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Isolation and Preliminary Characterization of Temperature-Sensitive Mutants of Encephalomyocarditis Virus

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Thirty temperature-sensitive mutants of encephalomyocarditis virus have been isolated and partially characterized. Fifteen of these mutants are phenotypically RNA^+, thirteen are RNA^-, and two are $RNA^\pm$. Six RNA^+ mutants, one RNA^- mutant, and one $RNA^\pm$ mutant have virions which are more thermosensitive at 56°C than the wild-type virions. HeLa cells infected at the nonpermissive temperature with any of the RNA^+ mutants produced neither infective nor noninfective viral particles. The cleavage of the precursor polypeptides in cells infected with 11 of the RNA^+ mutants was defective at the nonpermissive temperature. This defect in cleavage occurred only in those precursor polypeptides leading to capsid proteins.

Temperature-sensitive virus mutants have been isolated in many virus families. Within the *Picornaviridae*, temperature-sensitive mutants have been isolated from poliovirus (7), foot-and-mouth disease virus (13, 20, 21), and mengovirus (2, 10). I have isolated temperature-sensitive mutants of encephalomyocarditis (EMC) virus because I wished to find mutants where the cleavage of viral precursor proteins is defective in mutant-infected cells at the nonpermissive temperature. EMC virus was chosen for this purpose because the cleavage events occurring during virus infection have been extensively studied by Butterworth and Rueckert (4, 5) and appear easier to interpret than with other picornaviruses such as poliovirus (1) and rhinovirus (T. J. Matthews, D. R. Omilianowski, and R. R. Rueckert, Fed. Proc. **36**:919, 1977). This paper presents the isolation and partial characterization of 30 mutants of EMC virus and shows that cleavage of the viral precursor polypeptides is defective in HeLa cells infected with 11 of the RNA^+ mutants at the nonpermissive temperature.

MATERIALS AND METHODS

Media and buffers. Medium A is Eagle minimal essential medium with Earle saline supplemented with 0.1 mM nonessential amino acids (11). Medium F is Eagle minimal essential medium with Earle saline lacking calcium and magnesium and supplemented with 0.1 mM serine and glycine and 0.1% Pluronic F68 (11). Medium P5 is Eagle minimal essential medium with Earle saline supplemented with 0.1 mM nonessential amino acids, 0.1% bovine serum albumin, 40 mM magnesium chloride, and 75 μg of DEAE-dextran per ml (11). To help maintain a pH of 7.4, the following organic buffers were added to medium P5 to the indicated final concentrations: HEPES (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid; 10.7 mM), BES [*N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; 7.1 mM], and TES [*N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid; 7.1 mM]. Medium AHL is medium A lacking amino acids but supplemented with HEPES, BES, and TES as described above. Newborn calf serum is added to complete medium A and medium P5 to give 9.1% (vol/vol) and to complete medium F to give 5.7% (vol/vol). All media for cell and virus growth contain 100 U of penicillin G per ml and 100 μg of streptomycin sulfate per ml. PBSA is Dulbecco's phosphate-buffered saline plus 0.1% (vol/vol) bovine serum albumin.

Materials. Powdered tissue culture media and newborn calf serum were obtained from KC Biological, Inc. Amino acids, bovine serum albumin, and all organic buffers were purchased from Sigma Chemical Co. Actinomycin D was purchased from Calbiochem or Sigma Chemical Co. 5-Fluorouracil was a generous gift of W. E. Scott of Hoffmann-LaRoche, Inc.

5-[^{3}H]uridine (25 to 30 Ci/mmol) was obtained from New England Nuclear, and L-[^{35}S]methionine (400 to 1,000 Ci/mmol) was purchased from Amersham/Searle. Bio-Gel A-5m is a product of Bio-Rad Laboratories. Sodium dodecyl sulfate (SDS), specially pure, was purchased from British Drug Houses, Ltd. Acrylamide (97%), 3-mercaptopropionic acid, 1-chloro-3-tosylamido-7-amino-2-heptanone hydrochloride (TLCK), and *N,N'*-methylenebisacrylamide (BIS) (99+%) were obtained from Aldrich Chemical Co. The acrylamide was recrystallized once from chloroform before use. All other chemicals were reagent grade.

Cells. HeLa cells were obtained from R. R. Rueckert of the University of Wisconsin and propagated in suspension in siliconized Florence flasks at 37°C in medium F with serum. The cells grew with a generation time of 20 to 24 h and were maintained at a cell

density of 5×10^4 to 5×10^5 cells per ml. Periodic assays of the HeLa cells on Hayflick's medium (6) for mycoplasma were negative.

Virus. EMC virus was obtained from R. R. Rueckert of the University of Wisconsin, who originally obtained it from C. Fuerst of the University of Toronto. The virus was cloned by adding virus to HeLa cells in suspension in medium P5 with serum to a final concentration of 3×10^7 cells per ml and 6×10^2 PFU/ml. After 30 min at room temperature, the infected cells were diluted with medium P5 containing serum and dispensed in Falcon Microtest plates to give 5,000 HeLa cells and 0.1 PFU in each 0.01-ml well. With these conditions, approximately 5 of each 60 wells showed complete cytopathic effect after 48 h at 37°C. The contents of one well were removed, and the cloning procedure was repeated. A working stock of wild-type EMC was prepared from twice-cloned virus by infection of confluent HeLa cell monolayers at 0.01 PFU/cell and 37°C. When all the cells showed cytopathic effect, the cultures were frozen and thawed three times, and the cell debris was removed by low-speed centrifugation. All virus was stored at −50 or −70°C.

To prepare a purified virus stock for mutagenesis, HeLa cell monolayers were infected with wild-type EMC at 0.01 PFU/cell, the virus was allowed to adsorb for 30 min at room temperature, and the infected cells were incubated at the nonpermissive temperature of 39.5°C in medium P5 with serum. After the cultures showed complete cytopathic effect, they were frozen and thawed three times. The virus in the cell lysates was concentrated as described by Yamamoto et al. (28) with 10% (wt/vol) polyethylene glycol and 0.5 M NaCl and resuspended in 1/50 the original lysate volume in PBSA plus 0.1% 2-mercaptoethanol. The virus was sedimented at 25,000 rpm in the Spinco SW 50L rotor for 4.5 h at 5°C through 0.5 ml of 30% (wt/vol) sucrose containing 1 M NaCl–0.02 M Tris-hydrochloride, pH 7.4. The virus pellet was resuspended in 0.5 ml of PBSA plus 0.1% 2-mercaptoethanol. Isopycnic centrifugation in CsCl followed by gel filtration of the gradient fractions containing virus was basically as described by Medappa et al. (17).

