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Preliminary Characterization of Coxsackievirus B3 Temperature-Sensitive Mutants

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Prototype temperature-sensitive (*ts*) mutants of a coxsackievirus B3 parent virus capable of replication to similar levels at 34 or 39.5°C were examined for the nature of the temperature-sensitive event restricting replication in HeLa cells at 39.5°C. The *ts* mutant prototypes represented three different non-overlapping complementation groups. The *ts*1 mutant (complementation group III) synthesized <1% of the infectious genomic RNA synthesized by the coxsackievirus B3 parent virus at 39.5°C and was designated an RNA^- mutant. Agarose gel analysis of glyoxal-treated RNA from cells inoculated with *ts*1 virus revealed that cell RNA synthesis continued in the presence of synthesis of the small amount of viral RNA. This mutant was comparatively ineffective in inducing cell cytopathology and in directing synthesis of viral polypeptides, likely due to the paucity of nascent genomes for translation. The *ts*5 mutant (complementation group II) directed synthesis of appreciable quantities of both viral genomes (RNA^+) and capsid polypeptides; however, assembly of these products into virions occurred at a low frequency, and virions assembled at 39.5°C were highly unstable at that temperature. Shift-down experiments with *ts*5-inoculated cells showed that capsid precursor materials synthesized at 39.5°C can, after shift to 34°C, be incorporated into *ts*5 virions. We suggest that the temperature-sensitive defect in this prototype is in the synthesis of one of the capsid polypeptides that cannot renature into the correct configuration required for stability in the capsid at 39.5°C. The *ts*11 mutant (complementation group I) also synthesized appreciable amounts of viral genomes (RNA^+) and viral polypeptides at 39.5°C. Assembly of *ts*11 virions at 39.5°C occurred at a low frequency, and the stability of these virions at 39.5°C was similar to that of the parent coxsackievirus B3 virions. The temperature-sensitive defect in the *ts*11 prototype is apparently in assembly. The differences in biochemical properties of the three prototype *ts* mutants at temperatures above 34°C may ultimately offer insight into the differences in pathogenicity observed in neonatal mice for the three prototype *ts* mutants.

Temperature-sensitive (*ts*) virus mutants have been isolated from several members of the *Picornaviridae* family (3, 7, 11, 20, 23, 26, 28), and biochemical studies of prototype viruses with different temperature-restrictive lesions have been performed with the goal of providing insight into the biochemical basis of virulence for a particular virus-induced syndrome or disease. This goal is rarely met because most *ts* mutants are avirulent in the animal model in which they are tested; this is likely due to the body temperature of the host. We have used neonatal mice, which are poorly regulated homoiotherms (16, 30), to assess virulence of *ts* mutants of coxsackievirus B3 (CVB3). Different degrees of virulence were found for different *ts* mutants (32), and, in fact, specific *ts* mutants were found to induce forebrain anomalies such as hydranencephaly and porencephaly with high frequency (D. C. Jones, R. J. Gudvangen, H. W. Huntington, and C. J. Gauntt, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, T45, p. 245). We therefore examined several *ts* mutants of CVB3 in an attempt to determine the nature of the *ts* lesion with the long-term goal of associating some product(s) with virulence (death) or induction of a particular disease.

Ten *ts* mutants of CVB3 were isolated in this laboratory from a myocarditic parent strain capable of replication to similar yields at either 34 or 39.5°C (31). Eight mutants were isolated after mutagenesis with 5-fluorouracil, and two were spontaneous mutants. Of the 10 *ts* mutants, 9 were assigned to three non-overlapping groups after complementation tests (31). In this report,

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we provide data on biochemical aspects of the *ts* replication defect at 39.5°C in prototype viruses selected from each complementation group (*ts*1 from group III, *ts*5 from group II, and *ts*11 from group I) and examined at the nonpermissive temperature (31). The results suggest that the *ts* lesions are associated with an inhibition of synthesis of viral RNA genomes, synthesis of an aberrant capsid polypeptide which results in an instability in the virion capsid, and a defect in particle assembly, respectively, for prototype viruses *ts*1, *ts*5, and *ts*11.

MATERIALS AND METHODS

Cell culture and media. HeLa cells used in this study were obtained from the American Type Culture Collection, and stocks were cultured as monolayer cells in minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 μg of streptomycin per ml, 100 U of penicillin per ml, and 0.056% $NaHCO_3$. Cell culture reagents were purchased from GIBCO Laboratories, Grand Island, N.Y., or Flow Laboratories, Inc., Rockville, Md. Cells inoculated with viruses were incubated in virus growth medium (VGM; MEM containing 1% heat-inactivated fetal bovine serum, glutamine, antibiotics, and $NaHCO_3$ as described above).

Viruses. The origin and methods for propagation and plaque assay of the parent myocarditic coxsackievirus B3 strain ($CVB3_m$), herein designated wt, and prototype *ts* mutants (*ts*1, *ts*5, and *ts*11) derived from wt virus were previously described (31). All virus stocks were prepared in HeLa cells at 34°C and stored at −70°C until used. All viruses have equivalent high stabilities at −70, −20, or 0°C in VGM or in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline. Purification of virus particles, including a step of banding in CsCl gradients, was carried out by a previously published method (13). In all experiments, HeLa cell cultures were inoculated with 20 to 25 PFU per cell of a given virus. Zero time is regarded as the instant of adding virus to the monolayer cell cultures. Attachment of virus was allowed to proceed for 30 min at 25°C, after which the inoculum was removed and the cell cultures were washed three times with MEM, immersed in VGM, and incubated at 39.5°C.

Preparation and analysis of radiolabeled viral polypeptides. Virus-inoculated HeLa cell cultures (3×10^6 to 6×10^6 cells) were incubated with Earle's balanced salt solution supplemented with vitamins at concentrations found in MEM, amino acids at one-tenth their normal concentrations in MEM, and 1% heat-inactivated fetal bovine serum. Reconstituted ^{14}C-labeled protein hydrolysate (Schwarz/Mann, Orangeburg, N.Y.) at 1 to 10 μCi/ml was added at 4.5 or 3.5 h post-inoculation (p.i.) to cell cultures that were then incubated for 1 h more at 39.5°C. The radiolabeled virus-cell polypeptides were analyzed by polyacrylamide gel electrophoresis as previously described (8).

