Steady-State Infection by Echovirus 6 Associated with Nonlytic Viral RNA and an Unprocessed Capsid Polypeptide

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We established a human cell line which was persistently infected (PI) by the normally cytolytic echovirus 6. All of the cultured PI cells contained genome-size viral RNA which was synthesized continuously and incorporated into virus particles. This steady-state infection has been maintained for more than 6 years. In contrast to RNA of wild-type echovirus 6, the viral RNA from PI cells was not lytic when transfected into uninfected, susceptible cells. The capsid polypeptides of the virus particles produced during lytic infections were compared with those of virus particles from PI cells. Wild-type virions contained five polypeptides with molecular masses of 31.5, 27, 25.8, 21.2, and 9.5 kilodaltons. Comparison of polypeptide profiles of virions and empty immature capsids along with peptide analyses by immunoblotting and partial proteolysis of isolated viral proteins identified the cleavage products of the 31.5-kilodalton polypeptide (VP0) as the two smaller polypeptides (VP2 and VP4). The virus particles produced by PI cells as well as cellular extracts of PI cells contained only the three largest proteins (VP0, VP1, and VP3), indicating that VP0 was not processed during persistent infection. The lack of VP2 and VP4 in the defective virus particles coincided with their inability to attach to uninfected, susceptible cells. The maintenance of the steady-state infection of echovirus 6 was not dependent upon the release of virus particles from PI cells.

Previously, we reported the establishment of a persistent infection in a cloned human WISH cell line by the lytic virus echovirus 6 (6). This viral persistence has been maintained in culture for approximately 6 years and differs from other persistent enterovirus infections by the following characteristics. (i) All of the cells are infected. (ii) The persistently infected (PI) cells are resistant to superinfection by 30-fold-higher virus concentrations, which usually lyse uninfected cells. (iii) The PI cells produce virus particles continuously without cellular destruction. (iv) The virus yield from PI cells is similar to that recovered from lytic infections (approximately 1,500 particles per cell). (v) The virus particles from PI cells do not interfere with infection of parental uninfected cells by standard lytic virus. (vi) Viral persistence is not temperature sensitive. (vii) The steady-state infection is not dependent upon exogenous factors, such as antiviral antibody or interferon. (viii) The particles from PI cells are unable to attach to uninfected parental WISH cells.

The present report addresses the nature of the persistent viral genome and compares the properties of lytic virus particles recovered from acutely infected WISH cells with those of nonlytic virus particles produced during a steady-state infection. In our previous report, we demonstrated that the defective particles from PI cells were missing at least one polypeptide, designated VP3 on the basis of its migration in sodium dodecyl sulfate (SDS)-polyacrylamide gels. The smallest polypeptide, VP4, usually migrated with the dye front and was not easily detected. By utilizing specific antiviral sera and partial peptide proteolysis and examination of the proteins of natural empty immature capsids in the present study, we were able to identify the capsid cleavage products of the precursor polypeptide (VP0) and to demonstrate that VP3 migrates ahead of VP2 in preparations of lytic virus. The nonlytic particles from PI cells were missing VP2 and VP4 instead of VP3. These results indicate that a

capsid precursor protein, VP0, does not undergo cleavage in particles produced by PI cells. Our results also indicate that PI cells produce genomic-size viral RNA. The RNA is defective, since transfection of susceptible cells does not result in production of lytic virus particles. Some of the viral RNA is incorporated into defective virus particles. Since the defective particles do not attach to susceptible cells, the release of virus particles is not necessary for either transmission or maintenance of viral RNA in this persistent infection.

MATERIALS AND METHODS

Cell cultures. The human WISH cell line was obtained from the American Type Culture Collection and was grown and maintained as described previously (14). The PI WISH cell line was established from one clone of echovirus 6-infected cells (6) and has been maintained in monolayer cultures for 6 years.

Virus preparations and assays. The plaque-purified *m+* mutant of echovirus 6 (2) was passaged in WISH cells and used as a wild-type lytic virus. PI cells produced defective nonlytic viruses which were recovered either from supernatant media which had been in contact with monolayers for at least 3 days or from mechanically disrupted PI cells. Virus preparations were purified further by density gradient centrifugation in sucrose (15 to 45%) or in CsCl (14). Virus assays were performed by the agar overlay method with three or four monolayers for each sample (14).

Radioactively labeled purified echovirus 6 was prepared as described previously (6). [³⁵S]methionine (13 μ Ci/ml, 1,100 Ci/mmol) was added to monolayers at 4 h after virus inoculation (20 PFU/cell). Virus was produced in 6 to 9 h after infection and purified.