Virus assays. Virus infectivity assays were performed essentially as described by Hall and Rueckert (11). The overlay medium for incubation was 5 ml of medium P5 with serum and 0.4% (wt/vol) Noble agar. The plates were incubated for 40 to 44 h at 33°C or 22 to 26 h at 37 or 39.5°C in a humidified, 5% CO_2 in air atmosphere. At the end of the incubation, 2.5 ml of 10% Formalin was added to each plate for 1 h at room temperature. The agar overlay and Formalin were then removed, and the cell monolayers were stained with 0.1% crystal violet in 20% ethanol.

Hemagglutination assays for EMC virus were performed with human AB cells essentially as described by Martin et al. (15), but in microtiter plates with V-shaped wells. EMC virus at 4×10^9 PFU/ml gave 1,600 to 3,200 hemagglutinating units.

Isolation of mutants. Purified EMC virus grown at 39.5°C was added to a confluent HeLa cell monolayer in 0.2 ml of PBSA to give 10 PFU/cell. After 30 min at room temperature, 5 ml of medium P5 with serum and containing 0.25 mM (32.5 μg/ml) 5-fluorouracil was added, and the infected cells were incubated at 37°C in 5% CO_2 in air until they showed cytopathic effects (7, 8). The culture was then frozen and thawed three times, and the cell debris was removed by low-speed centrifugation. Temperature-sensitive mutants of EMC were isolated from this virus stock essentially by the method of Robb and Martin (22). HeLa cells in suspension in PBSA at 6×10^7 cells per ml were added to an equal volume of virus at 3,600 to 7,800 PFU/ml. After 30 min at room temperature, medium P5 with serum was added, and the cells were distributed into Falcon Microtest plates to give 7,500 cells per well and 0.5 to 1 PFU/well. After incubation for 72 h at 33°C, wells containing cells showing obvious cytopathic effects were scored. With a replica-plating device, aliquots of these master plates were transferred to two identical Microtest plates containing 15,000 HeLa cells per well. One plate was incubated at 33°C for approximately 36 h, and the other was incubated at 39.5°C for approximately 24 h. After staining the plates with 0.1% crystal violet, comparison of the two replica plates made from a single master plate showed which virus clones could propagate in HeLa cells at 33°C but not at 39.5°C. The contents of the wells containing potential temperature-sensitive mutants were removed and added to 0.5 ml of PBSA. After a viral infectivity assay at 33 and 39.5°C, mutant samples giving less than 10^{-4} the number of plaques at 39.5°C relative to 33°C were used to grow 5- to 10-ml stocks of the mutant on monolayers of HeLa cells in medium P5 with serum. The multiplicity of infection for mutant stock growth was 10^{-3} PFU/cell. Mutant samples giving more than 10^{-4} the number of plaques at 39.5°C relative to 33°C were cloned once or twice before growth of stock samples.

Incorporation of [^{3}H]uridine into virus-specific RNA. HeLa cells in suspension in PBSA were added to mutant or wild-type EMC virus to give 3×10^7 cells per ml and 10 PFU/cell in 0.2 ml. After 15 min at room temperature with constant mixing, 1.8 ml of medium P5 with serum and 5.5 μg of actinomycin D per ml were added, and one-half of each culture was placed at 39.5°C and the other half at 33°C. The cultures, in tightly capped culture tubes (20 by 125 mm) gassed with 5% CO_2 in air, were shaken in a water bath maintained at the appropriate temperature with a Braun Thermomix heater-circulator. At 75 min post-infection (p.i.), 0.2 ml of 5-[^{3}H]uridine was added to give 2 to 5 μCi/ml for most experiments. Aliquots of 0.1 ml were removed from the infected cultures at subsequent intervals, added to 0.4 ml of cold PBS containing 0.02% bovine serum albumin, and frozen until analysis. Before analysis, the samples were thawed, and 0.5 ml of cold 20% trichloroacetic acid was added. After 15 to 30 min the precipitates were collected by filtration on Whatman GF/A filters and were washed twice with 5 ml of cold 2% trichloroacetic acid. The dried filters were assayed for ^{3}H counts per minute in toluene containing 4 g of PPO (2,5-diphenyloxazole) and 0.05 g of dimethyl POPOP {1,4-bis-[2-(4-methyl-5-phenyl-1,3-oxazolyl)]benzene} per liter. Cells infected with RNA$^+$ mutants at 39.5°C incorporate [^{3}H]uridine similarly to wild-type-infected cells. Cells infected with RNA$^-$ mutants at 39.5°C incorporate [^{3}H]uridine similarly to noninfected cells.

Thermosensitivity of virus mutants. The thermosensitivity of mutants grown at 33°C was determined at 56°C. The mutant virus stocks or wild-type virus stock were diluted by 100 in PBSA. Identical aliquots were incubated at 56°C for varying amounts of time. Each aliquot was subsequently assayed for viral infectivity in the normal plaque assay to determine the surviving fraction of virus in that aliquot. For most of the thermosensitive mutants and for some of those with the thermosensitivity of wild-type virus, the experiments were repeated to verify the results.

Production and partial purification of mutant virus particles grown at 39.5°C. A 0.1-ml amount of HeLa cells in PBSA at 6×10^7 cells per ml was infected with an equal volume of wild-type or mutant virus at 6×10^8 PFU/ml. After 15 min at room temperature with mixing, the cultures were diluted with 1.8 ml of medium AHL containing 5 μg of actinomycin D per ml and all essential amino acids except methionine and were placed at 39.5°C. At 3.5 h p.i., 0.1 ml of medium containing 20 μCi of [^{35}S]methionine and 5 μCi of 5,6-[^{3}H]uridine per ml was added. At 7 h p.i., the cultures were frozen and thawed three times, and the debris was sedimented from the suspension. The virus was then sedimented by centrifugation at 25,000 rpm for 4.75 h at 4°C in the Spinco SW50.1 rotor. The centrifuge tubes contained 0.5 ml of 30% (wt/vol) sucrose in PBSA on the bottom. The pellet was allowed to resuspend overnight at 4°C in 0.1 ml of PBSA before passage through a small column (5 by 0.5 cm) of Bio-Gel A-5m (100 to 200 mesh).