Preparation and analysis of radiolabeled viral RNA species. Virus-inoculated HeLa cell cultures were pulse-labeled with [^3H]uridine (>25 Ci/mmol; Schwarz/Mann) at 5 μCi/ml for 1 h at 3.5 to 4.5 h p.i., for cell cultures incubated at 39.5°C. Virus (wt)-inoculated cell cultures to be incubated with ^{32}P (carrier-free in 0.02 N HCl; ICN Chemical and Radioisotope Division, Irvine, Calif. or Schwarz/Mann) were maintained on phosphate-free MEM until 3 h p.i., at which time ^{32}P was added to 10 μCi/ml and the cells were incubated at 34°C for an additional 15 h. Cells were harvested by scraping with a rubber policeman, washed once in MEM, and taken up in reticulocyte standard buffer (0.01 M Tris [pH 7.4]–0.01 M NaCl–1.5 mM $MgCl_2$). RNA was extracted with phenol and sodium dodecyl sulfate as previously described (13). The ethanol-precipitated nucleic acids were harvested by centrifugation at 20,000 × *g* for 30 min at 4°C. The precipitates were dissolved in 0.3 M STE (0.3 M NaCl–0.001 M sodium EDTA–0.01 M Tris, pH 7.6) and treated with glyoxal (Aldrich Chemical Co., Milwaukee, Wis.) as previously described (24). Immediately before use, the glyoxal was passed over a mixed bed resin (B10-REX RG501-X8; Bio-Rad Laboratories, Richmond, Calif.) to remove oxidized products. RNA was analyzed on 1.5% agarose columns, using the conditions of Naeve and Trent (25), except that the running time was 4.5 h at 0.2 mA per gel at 25°C. Electrophoresis reagents were purchased from Bio-Rad Laboratories. The gels were scanned at 260 nm in a Beckman Instruments model 240 spectrophotometer equipped with a linear transporter and recorder (Beckman Instruments, Inc., Irvine, Calif.). All gels were sliced into 2-mm segments and solubilized with Protosol (New England Nuclear Corp., Boston, Mass.) at 50°C for 2 h before the addition of toluene-Liquifluor (New England Nuclear) liquid scintillation fluid. Radioactivity was measured in a Beckman Instruments LS7800 spectrometer.

Samples of RNA to be assayed for infectious RNA were diluted in Auto-Pow MEM containing 500 μg of diethylaminoethyl dextran per ml (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.). HeLa cell monolayers used in plaque assay of infectious RNA were pretreated for 20 min at 25°C with the same diluent before inoculation. The standard nutrient agar overlay was added (31), and the cultures were incubated at 34°C for 3 days to permit plaque development.

Assay of cytoplasmic contents of infected cells inoculated at 39.5°C for nascent virus particles. The following procedure was used to determine whether HeLa cells inoculated with *ts* mutant viruses and incubated at 39.5°C produced any virus particles. Approximately 20×10^6 to 25×10^6 HeLa cells were challenged with 50 PFU per cell of virus; after adsorption at 34°C, the inoculum was removed and the cell cultures were washed and returned to 39.5°C. [^3H]uridine was added to 20 μCi/ml at 4 h p.i., and the cultures were incubated for an additional 4 h. At 8 h p.i., the cells were washed, harvested by scraping, suspended in reticulocyte standard buffer, and disrupted by Dounce homogenization; the nuclei were removed by centrifugation at 350 × *g* for 10 min at 4°C. The cytoplasm of each sample was immediately layered over a linear (1.27 to 1.37 g/ml) gradient of CsCl in TST buffer (0.2% tryptose-phosphate broth–0.1 M NaCl–0.05 M Tris, pH 7.4) and centrifuged at 33,000 rpm in a Beckman SW50.1 rotor at 4°C for 14 h. Aliquots (50 μl) of each fraction were applied to Whatman 3MM filter paper disks (A. H. Thomas Co., Philadelphia, Pa.) that were then dried and treated with 5% cold trichloroacetic acid (TCA) for 10 min at 0°C. After three washes in cold TCA, the disks were immersed in counting fluid and assessed for counts per minute (cpm). The density

of every fourth fraction was determined by using a Bausch and Lomb Abbe-3L refractometer.

Serum-blocking power test. The serum-blocking power test was used to determine whether capsid antigens were produced in cells inoculated with *ts* mutant viruses and incubated at 39.5°C. It was performed in a manner similar to that previously described (14), except that lower quantities of indicator virus were used. Specifically, however, the assay is for production of VP2, since this capsid polypeptide induces anti-CVB3-neutralizing antibody (2, 9). Samples of each cell lysate were frozen and thawed three times and sonicated, and 1.0-ml aliquots in 60-mm petri dishes were exposed to UV light irradiation (150-W GE germicidal lamp at 12 cm) for 5 min. Under these conditions, virus titers in the wt lysate were reduced from 10^8 PFU/ml to approximately 10^2 PFU/ml or less. Dilutions (0.5 ml) of each virus lysate were mixed with 0.5 ml of a dilution of hyperimmune mouse serum previously determined to neutralize approximately 90% of 1,000 PFU of $CVB3_m$. After incubation for 30 min at 37°C, 1,000 PFU of indicator $CVB3_m$ in 0.5 ml was added, and the suspension was incubated for an additional 30 min. Lower quantities of indicator virus were used to detect any virion antigens produced in *ts*1-inoculated HeLa cells incubated at 39.5°C. For controls, MEM was added in place of antibody. The dilution of virus lysate antigens that blocked approximately 90% of the antibody-neutralizing capacity was designated the endpoint; the reciprocal of that dilution was taken as the titer in serum-blocking power units of capsid antigens (VP2) for a particular virus lysate.

