protein VP1 was largely produced. However, VP1 of HAS-15 strain displayed higher electrophoretic mobility than VP1 of the MBB 11/5 strain. SDS-PAGE showed no differences between these two strains owing to poor separation of VP1 - VP3. Therefore, immunoprecipitation of $^{35}$S-labelled virus proteins was performed. The proteins of HAS-15 strain were formed at higher rate than those of MBB 11/5 (Fig. 3, lanes 4 and 5). A reduction of human polyclonal antibody binding by the strain MBB 11/5 was observed (Fig. 3, lanes 6 and 7). The profiles of $^{35}$S-methionine labelled proteins revealed that sufficient amounts of virus antigens are synthesized in both HAS-15 and MBB 11/5 infected cells (Fig. 3, lanes 8 and 9). However, human covalescent sera elicited by whole virus failed to react with each virus protein and preferentially bound to VP1 protein. The prediced 252 kD polyprotein was not detected.

## *Discussion*

Adaptation of hepatitis A virus in tissue culture is extremely long (210 days, Gausse-Müller *et al.*, 1981; 308 days, Provost, 1984), and during this period defective particles can be formed (Nüesch *et al.*, 1988). In our isolates distinct types of individual virus particles were recovered and the majority of them remained attached to membrane-like folds. Replication of picornaviruses has been tightly membrane-bound (Koch and Koch, 1985). In poliovirus infection of Hela cells, viral RNA replication was physically associated with newly-synthesized cytoplasmic membranes. Phospholipid synthesis is required for the proliferation of these mebranes (Guinea and Carrasco, 1990). Formation of HAV was also membrane-dependent and detection of lipid - associated virions was not unexpected. Electrophoretic profiles of both infected cell lines proved that translation of virus mRNA was not inhibited. The mobility of HAS-15 VP1 protein was higher than that of MBB 11/5. This is consistent with the finding that VP1 protein of HAS-15 has a deletion of six amino acids in positions 26 to 31 (Cohen *et al.*, 1987). Electrophoretic separation of purified virion particles revealed that a large portion of proteins has a molecular weight between 45 and 55 kD. On the basis of sequence alignments with PV (Cohen *et al.*, 1987) we assigned them as uncleaved 1D2A precursor proteins (predicted total mass 53 kD), perhaps as a result of lowered autocatalytic competence of 2A protease.

As yet, we know little about picornavirus genome replication. The poliovirus replication complex is membrane-bound and its spatial organization is essential for virus transcription (Bienz *et al.*, 1990). Cohen and co-authors (1989) demonstrated that mutations in the P2-P3 region of virus genome and, to

a lesser degree, the 5'noncoding region of HAV RNA are responsible for adaptation. It has also been proved that viral RNA synthesis is not simply regulated by the supply of RNA polymerase $3D^{pol}$ and the uridylylated VPg protein serves as primer (Takegami *et al.,* 1983). This 5'-terminal VPg is absent from viral RNA serving as mRNA (Paul *et al.,* 1978*b*). We have observed free as well as membrane-bound virions in various degree of maturation in HAS-15 strain of HAV. Together with a large production of capsid proteins it is obvious that restricted replication of HAV RNA is the result of highly efficient encapsidation as suggested by Anderson and Ross (1990) while empty procapsids are still produced. However, this is not the case of the MBB 11/5 strain. Nearly exclusive formation of non-infectious procapsids demonstrates that viral RNA is serving only as mRNA and it is not encapsidated. This seems to be most probably due to the lack of VPg at the 5'end of RNA, caused by 5'NCR mutation or by P3 (3AB) coding for VPg itself. It follows that both mutations which might occure during adaptation lead to inability of the primer attachment. The molecular mechanism of empty procapsid formation in enteroviruses is the subject of further study.

## References


Anderson, D. A., and Ross, B. C. (1990): Morphogenesis of hepatitis A virus: isolation and characterization of subviral particles. *J. Virol.* **64**, 5284-5289.

Bienz, K., Egger, D., Troxler, M., and Pasamontes, L. (1990): Structural organization of poliovirus RNA replication is mediated by viral proteins of the P2 genomic region. *J. Virol.* **64**, 1156-1163.