Preparation of virus-specific proteins from infected cells. A 0.1- or 0.05-ml amount of HeLa cells in suspension was infected with wild-type EMC or one of the EMC temperature-sensitive mutants to give 3×10^7 cells per ml and 10 PFU/cell. After 15 min at room temperature for adsorption, 9 volumes of medium AHL containing 5 μg of actinomycin D per ml and all essential amino acids except methionine were added. The cultures infected with RNA$^+$ mutants were placed at 39.5°C and treated as follows. At 3.75 h p.i., [^{35}S]methionine was added to give 10 μCi/ml. At 4 h p.i., a 1,000× molar quantity of unlabeled methionine was added to each culture. One-half of each culture was then removed, TLCK was added to give 8×10^{-4} M, and the samples were placed on ice. The remainder of each culture remained at 39.5°C for an additional 1.5 h until TLCK was added, and these samples were then placed on ice.

The cells infected with RNA$^-$ mutants were placed at 33°C until 6.25 h p.i. and treated as follows. One-half of each culture was shifted to 39.5°C. At 6.75 h p.i., [^{35}S]methionine was added to each culture to give 10 μCi/ml. After a 15-min pulse of label for the infected cultures at 39.5°C and a 25-min pulse for the infected cultures at 33°C, a 1,000× molar quantity of unlabeled methionine was added. A portion of each was then removed and placed on ice. The remainder of each culture labeled for 25 min at 33°C was then shifted to 39.5°C for a 1.5-h chase, and the remainder of each culture labeled for 15 min at 39.5°C remained at 39.5°C for the 1.5-h chase. At the end of the respective chase periods, all samples were placed on ice. All the infected cell samples were now centrifuged at 2,000 × *g* for 5 min. The pellets of infected cells were resuspended in 0.075 or 0.150 ml of 4 M urea, 1% (wt/vol) SDS, 1% (vol/vol) 2-mercaptoethanol, and 0.075 M Tris-sulfate, pH 9.0, and were heated in a boiling-water bath for 5 min. These extracts were dialyzed versus at least 1,000 volumes of 0.5 M urea, 0.1% SDS, 0.1% 2-mercaptoethanol, and 0.075 M Tris-sulfate, pH 9.0.

Slab gel electrophoresis. Slab gel electrophoresis was performed with the apparatus designed by Studier (24) and with the gel formulations and buffers described by Tegtmeyer et al. (26) and Maurer and Allen (16), respectively. The separating gel contained 0.375 M Tris-sulfate (pH 9.0), 20% (wt/vol) acrylamide, 0.1% (wt/vol) BIS, and 0.1% (wt/vol) SDS, whereas the spacer gel contained 0.075 M Tris-sulfate (pH 9.0), 5% (wt/vol) acrylamide, 0.13% (wt/vol) BIS, and 0.1% (wt/vol) SDS. The 0.065 M Tris-borate tank buffer was pH 9.0 and contained 0.1% SDS and 0.01 M 3-mercaptopropionic acid. The gels were subjected to electrophoresis at room temperature for 20 to 24 h at 7.5-mA constant current. They were stained with Coomassie brilliant blue as described by Weber and Osborn (27) and destained as described by Swank and Munkres (25) and prepared for autoradiography (14) or fluorography (3). Kodak XR-5 film was used directly for autoradiography or was preexposed as described by Laskey and Mills (12) for fluorography. Autoradiograms or fluorograms were scanned with a Gilford model 2520 gel scanner to estimate the relative quantity of [^{35}S]methionine-labeled material in each band. Films for quantitation were exposed so that the full-scale absorbance above background was usually 1.5 or less but never more than 1.9.

RESULTS

Isolation of temperature-sensitive mutants. The replica-plating technique described by Robb and Martin (22) was used for the isolation of temperature-sensitive mutants of EMC virus. With the Falcon Microtest plates used in this method, 3,551 of a total of 7,080 sample wells were infected with the mutagenized virus, whereas 3,529 wells remained uninfected. From the Poisson distribution, about 35% of the total number of wells, i.e., 2,457, must then have been infected with one virion per well. The virus clones from 46 of the infected wells were found to be temperature sensitive; i.e., they replicate at the permissive temperature of 33°C but poorly or not at all at the nonpermissive temperature of 39.5°C. Only virus clones giving less than 10^{-4} the number of plaques at 39.5°C relative to 33°C were used to grow 5- to 10-ml virus stocks. With this criteria, only 30 of the mutant clones were suitable for further investigation. Since the temperature-sensitive mutants were likely derived from only the singly infected wells, the frequency of suitable mutant isolation is estimated to be 30 of 2,457 or 1.2%.

Infectivity characteristics of mutants. Stocks of the temperature-sensitive mutants were grown at 33°C and have titers from 10^8 to

5×10^9 PFU/ml. Twenty-one of these stocks have titers of 10^9 PFU/ml or greater and compare well with normal wild-type virus lysates with titers of 2×10^9 to 5×10^9 PFU/ml. The mutant infectivity titers were also determined at 39.5°C and at an intermediate temperature of 36.5°C. The titer at 36.5°C was determined to help indicate if the temperature-sensitive function is defective above or below 36.5°C. Table 1 presents the ratio of plaques produced at 39.5 or 36.5°C relative to 33°C for all the mutants. Note that the number and size of plaques produced by wild-type EMC virus is similar at all these temperatures.

At 39.5°C, all the mutants except two have a $PFU_{39.5°C}/PFU_{33°C}$ ratio of less than 10^{-3}. About one-half the mutants have a ratio higher than the ratio of 10^{-4} used as a criteria for originally choosing suitable mutants. Since the plaque size found with most of the mutants at 39.5°C is similar to that found for wild-type virus at 39.5°C, it is likely that these plaques are due to the appearance of revertant viruses during the growth of the mutant stocks.