Methods for assessing virus-induced cell damage. HeLa cells were incubated in complete MEM containing 2 μCi of ^{51}Cr per ml for 3 h at 37°C in a CO_2 incubator. Cell cultures of approximately 2.5×10^5 cells in 35-mm petri dishes were washed three times and then were challenged with 50 to 75 PFU per cell in 0.1 ml, or with VGM for the controls. After 45 min of incubation at 34°C, the inocula were removed, and the cells were washed three times before the addition of 1 ml of VGM per culture. All cultures were placed in a humidified CO_2 incubator at 39.5°C. Fluids were individually removed from duplicate cell cultures at each time point and frozen at −20°C. Distilled water (1 ml) was added to each cell monolayer, and all were then frozen and thawed three times. The supernatant fluids of the 9-h p.i. cell culture harvests were centrifuged to remove any detached cells, which were then combined with the distilled water and remaining cells. ^{51}Cr levels were measured in a Beckman Biogamma 310 counter. Under the above conditions, each culture took up a total of approximately 500,000 cpm, and ^{51}Cr release from uninfected cells was approximately 2% of the total per hour.

In the trypan blue dye assay, cells were scraped from the surface of the dish into the VGM and mixed 1:1 with a 0.4% solution of trypan blue dye in phosphate-buffered saline. After 2 min at 25°C, stained (dead) cells were counted with the aid of a hemocytometer at 100×. Between 700 and 1,000 cells were counted per sample.

The visual cytopathology assay was performed at 40 and 100× under direct illumination with an Olympus model CK inverted microscope. At least 10 fields were examined in each of duplicate cell cultures, and a cytopathology score was assigned according to the scale given in the legend to Table 3.

RESULTS

Synthesis of RNA in HeLa cells inoculated with *ts*1, *ts*5, *ts*11, or wt viruses and incubated at 39.5°C. Suppression of host cell RNA synthesis has been effected in several picornavirus-host cell systems by including actinomycin D (Act D) in the virus inoculum and removing it by washing at the end of the 1-h adsorption period. We found that inclusion of Act D at 1 μg/ml (three sources) in the inoculum during challenge of HeLa cell monolayer cultures inhibited replication of the parent and prototype *ts* mutant viruses by 40 to 80% at 34°C; the order of sensitivity, from most to least sensitive, was *ts*1 > *ts*11 > wt > *ts*5 (data not shown). As shown below, all virus variants except *ts*1 effectively shut off host cell RNA synthesis at 39.5°C; therefore, Act D was not included in subsequent experiments unless noted because of the considerable inhibition it effected on virus yields.

The capacity of several picornaviruses to actively shut off host cell RNA synthesis is a well-known phenomenon (21). This also occurs in the wt virus-HeLa cell system at 39.5°C (Fig. 1). Species of RNA synthesized in HeLa cells inoculated with $CVB3_m$ or prototype *ts* mutants and incubated at 39.5°C were pulse-labeled from 3.5 to 4.5 h p.i. and extracted with sodium dodecyl sulfate and phenol. Similar counts in [^{3}H]uridine-labeled TCA-precipitable RNA were mixed with ^{32}P-labeled genomic RNA extracted from purified $CVB3_m$ particles, and after glyoxal denaturation, each mixture was co-electrophoresed into a 1.5% agarose gel (Fig. 1). In wt-infected cells, the predominant species of newly synthesized RNA comigrates with the ^{32}P-labeled wt genomic marker RNA from purified virions. Heterogeneous RNA species migrating between the 28 and 18S ribosomal markers are presumed to represent incomplete genomic RNA strands. The heterogeneous RNA is ribonuclease sensitive (data not shown). Because *ts*1 virus does not shut off host macromolecule synthesis (see below), we examined synthesis of RNA in the absence and presence (2 μg/ml) of Act D. Act D was included in the inoculum, and the cells were washed before incubation in VGM. In the absence of Act D, *ts*1 directed synthesis of a small amount of viral RNA in the presence of continued synthesis of ribosomal RNA. In these cultures, we always extracted larger amounts of the presumably incomplete species of viral RNA than of genomic RNA. Very little viral RNA, and no detectable cell RNA, was synthesized in the presence of Act D. Other *ts* mutants which belonged to complementation group III (20), including *ts*6,

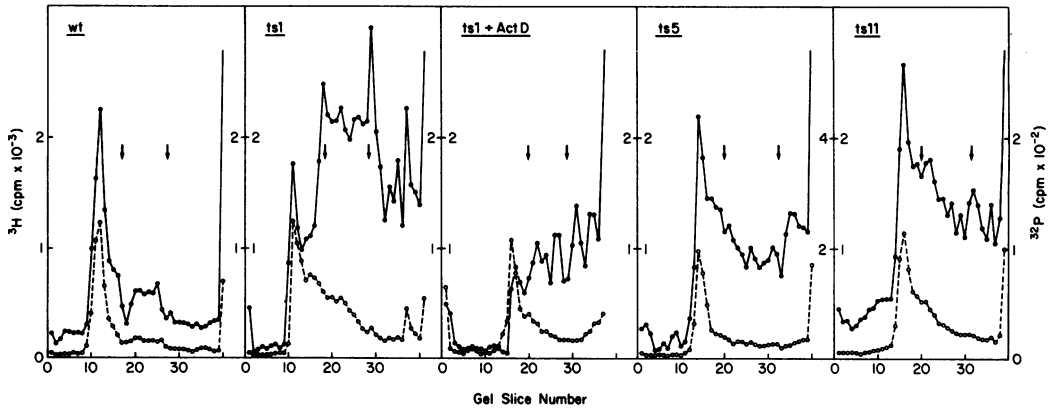

FIG. 1. Viral RNA species synthesized in HeLa cells inoculated with wt and prototype *ts* mutant viruses and incubated at 39.5°C. RNA was extracted from these cell cultures after labeling with [^{3}H]uridine from 3.5 to 4.5 h p.i. during incubation at 39.5°C. One culture contained actinomycin D at 2 μg/ml in the *ts*1 virus inoculum; the actinomycin D was removed with the inoculum, and this and other cultures were washed twice before addition of VGM and subsequent labeling. Equivalent cpm in [^{3}H]uridine-labeled RNA extracted from each of the virus-inoculated cell cultures were mixed with ^{32}P-labeled genomic RNA extracted from purified wt virus and treated with glyoxal as described in the text before electrophoresis into 1.5% agarose gels. Symbols indicate cell-associated [^{3}H]uridine-labeled RNA (●) and purified ^{32}P-labeled genomic RNA (○). The arrows indicate positions of 28 and 18S HeLa cell ribosomal RNA as measured by optical density scan (absorbancy at 260 nm). Electrophoresis was from left to right.