Anti-echovirus 6 antiserum. Monkey anti-echovirus 6 (D'Amori) serum was obtained from the National Foundation of Infantile Paralysis, control no. 6L-61429. This serum had a 50% plaque neutralization titer against echovirus 6 in

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WISH cells of 1:4,000. It did not neutralize echovirus 4 or poliovirus type 1 (Mahoney or LSC2ab strains). Mouse anti-echovirus 6 antisera were obtained by inoculating BALB/c mice intraperitoneally with three doses of 1×10^9 to 2×10^9 PFU (approximately 10^{12} particles) at weekly intervals. The first dose was suspended in complete Freund adjuvant, and subsequent doses were administered with incomplete Freund adjuvant. Polyclonal antisera were obtained, which reacted with all five capsid virus polypeptides in immunoprecipitation assays and did not react with cellular proteins. These antisera had endpoint titers of between 1:6,400 and 1:12,800 in enzyme-linked immunosorbent assays (Zymed MonAB-Screen P Kit; Zymed Laboratories Inc., South San Francisco, Calif.), and 50% plaque reduction neutralization titers of between 1:2,000 and 1:4,000. An anti-echovirus antiserum, which reacted strongly with VP0 as well as with the two smallest viral capsid polypeptides (Fig. 6B), was obtained after inoculation of mice with a heat-inactivated (56°C, 30 min) echovirus 6 preparation, which had a high concentration of VP0 and no demonstrable VP1 and VP3 (as detected by silver stain of viral polypeptides on SDS-polyacrylamide slab gels). The mouse antiserum did not react with either VP1 or VP3 in immunoprecipitation assays or in immunoblots. It had an endpoint titer of 1:6,400 in the enzyme-linked immunosorbent assays and had a 50% plaque reduction neutralization titer of 1:4,000. The serum was specific for echovirus 6, since it did not react with cellular proteins and did not neutralize poliovirus type 1 (LSC2ab strain).

Isolation of echovirus 6 RNA and cellular mRNA. Purified virus preparations were dialyzed in NTE (100 mM NaCl, 10 mM Tris chloride, pH 7.5, 1 mM EDTA), brought to 0.5% SDS, and extracted twice with an equal volume of phenol-chloroform-isoamyl alcohol (24:24:1). The final aqueous layer was chilled overnight in 2.5 volumes of ethanol containing 0.3 M sodium acetate. The RNA was pelleted by centrifugation in a microcentrifuge at $14,000 \times g$ for 30 min at 5°C, and the pellet was suspended in 70% ethanol containing 0.3 M sodium acetate, washed by centrifugation, and suspended in a small volume of water treated with diethyl pyrocarbonate. mRNA was isolated from cellular monolayers (4×10^7 cells) by the procedure described by Maniatis et al. (11).

Assay of infectivity of viral RNA. Cell monolayers were washed three times with 0.15 M NaCl before treatment with 1 mg of DEAE-dextran (2×10^6 daltons) per ml for 15 min at 23°C. The monolayers were rewashed three times, inoculated with RNA samples, and incubated for an additional 15 min at 23°C before agar overlay. Purified virus preparations, used for RNA extraction, served as controls for the plaque assays. Also, samples of RNA and virus were pretreated with pancreatic RNase (100 µg/ml) for 30 min at 23°C as controls for viral RNA infectivity. Plaques were visible after 4 days of incubation at 36°C.

Preparation of viral cDNA probe. Salmon sperm DNA (type III Na salt; Sigma Chemical Co., St. Louis, Mo.) was used as a source of random primer for preparation of cDNA from echovirus 6 RNA. The DNA (5 mg/ml) was fragmented by DNase I (70 µg/ml in the presence of 10 mM Tris, pH 8.3, and 10 mM MgCl₂) by incubation at 37°C for 2 h before being heated to 120°C in an ethylene glycol-monoethyl ether bath for 10 min. The denatured DNase was removed by centrifugation. Echovirus 6 RNA was incubated with the salmon sperm random primer fragment in the presence of 8 mM dithiothreitol, 50 mM Tris, pH 8.4, 8 mM MgCl₂, dATP, dGTP, dTTP, and ³²P-labeled dCTP, and 400 U of Moloney

murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Bethesda, Md.). Maximum incorporation of the label occurred in 30 min. The cDNA probe was isolated after addition of 20% SDS on a Sephadex G-50-150 column in TNE (5 mM Tris, 5 mM NaCl, 0.5 mM EDTA), clarified, and brought to 0.1 N NaOH.