TABLE 1. *Properties of temperature-sensitive EMC mutants*

Mutant	$PFU_{39.5°C}/PFU_{33°C}$	$PFU_{36.5°C}/PFU_{33°C}$[a]	RNA synthesis at 39.5°C[b]	Thermosensitivity[c]	$\epsilon_{mutant}/\epsilon_{wild\ type}$[d]
1	4×10^{-5}	0.1	0	1	—
2	2×10^{-4}	0.1	1.46	3	0.23
3	3×10^{-4}	0.6	2.19	1	0.16
4a	3×10^{-5}	0.5	0.03	1	—
15	3×10^{-5}	0.3	2.04	1	0
17	9×10^{-5}	0.1	0.05	1	—
21b	3×10^{-5}	0.1	0.23	1	—
31b	4×10^{-4}	4×10^{-3}	1.42	1	—
33b	4×10^{-4}	0.2	0	1	—
36a	10^{-4}	10^{-4}	0.02	1	—
42	2×10^{-3}	2×10^{-3} 0.1	0	1	—
46a	7×10^{-4}	10^{-3} 3×10^{-2}	0.02	2	—
54	4×10^{-5}	4×10^{-3}	1.55	1	0
55a	10^{-4}	0.4	0.69	3	1.03[e]
59a	10^{-3}	7×10^{-2}	0.05	1	—
60	2×10^{-4}	0.2	1.37	1	—
61a	9×10^{-6}	0.2	0.01	1	—
62a	2×10^{-3}	4×10^{-3}	1.44	1	0.17
65	10^{-4}	0.6	0.09	1	—
67	4×10^{-4}	0.3	1.36	1	—
68	2×10^{-4}	0.2	2.07	2	0
72	3×10^{-4}	0.3	0.15	1	—
73	7×10^{-6}	10^{-3}	0.13	1	—
74a	10^{-4}	8×10^{-5} 5×10^{-4}	2.05	1	0.43
77b	$<6 \times 10^{-6}$	2×10^{-3}	0.22	2	—
83	8×10^{-5}	0.1	1.87	3	0.08
84	10^{-4}	0.5	0.84	2	—
86a	4×10^{-4}	0.1	0.04	1	—
100	4×10^{-5}	0.5	1.72	1	0.14
106aa	5×10^{-4}	10^{-3} 4×10^{-2}	1.69	2	0.11

[a] The first value for $PFU_{36.5°C}/PFU_{33°C}$ for a mutant gives the titer for normal or small size plaques; the second value is for minute plaques seen in the same assay.

[b] The amount of 5-[^{3}H]uridine incorporated into a mutant-infected HeLa cell culture relative to a wild-type-infected culture. The cumulative incorporation curves were integrated and normalized to those of the wild-type-infected culture after subtraction of the integrated incorporation into noninfected cultures.

[c] The numbers in the column represent the thermosensitivity of each mutant relative to wild-type EMC. A mutant in group 1 has a thermosensitivity similar to that of the wild type, whereas a mutant in group 3 is very thermosensitive, as described in the text.

[d] The ratio represents the fractional amount of [^{35}S]methionine in protein ϵ among all viral polypeptides in mutant-infected cells relative to the same in wild-type-infected cells after a chase at 39.5°C. The dashes represent experiments where the cleavage is similar to that in wild-type-infected cells.

[e] The amount of ϵ found in ts 55a-infected cells at 39.5°C is similar to that made in wild-type-infected cells. However, ϵ is not cleaved properly to give β and δ.

At 36.5°C, 20 of the mutants are leaky and have $PFU_{36.5°C}/PFU_{33°C}$ ratios of 0.1 to 1, with the average plaque size for individual mutants varying from minute to approximately one-half the diameter of wild-type plaques. For the remaining 10 mutants, infectivity assays at 36.5°C give ratios less than 0.1. With some of these mutants, it is likely that most of the plaques are due to revertant viruses, because their size is similar to that for wild-type virus and because the 36.5 and 39.5°C titers are similar. With other mutants, the plaques produced are apparently due to leaky virus growth or a combination of leaky virus growth and revertant virus growth at 36.5°C.

Division of EMC mutants into RNA^+ and RNA^- phenotypic classes. The temperature-sensitive mutants were further characterized by placing them into the two phenotypic classes RNA^+ and RNA^-. A typical experiment is pre-

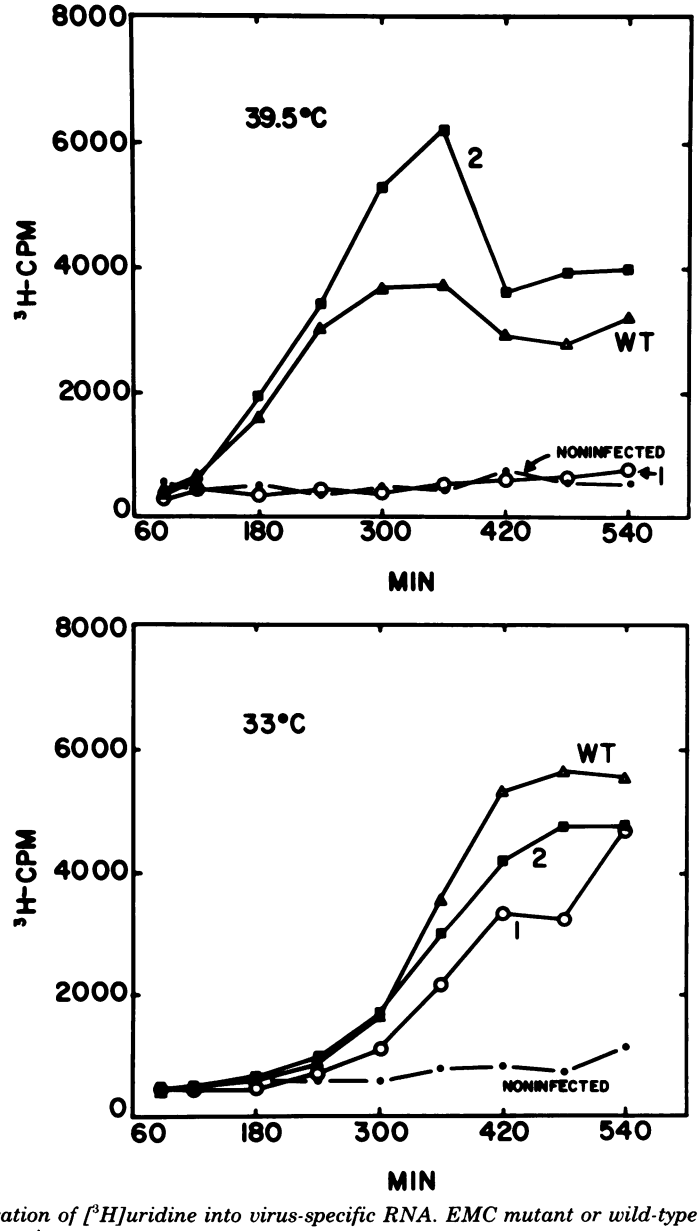


FIG. 1. *Incorporation of [³H]uridine into virus-specific RNA. EMC mutant or wild-type virus was added to HeLa cells at zero time.*