*ts*7, and *ts*10, synthesized very little genomic RNA and comparatively larger amounts of RNA migrating between 28 and 18S at 39.5°C (data not shown). Cells inoculated with *ts*5 or *ts*11 synthesized RNA, and gel patterns on these RNA species were similar to that found for RNA extracted from cells inoculated with wt virus. Gel patterns of total RNA not denatured were very similar, except that a slow-migrating viral RNA species was always detectable in patterns of RNA from cultures inoculated with all virus variants except *ts*1, where this species was usually not detected above background counts (data not shown). The slow-migrating RNA species had some properties similar to those described classically for replicative intermediate or replicative forms of RNA: it was only partially soluble in 2 M LiCl and partially sensitive to enzymatic digestion with either pancreatic, T_1, or A ribonuclease, and with S1 nuclease (data not shown). A similar RNA species with both replicative form and replicative intermediate-like properties was found in HeLa cells that had been infected with a poliovirus type 1 *ts* mutant and incubated at nonpermissive temperature (17).

The extent of synthesis of genomic RNA by *ts* mutant prototype and wt viruses in HeLa cells incubated at 39.5°C was assessed by assay of infectious RNA extracted from these cell cultures. Cells were challenged with 25 to 100 PFU per cell, and after 1 h for adsorption of virus at 25 or 34°C, the inocula were removed and the cell cultures were washed thrice with MEM and incubated in VGM at 39.5°C until 5 h p.i. At this time, the cells were harvested, and RNA was extracted from the cytosol with phenol and sodium dodecyl sulfate. Samples were assayed for infectious RNA by a plaque method on HeLa cells. The results of three experiments are shown in Table 1. Using specific infectivity (PFU × 10^4 per unit of optical density at 260 nm) of wt RNA as the standard for the maximum level produced in each experiment, it was apparent that *ts*1 virus produced little to no infectious RNA (<0.3% of wt yields of infectious RNA). The other two prototype *ts* mutants, *ts*5 and *ts*11, directed synthesis of reduced levels of infectious RNA at 39.5°C, although the levels produced varied from approximately 10 to 30% of the level directed by wt virus at 39.5°C. Thus, all three prototype *ts* mutants are defective in synthesis of viral RNA, but *ts*1 virus is clearly an RNA$^-$ mutant.

Viral polypeptide synthesis by *ts* mutants at 39.5°C. Viral polypeptides synthesized in HeLa cells incubated at 39.5°C in the presence of a ^{14}C-labeled amino acid mixture were extracted and analyzed in a 10% polyacrylamide gel slab (Fig. 2). Polypeptides from purified wt virus particles were utilized as markers for structural viral polypeptides (VP) in this gel. Eighteen newly synthesized polypeptides were reproducibly distinguished in the cytoplasm from cells inoculated with wt, *ts*5, or *ts*11 viruses and incubated at 39.5°C. The profile of polypeptides

TABLE 1. Infectious RNA extracted from HeLa cells infected with wild-type and prototype temperature-sensitive mutants of CVB3 and incubated at 39.5°C[a]

Expt no.	Infectious RNA extracted from HeLa cells inoculated with:	Specific infectivity (PFU × 10^4/OD_{260} unit)[b]	% of specific infectivity of wt RNA
1	wt	6.450	100
	*ts*1	0.0076	0.1
	*ts*5	1.622	25.2
	*ts*11	2.108	32.6
2	wt	9.001	100
	*ts*1	0.009	0.1
	*ts*5	1.353	15.0
	*ts*11	2.301	25.6
3	wt	1.328	100
	*ts*1	<0.004	<0.3
	*ts*5	0.223	16.8
	*ts*11	0.136	10.2

[a] Cells challenged with each virus (25, 100, and 50 to 75 PFU per cell, respectively, for experiments 1, 2, and 3) were incubated at 39.5°C until 5 h p.i., at which time they were harvested, and RNA was extracted from the cytoplasmic fraction by sodium dodecyl sulfate and phenol.

[b] Extracted RNA was diluted into MEM containing DEAE-dextran at 500 μg/ml and assayed for infectious RNA on HeLa cell monolayer cultures at 34°C (13).

synthesized in *ts*1 virus-inoculated HeLa cells presented the least distinct separation due to a relatively high background, suggesting that shut-off of host polypeptide synthesis by *ts*1 was incomplete at 39.5°C. The sizes of the noncapsid viral polypeptides found in infected cells, as estimated from the molecular weight markers, varied from approximately 90,000 to 12,000. These results are similar to those previously reported for poliovirus (22). Cells inoculated with wt, *ts*5, or *ts*11 viruses and incubated at 39.5°C contained newly synthesized noncapsid viral polypeptides of similar sizes, albeit not in the same amounts.

Purified wt virus particles contained four structural VPs with molecular weights of 35,000 (VP1), 30,000 (VP2a), 28,000 (VP2b), and 23,000 (VP3), values that are slightly higher than those reported by Crowell and Philipson (10) but slightly lower (except for VP1) than those reported by Chatterjee and Tuchowski (4). Although slight differences in techniques of polyacrylamide gel electrophoresis could account for the observed differences, it is also likely that the differences are due to the different variants of CVB3 used in the three studies. VP4, with an estimated molecular weight of 5,000 (21), was not observed under these conditions of electrophoresis. A small polypeptide (molecular weight approximately 5,000) was found in electropherograms of polypeptides from purified wt virus particles in 15% polyacrylamide gels (data not shown). Synthesis of the four capsid polypeptides (VP1, VP2a, VP2b, and VP3) identified in wt virus-inoculated cells were easily detected in cells inoculated with *ts*5 or *ts*11 viruses and incubated at 39.5°C. Newly synthesized capsid polypeptides were not detected in the cytoplasm of cells inoculated with *ts*1 and incubated at 39.5°C.