Northern (RNA) blot hybridization. RNA was electrophoresed through 1% agarose gels containing methyl mercuric hydroxide (0.005%) and transferred to diazotized paper (1). The Northern blots were treated with prehybridization buffer (4 h, 42°C) containing 50% deionized formamide, 10% sodium dextran sulfate (500 kilodaltons [kDa]), 1 M NaCl, 50 mM Tris, pH 7.5, 0.2% each of bovine serum albumin, polyvinylpyrrolidone, and Ficoll (Pharmacia Fine Chemicals, Piscataway, N.J.) (10× Denhardt solution), 0.1% sodium pyrophosphate, and 250 µg of sheared salmon sperm DNA per ml with agitation at 42°C. After preincubation, ³²P-labeled cDNA probe of echovirus 6 was added at a concentration of 1 to 3 ng/ml and incubated for an additional 16 h at 42°C. Nonspecifically bound [³²P]cDNA was removed by five washes for 30 min each at 62°C with decreasing concentrations of 2× SSC (0.3 M NaCl, 0.03 M trisodium citrate) to a final concentration of 0.1× SSC. All washes except the last wash contained 0.1% SDS. The washed blots were air dried and exposed to Fuji XR film at -70°C with 2 Quanta intensifying screens.

Polyacrylamide gel electrophoresis. Viral and cellular proteins were resolved on 10, 12.5, and 15% polyacrylamide slab gels after electrophoresis by the Laemmli system (10) as described previously (6). Sample buffer consisted of 4 M urea, 1% SDS, 20% glycerol, 1% 2-mercaptoethanol, 10 mM Tris base, 1 mM EDTA, and 0.05% bromphenol blue. Gels were also run with a modification of the Laemmli system, using 1 M Tris hydrochloride (pH 8.7) buffer rather than the 1.5 M Tris hydrochloride buffer, to enhance resolution of proteins below 40 kDa in size. Resolved proteins were visualized by silver staining (13) or by fluorography using Kodak X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.) and Cronex Lightning-Plus (DuPont Co., Wilmington, Del.) intensifying screens at -70° (6).

Partial proteolysis with two-dimensional gel electrophoresis. Viral proteins were first resolved by electrophoresis on either 12.5 or 15% SDS-polyacrylamide slab gels. This first-dimension gel strip, containing all of the viral proteins as indicated by prestained low-molecular-weight protein standards (Bethesda Research Laboratories), was placed horizontally into the stacking gel of a 15% SDS-polyacrylamide slab gel and sealed with molten 1% agarose before the gel plates were placed in the electrophoretic apparatus. The gel strip was overlaid with 100 µl of protease sample buffer (0.125 M Tris, pH 6.8, 0.1% SDS, 20% glycerol, 0.1% bromphenol blue) containing 0.5 µg of *Staphylococcus aureus* V8 protease (Miles Laboratories, Naperville, Ill.). Samples were electrophoresed with the discontinuous buffer system at 65 mA (200 V) until the dye front moved through the first-dimension gel strip and well into the stacking gel. The current was turned off for 1 h to allow proteolysis to occur and then resumed at 50 mA (150 V) for the remainder of the gel run. Resolved digested viral proteins were visualized by means of silver staining or fluorography and compared with marker proteins.

Immunoprecipitation of viral proteins. Polyclonal anti-echovirus 6 serum (prepared in monkeys or mice) or mouse serum recovered from mice immunized with the 31.5-kDa viral polypeptide (VP0) was used. Labeled echovirus 6 (approximately 10^5 cpm) was brought to 0.4 ml with immu-

noprecipitation buffer (140 mM NaCl, 20 mM Tris hydrochloride, pH 7.8, 1% Nonidet P-40, 0.5% sodium desoxycholate) supplemented with 1 mg of ovalbumin per ml and 0.5 mM phenylmethylsulfonyl fluoride. The appropriate serum (10 μ l) was added to labeled virus, and the mixture was rotated for 16 h at 4°C. A suspension (50 μ l) of 40% protein A-Sepharose beads (Sigma) was added and incubated with shaking for 1 h at 4°C. Immunoglobulin G-antigen complexes were pelleted by centrifugation and washed twice with immunoprecipitation buffer containing 0.1% SDS, followed by two washes with 140 mM NaCl–20 mM Tris hydrochloride–0.1% Nonidet P-40 and two washes with 10 mM Tris, pH 7–0.1% Nonidet P-40. The washed pellet was heated in sample buffer (100°C, 5 min) before electrophoresis.

Immunoblot detection of viral proteins. SDS-polyacrylamide slab gels containing resolved proteins and prestained marker proteins were transferred to 0.45- μ m-thick nitrocellulose membranes as described by Towbin et al. (18) by using an electrophoretic transfer chamber (E-C Apparatus Corp., St. Petersburg, Fla.). The transfer buffer consisted of 25 mM Tris, 192 mM glycine, pH 8.3, and 20% (vol/vol) methanol. Electroblothing was completed after 16 h at 225 mA. Marker proteins were included to monitor completion of transfer.