sented in Fig. 1, with the results for all the EMC temperature-sensitive mutants given in Table 1. HeLa cell cultures infected with any of 15 of the RNA^+ EMC virus mutants incorporated from 69 to 219% as much [^{3}H]uridine at 39.5°C as cultures infected with wild-type EMC virus. HeLa cells infected with any of the remaining 15 mutants incorporated substantially less [^{3}H]uridine at 39.5°C than HeLa cells infected with wild-type EMC virus. Thirteen of these mutants were defined as RNA^- because incorporation ranged from 1 to 15% of the amount incorporated into wild-type-infected cells. With the remaining two mutants, ts 77b and ts 21b, the [^{3}H]uridine incorporation into infected cultures was 22 and 23%, respectively, and these were defined as $RNA^\pm$ mutants.

Thermosensitivity of mutant virions at 56°C. If the virions of a temperature-sensitive mutant are thermosensitive relative to the wild-type virus, the defect in the mutant is likely in one of the capsid proteins. Thus, the thermosensitivity at 56°C of the EMC temperature-sensitive virions made at 33°C was determined and is presented in Fig. 2. The mutants can be placed into three groups. One group contains 22 mutants with inactivation curves similar to that of wild-type EMC virus. Only the end points of the complete curves for most of these mutants are presented in the figure. Mutant ts 60 is in this group but is not presented in the figure because interpretation of the inactivation curve has not been completed. A second group contains five mutants which were inactivated more rapidly than wild-type EMC virus and had approximately 0.1 as many survivors as the wild type after 60 min at 56°C. The third group contains three very thermosensitive mutants which had only 0.001 to 0.002 as many survivors as the wild-type virus after 60 min at 56°C. Six RNA^+ mutants are found in the second and third groups. One RNA^- mutant, ts 46a, is found in group 2, as is one of the $RNA^\pm$ mutants, ts 77b. Therefore, 8 mutants of 30 apparently have some defect in their virions which render them more thermosensitive than wild-type virus.

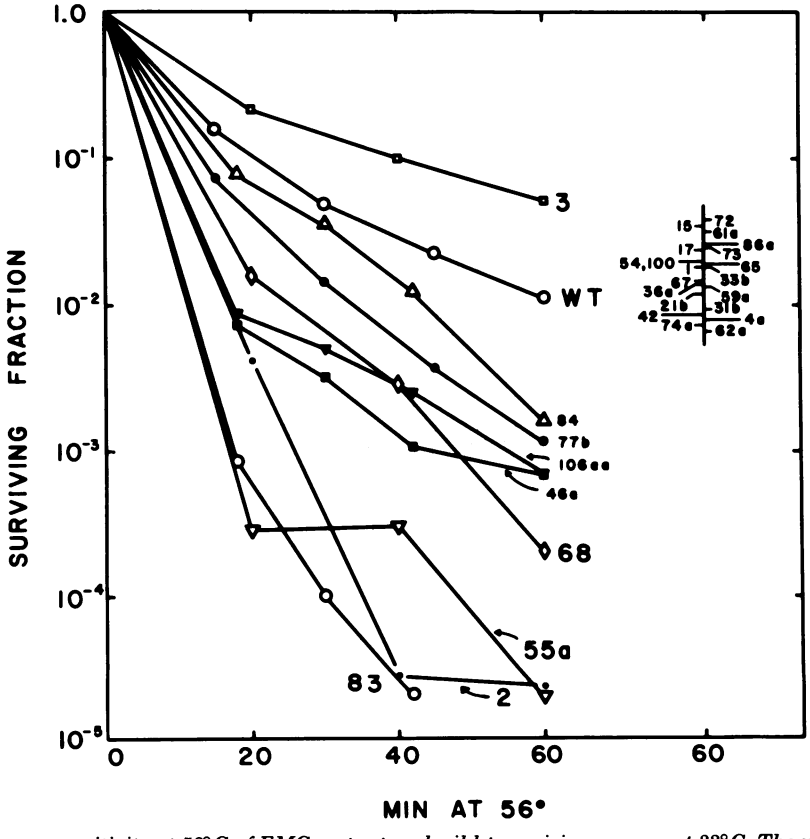


FIG. 2. *Thermosensitivity at 56°C of EMC mutant and wild-type virions grown at 33°C. The values for the surviving fraction at 60 min of 20 of the mutants with curves similar to those of the wild type and ts 3 are given on the right-hand side of the figure.*