Estimates of viral capsid polypeptide levels synthesized by *ts* mutants in HeLa cells at 39.5°C were made by using the serum-blocking power test, which is an assay for VP2, the capsid immunogen involved in specific neutralization of virions (2, 9). The results (Table 2) show that cells inoculated with *ts*5 or wt viruses produced similar levels of capsid antigens at 39.5°C, whereas *ts*11 virus-inoculated cells produced fewer capsid antigens. In contrast, cells inoculated with *ts*1 virus produced minimally detectable levels of capsid antigens. Cells that were inoculated with wt virus and harvested at 1 h p.i. contained capsid antigens detectable at dilutions of <10 and 10 in two experiments (data not shown). This result confirms the data obtained by polyacrylamide gel electrophoresis-radioautography on the extremely limited production of viral proteins in HeLa cells challenged with *ts*1 virus and incubated at 39.5°C.

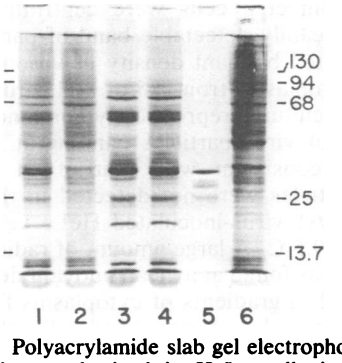

FIG. 2. Polyacrylamide slab gel electrophoresis of polypeptides synthesized in HeLa cells inoculated with wt or prototype *ts* mutant viruses and incubated at 39.5°C. Equivalent ^{14}C cpm in TCA-precipitable materials from each virus-inoculated cell culture were loaded into different wells of a slab gel. Virus-inoculated cell cultures were incubated at 39.5°C between 3.5 and 4.5 h p.i. in the presence of ^{14}C-labeled amino acids. Lanes 1 to 4 represent polypeptides synthesized in cells inoculated with wt, *ts*1, *ts*5, or *ts*11 viruses, respectively. Purified wt virus polypeptides were electrophoresed into lane 5. The uninoculated HeLa cell polypeptides synthesized at 39.5°C were electrophoresed into lane 6. Positions of migration of marker polypeptides are indicated on the right.

TABLE 2. Serum-blocking power test with antigens in HeLa cell lysates from cell cultures inoculated with *ts* mutant viruses and incubated at 39.5°C[a]

Expt no.[b]	Virus lysate	Dilution of virus lysate at endpoint[c]
1	*ts*1	20
	*ts*5	320
	*ts*11	80
	wt	640
2	*ts*1	40
	*ts*5	>640
	*ts*11	160
	wt	>640

[a] For the serum-blocking power test procedure, see the text.

[b] Virus-inoculated cell cultures were harvested at 8 or 24 h p.i. in experiments 1 and 2, respectively.

[c] Endpoint refers to that dilution of cell lysate antigens which binds 10% or less of the antiserum, resulting in neutralization by the unblocked antibodies of 90% or greater of the number of indicator PFU.

Production of virions by *ts* mutant prototypes at 39.5°C. The cytoplasmic contents of cell cultures inoculated with wt virus or a prototype *ts* mutant and incubated in the presence of [^{3}H]uridine at 39.5°C were subjected to isopycnic centrifugation in CsCl gradients in search of virus particles with a buoyant density similar to that of virions. The results (Fig. 3) showed that the gradient into which cytoplasmic materials from wt virus-infected cells were centrifuged contained a readily detectable band of particles at the expected buoyant density of virions (1.345 g/ml). Cytoplasms from *ts*5 or *ts*11 virus-inoculated cell cultures reproducibly contained small amounts of virus particles banding at buoyant densities consistent with that of wt virions. Virus particles were not detected in the cytoplasm of *ts*1 virus-inoculated HeLa cells incubated at 39.5°C. A large amount of radiolabeled material was found at a mean buoyant density of 1.233 g/ml in gradients of cytoplasms from wt-infected cells. In *ts*1-inoculated cells, the cytoplasm contained a large amount of material at 1.228 g/ml. Neither peak contained appreciable amounts of infectious virus ($<10^3$ PFU/ml), and we suspect that both of these less dense peaks of materials were of cell origin, since material of similar buoyant density was found in uninfected cells incubated at 34 or 39.5°C (data not shown).

Stability of *ts* mutant viruses at 39.5°C. Both *ts*5 and *ts*11 synthesized large amounts of viral polypeptides and appreciable amounts of genomic (infectious) RNA at 39.5°C, yet assembly into particles occurred with low efficiency (Fig. 3). Since lability of assembled virus particles at 39.5°C could, in part, account for the low titers found at 39.5°C, we assessed the stability of virions of the prototype *ts* mutants at this temperature. Viruses were diluted into VGM and incubated at 39.5°C for 6 (the time required for one complete growth cycle [reference 31]), 24, or 48 h. Typical results (Fig. 4) revealed that stability in virions of the mutants is, in order of decreasing stability, wt > *ts*11 > *ts*1 > *ts*5. Virions of *ts*5 were extremely labile to incubation at 39.5°C; 6 h of incubation resulted in a loss in titer of over 5,000-fold, compared with 2-fold losses for wt and *ts*11 viruses. The stability of *ts*1 was less than that of *ts*11 and wt viruses but greater than that of *ts*5 virus. Therefore, it appears that the low yields of *ts*5 at 39.5°C could also be attributed to capsid instability. Inclusion of phenylmethysulfonyl fluoride at 3.5 mM in VGM with *ts*5 virus during incubation at 39.5°C for 4 h did not prevent inactivation (~1,000-fold titer decrease; data not shown). Also, the buoyant density of a preparation of *ts*5 virus particles that had been incubated at 39.5°C for 4 h (99.8% loss in titer) did not change, and recovery of radiolabeled particles was similar to recovery