Bradley, D. W., Schable, Ch. A., McCaustland, K.A., Cook, E. H., Murphy, B. L., Fields, H. A., Ebert, J. W., Wheeler, C., and Maynard, J. E. (1984): Hepatitis A virus: growth characteristics of *in vivo* and *in vitro* propagated wild and attenuated virus strains. *J. med. Virol.* **14**, 373-386.

Cohen, J. I., Ticehurst, J. R., Purcell, R. H., Buckler-White, A., and Baroudy, B. M. (1987): Complete nucleotide sequence of wild-type hepatitis A virus: comparison with different strains of hepatitis A virus and other picornaviruses. *J. Virol.* **61**, 50-59.

Cohen, J. I., Rosenblum, B., Feinstone, S. M., Ticehurst, J., and Purcell, R. H. (1989): Attenuation and cell culture adaptation of hepatitis A virus (HAV): a genetic analysis with HAV cDNA. *J. Virol.* **63**, 5364-5370.

Frösner, G. G., Deinhardt, F., Scheid, R., Gauss-Müller, V., Holmes, N., Messelberger, V., Siegl, G., and Alexander, J. J. (1979): Propagation of human hepatitis A virus in a hepatoma cell line. *Infection* **7**, 303-306.

Gauss-Müller, V., Frösner, G. G., and Deinhardt, F. (1981): Propagation of hepatitis A virus in human embryo fibroblasts. *J. med. Virol.* **7**, 233-239.

Guinea, R., and Carrasco, L. (1990): Phospholipid biosynthesis and poliovirus genome replication, two coupled phenomena. *EMBO Journal* **9**, 2011-2016.

Gust, I. D., Coulepis, A. G., Feinstone, S. M., Locarnini, S. A., Moritsugu, Y., Najera, R., and Siegl, G. (1983): Taxonomic classification of hepatitis A virus. *Intervirology* **20**, 1-7.

Koch., F., and Koch, G. (1985): *Molecular biology of poliovirus,* Springer Verlag, New York.

Nüesch, J., Krech, S., and Siegl, G. (1988): Detection and characterization of subgenomic RNAs in hepatitis A virus particles. *Virology* **165**, 419-427.

Paul, A. V., Tada, H., von der Helm, K., Wissel, F., Kiehn, R., Wimmer, E., and Deinhardt, F. (1987*a*): The entire nucleotide sequence of the genome human hepatitis A virus (isolate MBB). *Virus Res.* **8** 153-171.

Paul, A. V., Yang, C. F., Jang, S. K., Kuhn, R. J., Toda, H., Nicklin, M., Kräusslich, H. G., Lee, C. K., and Wimmer, E. (1987*b*): Molecular events leading to poliovirus genome replication. *Cold Spring Harbor Symposia on Quantitative Biology,* Vol. LII, Cold Spring Harbor Laboratory, pp. 343-352.

Provost, P. J. (1984): *In vitro* propagation of hepatitis A virus. In R. Gerety (Ed.), *"Hepatitis A Virus",* Academic Press, Inc. (New York), pp. 245-261.

Rueckert, R. R., and Pallansch, M. A. (1981): Preparation and characterization of EMC virus. *Meth. Enzymol.* **78**, 315-325.

Takegami, T., Kuhn, R. J., Anderson, C. W., and Wimmer, E. (1983): Membrane- dependent uridylylation of the genome- linked protein VPg of poliovirus. *Proc. natn. Acad. Sci. U.S.A.* **80**, 7447-7451.

Weitz, M., Baroudy, B. M., Maloy, W. L., Ticehurst, J. R., and Purcell, R. H. (1986): Detection of a genome - linked protein (VPg) of hepatitis A virus and its comparison with other picornaviral VPgs. *J. Virol.* **60**, 124-130.

Wheeler, C. M., Robertson, B. H., Nest, G. R., Dina, D., Bradley, D. W., and Fields, H. A. (1986): Structure of the hepatitis A virion: peptide mapping of the capsid region. *J. Virol.* **58**, 307-313.

Wimmer, E. (1982): Genome - linked proteins of viruses. *Cell* **28**, 199-201.