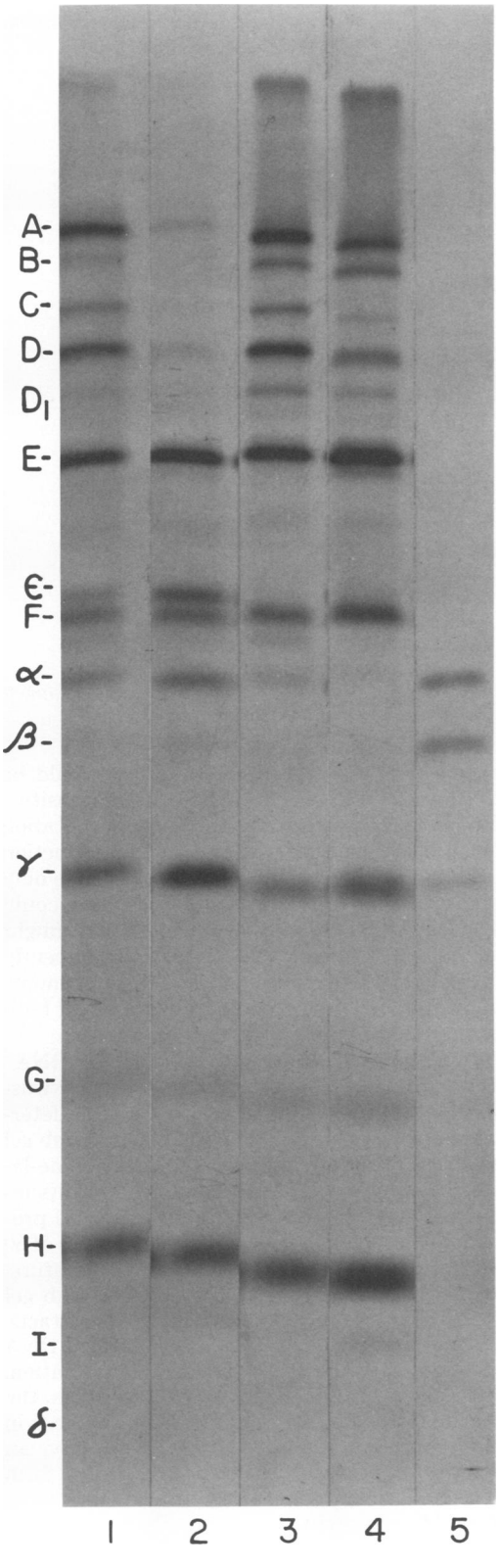
Production of noninfective virus particles at 39.5°C in mutant-infected cultures. HeLa cells in suspension were infected with the RNA^+ mutants at 39.5°C to determine if substantial numbers of noninfective virus particles are made at the nonpermissive temperature. The virus titer in a wild-type-infected lysate was 9.5×10^8 PFU/ml, whereas the titer in the mutant-infected lysates ranged from 3.4×10^4 to 4.0×10^5 PFU/ml, with one infected culture at 1.0×10^6 PFU/ml. The putative virus in these lysates was partially purified by sedimentation followed by gel filtration of the resuspended pellet. The hemagglutination titer in the semipurified wild-type virus sample was 640 hemagglutinating units, whereas the titer in all the mutant virus samples was less than the detectable level of 5 hemagglutinating units. About 10% as much [^{35}S]methionine-labeled material was found in the semipurified mutant virus samples relative to the semipurified wild-type virus sample. Electrophoresis on SDS-containing polyacrylamide slab gels of these labeled samples showed that the wild-type sample contained primarily EMC capsid proteins, whereas the mutant virus samples contained several species of noncapsid EMC protein and little if any of the EMC capsid proteins α, β, and δ or of the precursor to β and δ, ϵ.

Capsids lacking RNA are not normally made and preserved during EMC wild-type virus growth (23). However, to show that such EMC particles with an S value of approximately 80 (23) would have sedimented in the experiments described above, $Q\beta$ virus with an S value of 84 (19) was sedimented in two similar experiments. The recovery of the $Q\beta$ infective virus in the resuspended pellet was 35% in one experiment and 68% in another. EMC virus was included in only one of these experiments, but 94% of the input EMC virus was recovered in the resuspended pellet. This indicates that approximately 50% of any particles with an S value of about 84 would have sedimented in the experiments described here.

Thus, I conclude that the EMC RNA^+ mutants produce less than 0.1% as many infective virus particles at 39.5°C relative to wild-type virus in infected HeLa cells and less than 1% as many physical particles as measured by hemagglutination. The lack of the capsid proteins α, β, and δ and the precursor polypeptide ϵ in the partially purified mutant samples also indicates that insignificant amounts of viral capsid material with an S value of 80 or greater are assembled.

Mutants defective in cleavage of the precursor polypeptides. The synthesis of the picornaviruses involves a sequence of cleavages to give functional viral proteins from the initially translated polypeptide. I wished to determine if all the proper cleavages occur in the EMC mutants grown at the nonpermissive temperature of 39.5°C. A typical experiment is presented in Fig. 3 comparing one RNA^+ mutant, ts 15, with wild-type EMC virus. Figure 4 shows a densitometer tracing made from the fluorogram of the chase portions of the experiment shown in Fig. 3. As can be seen, little cleavage occurred during the pulse and chase periods at 39.5°C with the ts 15 precursor polypeptides A, B, and D1. As a result, little if any of the capsid polypeptides ϵ, α, and β are seen. Capsid protein δ is normally difficult to detect unless long fluorogram exposures are made. Capsid protein γ appears to be present but in reduced amounts. The stable noncapsid proteins E, F, G, H, and I appear to be present in normal amounts in cells infected with ts 15. Ten of the 15 RNA^+ mutants show similar characteristics, but the extent of cleavage is greater with some mutants than with ts 15. In cells infected at 39.5°C with the RNA^+ mutant ts 55a, all cleavages except one appear to occur normally. With this mutant, ϵ is produced but is not cleaved to give β and δ. A relative comparison of the extent of cleavage that occurs in mutant-infected cells at 39.5°C is given in Table 1 as a ratio of the relative amount of protein ϵ synthesized in mutant-infected cells to that made in wild-type-infected cells after a pulse-chase experiment. This measurement was made when it was evident by visual examination of the fluorograms that there was defective cleavage. The measurement also assumes that protein ϵ for all mutants contains the same number of methionine residues.

The cleavage steps in RNA^- and $RNA^\pm$ mutant-infected cells were also examined, but with slightly different experimental conditions. Because little viral RNA is made in RNA^- and $RNA^\pm$ mutant-infected cells at 39.5°C, the infections were allowed to proceed at 33°C until shortly before the pulse of radioactivity. The cultures were then labeled at 33°C or were shifted to 39.5°C immediately before labeling. All samples were at 39.5°C for the chase portions of the experiment. In HeLa cells infected with any of 8 of the 13 RNA^- mutants or either of the 2 $RNA^\pm$ mutants and pulse-labeled at 39.5°C, the viral protein profiles appear to be very similar to the profiles seen with wild-type virus-infected cells. An example of this is shown in Fig. 5 in channels 3 and 4. However, in cells infected with any of the five other RNA^- mutants, there may be some minor differences in the mutant viral protein profiles relative to the viral protein profile seen in wild-type-infected cells. Channels 5 and 6 of Fig. 5 present the viral



protein profile seen in mutant ts 72-infected cells. Several minor differences relative to channels 1 and 2, respectively, may be noted. In all the experiments with the RNA$^-$ mutants, the results were the same for infected cells pulse-labeled at 33 or 39.5°C before the chase at 39.5°C. In summary, there appears to be no serious defect in the cleavage of any viral protein precursors in cells infected with any of the RNA$^-$ or RNA$^\pm$ mutants with the experimental conditions described.