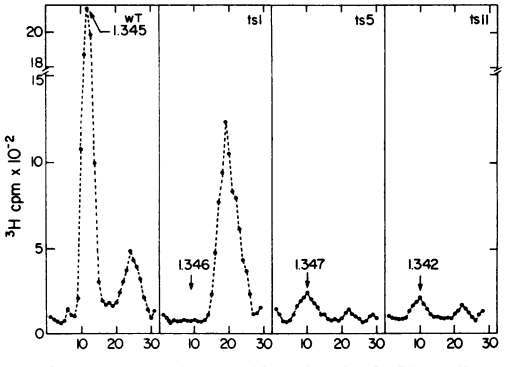

FIG. 3. Isopycnic centrifugation in CsCl gradients of the cytoplasmic contents of HeLa cells inoculated with wt, *ts*1, *ts*5, or *ts*11 viruses and inoculated at 39.5°C to detect nascent [^{3}H]uridine-labeled virus particles. Virus-inoculated HeLa cell cultures were exposed to [^{3}H]uridine between 3 and 8 h p.i. The cytoplasmic contents were layered onto a linear buffered CsCl gradient (1.27 to 1.37 g/ml) and centrifuged at 33,000 rpm in an SW50.1 rotor at 4°C for 14 h. Each fraction was assayed for TCA-precipitable cpm. The density of every fourth fraction was determined by refractometry, and the density (grams per milliliter) of the peak fraction representing virus particles (or the likely area) in each gradient is denoted by an arrow. Selected fractions in the area of virus particles were assayed for infectivity by plaque assay on HeLa cells. Specific infectivities at each arrow were 327, 96, 436, and 736 PFU/cpm, respectively, for virus particles in CsCl gradients onto which cytoplasms from wt, *ts*1, *ts*5, and *ts*11 virus-inoculated cells were layered and centrifuged to equilibrium.

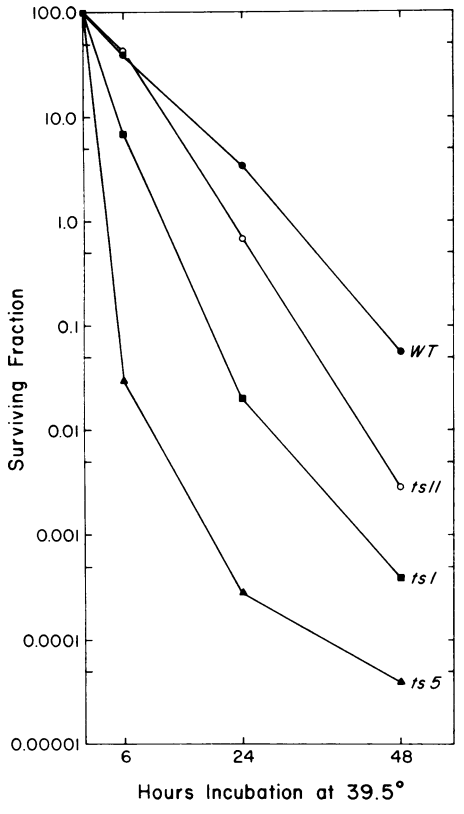

FIG. 4. Stability of virions of *ts*1, *ts*5, or *ts*11 to incubation at 39.5°C. Stocks of virus prepared at 34°C were diluted 1:10 in VGM, incubated in sealed tubes in a humidified incubator at 39.5°C for various periods, and stored at −70°C until assayed by the plaque method on HeLa cells.

for the control *ts*5 virus (data not shown). Attempts to disperse possible aggregates of *ts*5 virus particles that may have formed during incubation at 39.5°C were made by treatment of the virus suspension with proteinase K (Beckman; 10 μg/ml for 30 min at 34°C) or trypsin (0.25% under similar conditions) or by brief sonication; all were unsuccessful (data not shown).

Temperature shift-down effect on replication of *ts*5 virus. The instability of *ts*5 virus at 39.5°C suggested that a capsid polypeptide(s) was defective in maintaining the proper configuration needed in its structural role(s) in capsid stability at the nonpermissive temperature. Temperature "shift-down" experiments were conducted to determine whether the capsid polypeptides synthesized at 39.5°C could renature and function in assembly of virions upon shift of the cultures to 34°C. We also added cycloheximide (10 μg/ml) into several cultures at various times p.i. and immediately shifted these cultures to 34°C to determine whether capsid polypeptides synthesized at 39.5°C could, in the absence of further protein synthesis, be utilized in production of virions.

Cultures of HeLa cells were washed and challenged with approximately 50 PFU per cell. After 30 min for adsorption at 34°C, the inoculum was removed, and the cell cultures were washed three times with MEM and given 1 ml of VGM. Duplicate cell cultures were shifted from 39.5 to 34°C at 2, 3, 4, 5, or 6 h p.i.; after an additional 1 to 6 h of incubation at 34°C, the cultures were harvested by immediate freezing to −70°C. Some cultures were given cycloheximide in amounts up to 10 μg/ml at various times p.i. and then were immediately shifted to 34°C until they were harvested in duplicate at 8 h p.i. Virus contents of cell lysates were assessed by plaque assay after three cycles of rapid freezing and thawing.

The results of these experiments are shown in Fig. 5. Infected cultures shifted from 39.5 to

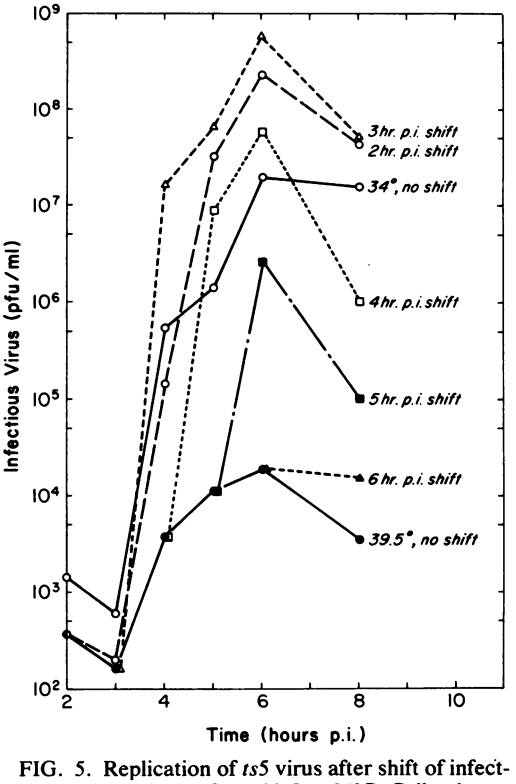

FIG. 5. Replication of *ts*5 virus after shift of infected HeLa cell cultures from 39.5 to 34°C. Cell cultures were challenged with ~50 PFU per cell and incubated for 30 min at 34°C. The cells were washed three times and incubated in VGM at 34 or 39.5°C. At harvest time, appropriate cell cultures were immediately frozen and then were freeze-thawed three times before the contents were assayed by the plaque method on HeLa cell cultures at 34°C.

34°C at 2, 3, or 4 h p.i. resulted in yields of infectious virus at 6 h p.i. that were higher than the yields measured for cell cultures incubated continuously at 34°C. This suggests that large amounts of capsid polypeptides are produced in these cells during these brief intervals at 39.5°C. Incubation of the cell cultures at 39.5°C for 2 or 3 h before shift-down to 34°C increased the 6-h yield by 10-fold or more. Shifting the cells from 39.5 to 34°C at 5 h p.i. and incubating them for only 1 h more resulted in a 100-fold increase in yield of infectious virus over that obtained from cell cultures incubated continuously at 39.5°C. Shifting cultures down at 6 h p.i. for 2 h of incubation at 34°C did not appreciably affect virus yields. Note that virus titers from all cell cultures that were shifted down to 34°C, except for the 6-h shift-down cell cultures, decreased in the 2-h period between 6 and 8 h p.i. by 80 to 98%. Infected cell cultures that were treated with cycloheximide and then shifted to 34°C at various times p.i. (2, 3, 4, 5, or 6 h p.i.) showed low titers of infectious virus (2.0×10^4 to 6.3×10^4 PFU/ml [data not shown]), suggesting that capsid polypeptides and polypeptide precursors were degraded after synthesis unless assembled into subunits, as found for vesicular stomatitis virus (19), or that assembly, as found for poliovirus, requires an unstable protein(s) whose continuous synthesis is prevented in the presence of cycloheximide (27). Growth curves of wt virus at 34 and 39.5°C were very similar to those previously reported (31) and are not shown.

Data from the experiment described above suggested that capsid precursor materials synthesized at 39.5°C were subsequently assembled into particles after the shift-down to 34°C. We confirmed this hypothesis by the following pulse-chase shift-down experiment (data not shown). Two cultures (25×10^6 cells) of HeLa cells were challenged with *ts*5, and after 45 min of incubation at 34°C, the cells were washed; one culture was returned to 34°C, and the other was placed at 39.5°C. Both cultures were pulsed with ^{14}C-amino acids at 0.1 μCi/ml from 1.0 to 3.0 h p.i. After washing, the cultures were immersed in VGM containing twice the normal amino acid concentration, and both were placed at 34°C until 8 h p.i. Cytosol was obtained from Dounce-homogenized cells swelled in reticulocyte standard buffer and layered onto (buffered) CsCl at 1.34 g/ml. After centrifugation to band virus particles, assessment of TCA-precipitable cpm revealed radiolabeled infectious peaks of virus at identical buoyant densities (1.346 and 1.347 g/ml). Also, specific infectivities of 5.23×10^4 and 5.07×10^4 PFU/cpm were found for control and shift-down cultures, respectively.

In vitro cytopathology of HeLa cells by *ts* mutants at 39.5°C. HeLa cells were challenged with 50 to 75 PFU per cell of each prototype *ts* mutant; after 45 min for adsorption at 34°C, the cells were washed three times and incubated in VGM at 39.5°C. The assay procedures were applied to cells harvested at 9 h p.i.; results of representative experiments are shown in Table 3. The ^{51}Cr-release method detected a virus-induced small increase in release of ^{51}Cr above that found for the cell control, and the release was similar for all viruses up to 4 h p.i.; however, by 6 h p.i., the progressive increase in ^{51}Cr release above that of the cell control was different for each virus over the next 3 h (data not shown). The ^{51}Cr-release assay appeared to be the least sensitive of the three methods for indicating cytopathology induced by any of the viruses. However, all three tests essentially agreed on the order of viruses relative to the extent of cell damage induced, i.e., in order of increasing cell damage induced, they were *ts*1 < wt < *ts*5 < *ts*11. Thus, *ts*1 virus induced little cell damage by 9 h p.i., whereas *ts*11 virus induced extensive cell damage. Although cells inoculated with *ts*1 virus at 9 h p.i. were quite similar in appearance to uninoculated HeLa cells, by 24 h p.i. all cells in the *ts*1 virus-inoculated culture were destroyed. Thus, these prototype viruses can be distinguished from each other on the basis of rapidity of induction of cytopathology in HeLa cells.

DISCUSSION

Complementation among picornaviruses has been demonstrated several times, although the complementation indices are low (3, 6, 7, 15). We have found that complementation can reproducibly be demonstrated among select pairs of 9 of 10 temperature-sensitive mutants of CVB3, with the result that 9 of these mutants could be assigned to three non-overlapping groups (31). Prototype *ts* mutants (*ts*1, *ts*5, and *ts*11) representing each of the three complementation groups were shown previously to differ in efficiency of plating at 37 and 39.5°C and in pathogenicity for newborn mice (32). Our study also shows that differences exist among the three prototype *ts* mutants in the biochemical processes being directed in HeLa cells at 39.5°C.

The prototype *ts*1 mutant directed synthesis of very little genomic viral RNA at 39.5°C, and comparatively larger amounts of presumed incomplete viral RNA were found migrating between the 28 and 18S ribosomal RNA markers, as determined by [^{3}H]uridine counts in agarose gels. In addition, synthesis of cell ribosomal RNA species continued in cells inoculated with *ts*1 virus in the absence of Act D. The heterogeneous size of the subgenomic RNA migrating between 28 and 18S, presumed to be viral RNA,

TABLE 3. Cell damage induced by *ts* mutants in HeLa cells incubated at 39.5°C[a]

Virus	Cytopathology assay		
	^{51}Cr release (%)[b]	Trypan blue dye uptake[c]	Visual estimate[d]
*ts*1	7.8	6–13	±
*ts*5	15.8	61–69	2+ to 3+
*ts*11	17.2	72–87	3+ to 4+
wt	8.8	55–62	2+

[a] Assessments were done at 9 h p.i. Titers in these cultures ranged from 2×10^4 to 4×10^4 or from 8×10^7 to 2×10^8 PFU/ml for *ts* mutants and wt virus, respectively.

[b] Corrected for release from uninfected cells (17%); values are averages of two cell cultures per group.