DISCUSSION

The procedure developed by Robb and Martin (22) to isolate temperature-sensitive mutants of simian virus 40 is also a useful method for the isolation of temperature-sensitive mutants of EMC. The rapid and complete cytopathogenicity seen with EMC-infected HeLa cells allows an easy determination of an infected well versus a noninfected well on a Microtest plate. Clones of viral mutants isolated from Microtest plates should remain uncontaminated from other viral clones because of the physical separation of the wells.

The concentration of 5-fluorouracil in the mutagenesis of EMC virus was 0.25 mM to minimize the appearance of multiple-site mutants as suggested by Cooper et al. (8). I obtained mutants with a frequency of at least 1.9% of the clones tested with a usable frequency of 1.2%. This rate is similar to the isolation frequency of 2 to 3% reported by Cooper et al. for poliovirus with a similar concentration of mutagen.

All the mutants were assayed at 33, 36.5, and 39.5°C. Very few plaques were, of course, produced at 39.5°C relative to 33°C, but about two-thirds of the mutants produced significant numbers of plaques at 36.5°C. These were usually minute or small relative to the plaques produced at 33°C by either the mutant or wild-type viruses

FIG. 3. *Fluorogram of polyacrylamide slab gel containing virus-specific polypeptides from HeLa cells infected with EMC wild-type (1 and 2) or ts 15 (3 and 4) virus at 39.5°C. Channels 1 and 3 show labeled virus-specific proteins synthesized during a 15-min pulse of [^{35}S]methionine, and channels 2 and 4 show the labeled proteins seen in extracts made after the 15-min pulse followed by a 1.5-h chase. The detailed procedure is given in the text. From 1.6 × 10^4 to 1.9 × 10^4 ^{35}S cpm were placed in each sample well. The samples were subjected to electrophoresis for 19 h at 7.6 mA. The gel was prepared for fluorography and exposed to X-ray film for 24 h. The letters were assigned to the bands with the nomenclature of Rueckert (23) by comparison with the parallel electrophoresis of purified ^{14}C-labeled EMC capsid polypeptides (channel 5) and unlabeled marker proteins of known molecular weight. The figure was constructed with channels from different regions of the same gel.*

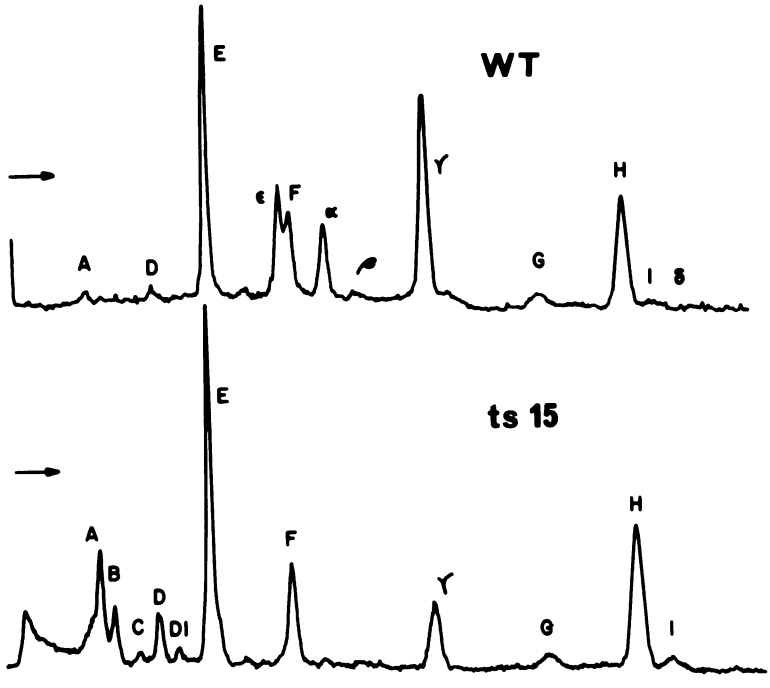


FIG. 4. *Absorbance tracings of channel 2, wild type, and channel 4, ts 15, made from the X-ray film shown in Fig. 3. The full-scale adsorbance for the wild-type tracing is 1.0, and that for the ts 15 tracing is 1.2.*

and likely represent the leakiness of the mutants at 36.5°C. One-third of the mutants produced very low numbers of plaques at 36.5°C, which probably represent either revertent viruses or a low efficiency of plating.

The temperature-sensitive mutants were divided into the two phenotypic groups, RNA^+ and RNA^-, by growth in the presence of [^{3}H]-uridine at the nonpermissive temperature. In most cases, the mutants were definitively placed into one group or the other, but two mutants were defined as $RNA^\pm$, because cells infected with either mutant incorporate 20 to 25% as much uridine as wild-type-infected cells. The incorporation of [^{3}H]uridine into cells infected with many of the RNA^+ mutants is as much as 2.2 times greater than the incorporation into wild-type-infected cells. This was a consistent observation throughout the experiments. One might speculate that defective control of RNA synthesis in the mutant-infected cells led to larger amounts of virus-specific RNA than might be expected in wild-type-infected cells.

Mutant EMC virions that are thermosensitive relative to the wild-type form of the virus likely contain mutant proteins. Six of the RNA^+ mutants are thermosensitive, whereas only one of the RNA^- mutants has this characteristic. Unless the capsid proteins are involved in the control and/or synthesis of viral RNA, one would expect that only the RNA^+ mutants would be thermosensitive. Thus, the thermosensitive RNA^- mutant might possibly have a mutation both in a capsid protein and in some function leading to RNA synthesis, or it might have only one mutation in a capsid protein which could also lead to defects in RNA synthesis. One might also suggest that a mutation in the recently discovered protein attached to the viral genome of picornaviruses (18) could lead to a defect both in the virus capsid and in RNA synthesis.

In HeLa cell cultures infected with the RNA^+ mutants at 39.5°C, substantial amounts of virus-specific RNA and protein are produced as determined by [^{3}H]uridine incorporation and slab gel electrophoresis of infected [^{35}S]methionine-labeled cell extracts, respectively. The results indicate, however, that few, if any, of these proteins are assembled into virus-like particles of 80 S or greater at the nonpermissive temperature, as measured by hemagglutination and slab gel electrophoresis of the semipurified extracts. With some picornaviruses, capsids lacking RNA are frequently detected during virus isolation, but this does not seem to be true with the cardioviruses (23) and did not seem to occur in the experiment described here. Evidently, an association between RNA and the proper form of the capsid proteins is essential before virus assembly can occur.

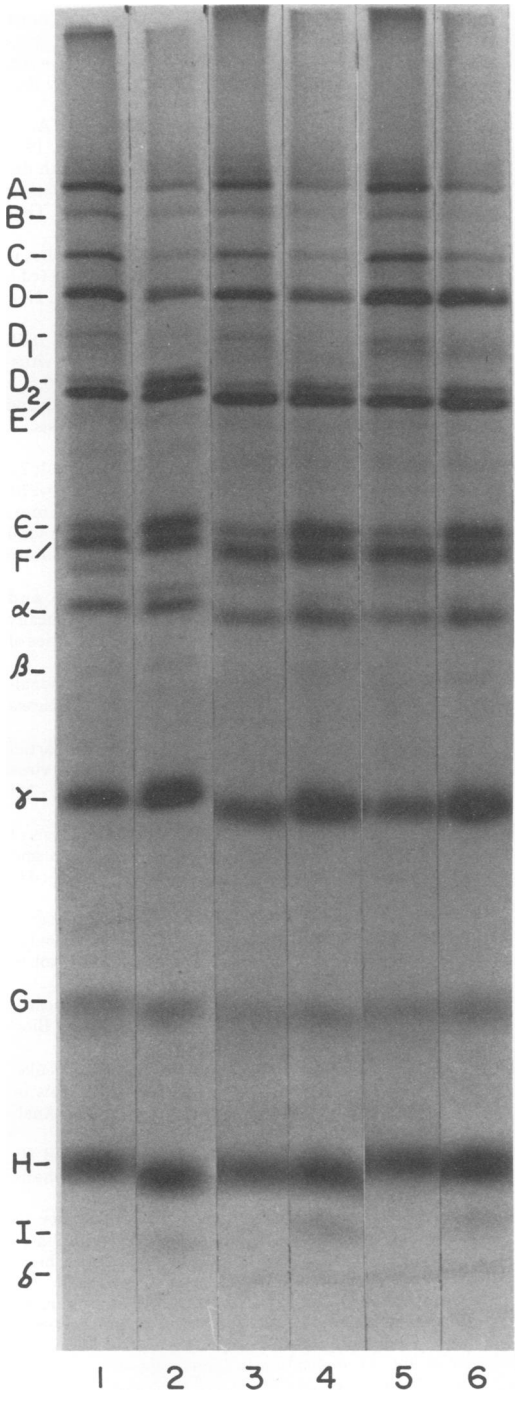


FIG. 5. *Autoradiogram of polyarylamide slab gel containing virus-specific polypeptides from HeLa cells infected with EMC wild-type virus (channels 1 and 2), RNA⁻ mutant ts 65 (channels 3 and 4), or RNA⁻ mutant ts 72 (channels 5 and 6). Channels 1, 3, and 5 show labeled virus-specific proteins synthesized during 15-min pulse of [^{35}S]methionine at 39.5°C, and channels 2, 4, and 6 show the labeled proteins seen in extracts made after a 1.5-h chase at 39.5°C. Incubation of the infected cells before the pulse was at 33°C. The detailed procedure is given in the text. From 7×10^4 to 9.4×10^4 ^{35}S cpm were placed in each sample well. The samples were subjected to electrophoresis for 18 h at 7.5 mA. The gel was prepared for autoradiography and exposed to X-ray film for 100 h. The figure was constructed with channels from different regions of the same gel for the two mutant extracts and with channels from a different gel for the wild-type extract. All extracts were made in the same experiment.*

The experiments described in this paper indicate that cleavage of viral capsid protein precursors is defective in cells infected at 39.5°C with 11 of the RNA⁺ mutants. In 10 of the 11 mutants, the defect in cleavage produced little if any of proteins α and β or the precursor to β and δ, ϵ. Protein δ is normally present in low or undetectable amounts. At the same time, larger amounts of the precursors to these proteins, A, B, and D1, remained uncleaved than were normally detected in wild-type-infected cells. Protein γ seems to always be present in mutant- or wild-type-infected cells at 39.5°C, but the relative amount in the cleavage-defective mutant-infected cells is approximately 50% that found in wild-type-infected cells. If the present schemes for the cleavage of picornavirus proteins are correct, γ should not be detected in extracts of cells infected with any of the cleavage-defective mutants because no ϵ, α, or β is detected and the gene for γ maps between ϵ and α (23). Possibly a labeled host or viral protein has the same mobility as γ in these gels. It would then be difficult to detect the absence of γ. The reason for the defect in cleavage of the viral precursor proteins A, B, and D1 in cells infected with the cleavage-defective mutants is unknown. It is tempting to speculate that a temperature-sensitive, virus-specific cleavage enzyme is involved, but it is also possible that the conformations of the capsid precursor proteins are a factor in the cleavage process. Thus, a changed amino acid sequence in precursor protein A might inhibit proper cleavage at the nonpermissive temperature.

Five of the cleavage-defective mutants are also thermosensitive. This would indicate that the mutation in these mutants is in a capsid protein rather than in a virus-specific cleavage enzyme, unless the cleavage enzyme is also a capsid protein.

The equestron theory for the control of picornavirus synthesis as proposed by Cooper et al. (9) indicates that a complex of cleaved capsid polypeptides is capable of controlling synthesis of virus-specific RNA. My results indicate that

the amount of virus-specific RNA made in HeLa cells infected with any of several of the cleavage-defective mutants at 39.5°C is similar to the amount made in cells infected at 39.5°C with several of the RNA$^+$ mutants with normal cleavage function. Thus, a complex of cleaved capsid precursors may not be necessary for the equestron theory. Perhaps the uncleaved precursors can function also in control. Alternatively, perhaps the results indicate indirectly that capsid proteins have no function in RNA synthesis control.

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