[c] Expressed as percentage of cells absorbing (dead cells) trypan blue; corrected for percentage of uninfected cells absorbing (2 to 4%) trypan blue. Results are from two experiments.

[d] Scale: ±, less than 5% cells with some cytopathology; 1+, 5 to 25% cells with cytopathology; 2+, 25 to 75% exhibit cytopathology; 3+, 75 to 95% exhibit cytopathology; and 4+, 95% or greater of cells exhibit cytopathology. Results are from two experiments.

suggested that the RNA was extracted from defective interfering particles, similar to those generated during undilute passage of poliovirus type 1 (5). We unsuccessfully sought an increase in RNA of this range of sizes in the cytoplasm of HeLa cells during 40 serial undilute passes of *ts*1 at 34°C; similarly, cytoplasms from cells inoculated with undilute passes 5, 12, 21, and 39 were devoid of particles having a buoyant density in CsCl gradients less than that of virions, and diminutions in virus yields with successive undilute passes were not observed (data not shown). Based on these results, synthesis of defective interfering particle RNA in *ts*1-infected cells does not appear to be a plausible explanation for the RNA migrating between 28 and 18S.

The *ts*1 mutant was relatively (compared with *ts*5 or *ts*11 mutants) ineffective in shutting down host protein synthesis at 39.5°C. Hewlett et al. (17) recently described an RNA$^-$ *ts* mutant of poliovirus type 1; however, this mutant was as effective as the parent wild-type virus in inhibiting host protein synthesis. Cells inoculated with *ts*1 virus and incubated at 39.5°C displayed a considerable delay in cytopathology after virus challenge compared with the parent or the other two prototype viruses. In earlier studies with *ts* mutants of poliovirus type 1, Garwes et al. (12) associated a defect in induction of late cytopathic effects (morphological changes in infected cells) with a defect in synthesis of double-stranded viral RNA. However, studies with guanidine and streptovitacin A in poliovirus-infected cells (1, 29) showed that cytopathology was not correlated with production of double-stranded RNA but, rather, was best correlated with quantity of virus-induced proteins. The relative inability of *ts*1 to induce cytopathology in HeLa cells could therefore fit the hypothesis that picornavirus-induced cytopathology depends on quantity of viral proteins produced which, in turn, depends on the availability of viral genomes for translation.

Both *ts*5 and *ts*11 viruses induced synthesis of reduced (compared with $CVB3_m$), albeit significant, levels of viral genomes at 39.5°C. These RNA$^+$ mutants synthesize equivalent or increased amounts of capsid polypeptides (compared with $CVB3_m$) at 39.5°C, and the polyacrylamide gel electrophoresis patterns of these proteins were indistinguishable. The results of temperature shift-down experiments with the *ts*5 mutant suggested that capsid polypeptides newly synthesized at 39.5°C could be subsequently assembled into virions at 34°C, although capsid integrity could not be maintained, possibly because of an alteration in one of the capsid polypeptides. Thus, the substitution of an amino acid in one capsid polypeptide could lead to the temperature-sensitive defect in *ts*5. Capsid polypeptides from virions of *ts* mutants of foot-and-mouth disease viruses that display single amino acid changes have been detected by high-resolution electrofocusing in studies of a number of *ts* mutants (18); this technique might prove useful in distinguishing capsid polypeptides of *ts*5 (and *ts*11) virus from $CVB3_m$.

The *ts*11 mutant appears to have a defect in assembly, and one possible explanation is that the *ts*11 virus has a defect in synthesis of a putative functional picornavirus morphopoietic factor (27). Poliovirus *ts* RNA$^+$ mutants with such apparent defects in morphogenesis have been isolated (27). As in the case of *ts*11, the poliovirus RNA$^+$ mutants direct synthesis of both virus particle and nonstructural polypeptides that have no identifiable alterations from similar proteins synthesized by the parent virus (27). Likewise, polyacrylamide gel electrophoresis profiles of polypeptides produced and processed by cells inoculated with *ts*11 virus and incubated at 39.5°C showed no appreciable differences from similar profiles of $CVB3_m$-induced polypeptides. Maturation-defective *ts* mutants of encephalomyocarditis virus have been isolated which exhibit problems in cleavage of the capsid precursor polypeptides (28).

At present, we do not understand how the relative virological inactivity of *ts*1 virus, compared with *ts*11 virus, in HeLa cells at 39.5°C could account for the slight increase in survival of neonates inoculated with *ts*1 virus (66%) over the survival of neonates inoculated with *ts*11 virus (40%) (32). The efficiency of plating and

leakiness values for *ts*1 and *ts*11 viruses (31) are not sufficiently different to explain this difference in pathology detected in neonatal mice. One possibility depends on the poor temperature regulation by murine neonates (16, 30), which probably permits some replication of the three prototype *ts* mutants at 35 to 37°C, and the extent of replication could contribute to the increased *ts*11 virus-induced fatalities over *ts*1 virus-induced fatalities among this population. Indeed, the efficiency of plating of *ts*11 virus at 37°C is 100-fold better than that of *ts*1 virus (31). The *ts*1 mutant and the *ts*6 mutant (also of complementation group III) exhibit unusual pathology in neonates surviving intracranial inoculation (Jones et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, T45, p. 245). Hydranencephaly and porencephaly were induced in 36 and 66% of the survivors within 14 to 105 days p.i. of *ts*1 or *ts*6 viruses, respectively. The *ts*5 mutant, like the parent $CVB3_m$, is highly lethal (>95%) for neonates (32), and the combination of partial replication at 35 to 37°C in neonates coupled with production of large quantities of viral proteins at any temperature could account for the virulence of *ts*5. The *ts*11 mutant has recently been found to induce both hydrocephalus ex vacuo and hydranencephaly (D. C. Jones, R. J. Gudvangen, H. W. Huntington, and C. J. Gauntt, unpublished data). The *ts*11 virus in vitro was highly cytopathogenic in HeLa cells at 39.5°C in the absence of production of virus; therefore, a product(s), perhaps viral proteins, could partially account for the virulence exhibited by this *ts* mutant. Thus, the biological and biochemical differences found among these prototype viruses in vitro provide some basis for beginning to understand the observed differences in pathogenicity in vivo.

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