Viral protein-antibody complexes were detected by using 125 I-protein A (labeled with Bolton and Hunter reagent, Amersham Corp., Arlington Heights, Ill.). Blotto (8) was used to block nonspecific antibody binding to the nitrocellulose, as a washing buffer, and for dilution of antibody. Air-dried nitrocellulose membrane strips (cut 1 cm wide) were incubated with anti-echovirus 6 sera (16 h, 25°C). Strips were washed six times before being labeled with 1.5 μ Ci of 125 I-protein A per strip (4 h, 25°C), rewashed, air dried, and exposed to Kodak X-OMAT AR film.

RESULTS

Presence of viral RNA in PI cells. mRNAs were recovered from cytoplasmic extracts of PI cells and from extracts of uninfected and acutely infected (10 PFU per cell, 4 h) WISH cells. Two monolayer cultures (2×10^7 cells per monolayer) were used to prepare each cellular extract. The PI cells had been maintained as monolayers for 30 days, and the WISH cells were used as soon as they reached confluency (4 days). Approximately 1 μ g of the cellular RNAs was electrophoresed in methyl mercury-agarose gels. RNA extracted from purified echovirus 6 virions (7 ng) was included as a positive control. The concentration of applied RNA was estimated by intensity of staining with ethidium bromide and comparison with RNA standard solutions. The RNAs were transferred to diazotized paper and probed with 32 P-labeled cDNA of echovirus 6 (Fig. 1). The cDNA hybridized with RNA from acutely infected cells (lane 2) and with RNA from persistently infected cells (lane 4). These RNAs migrated as one band to the same position as genomic viral RNA (lane 3). The cDNA did not hybridize to RNA from uninfected cells (lane 1) and was thus specific for viral RNA. Subgenomic RNA species were not detected even after extended exposure of fluorographs (2 weeks rather than 2 days).

Comparison of RNA in nonlytic and lytic viral particles. In order to determine whether the genome-sized viral RNA in PI cells and in lytically infected cells was incorporated into virus particles, RNAs from purified virus preparations were extracted and analyzed by electrophoresis in methyl mercury-agarose gels. Extracellular media of PI cell monolayers were collected 3 days after growth to confluency (7 days

FIG. 1. Presence of echovirus 6 RNA in cytoplasmic extracts of PI cells. 32 P-labeled echovirus 6 cDNA was hybridized to Northern blots of RNA recovered from uninfected cells (lane 1), acutely infected (10 PFU per cell; 4 h) cells (lane 2), purified echovirus 6 particles (lane 3), and persistently infected cells (lane 4). The RNAs (2.0×10^6 and 2.5×10^5 daltons) from the cowpea strain of tobacco mosaic virus were used as reference markers for molecular weights and are indicated by arrows.

after cell passage), clarified, and cushioned on CsCl (1.4 g/ml). The particles in the CsCl cushion were purified further in linear CsCl gradients and isolated in a band at a density of 1.33 g/ml. RNAs from linear gradients of wild-type echovirus 6 were included as positive controls. The same concentration of purified lytic and nonlytic virus (approximately 10^{12} particles), as determined by UV light absorption, was used for RNA extractions. The RNA from particles produced by PI cells (lane 2) migrated as a single band to the same position as RNA from purified echovirus 6 particles (lane 1) and had a molecular mass of approximately 2.6×10^6 daltons (Fig. 2). No bands were detected when the RNA preparations were pretreated with pancreatic ribonuclease A (100 μ g/ml, 1 h, 37°C) before electrophoresis (not shown).

Infectivity of viral RNA. We reported previously that the virus particles produced by PI cells were unable to attach to uninfected WISH cells (6). Thus, entry and expression of the viral RNA could not occur. Transfection experiments were performed to examine the possibility that the RNA from particles produced by PI cells was infectious when it was introduced directly into the cell. Uninfected WISH cells were inoculated with viral RNA in the presence of DEAE-dextran (1 mg/ml). Transfection of viral RNA isolated from wild-type echovirus 6 was performed under the identical conditions at the same time. Transfected monolayers were examined for plaque formation, cytopathic effect, and virus yields. Plaque assays were carried out in monolayers in 25-cm 2 flasks (four per sample). RNA (1.6 ng) from wild-type lytic virus produced 630 plaques (approximately 400 PFU/ng of RNA). Pretreatment of this viral RNA with ribonuclease

FIG. 2. Genome-sized viral RNA in particles recovered from PI cells. RNAs from purified lytic (wild-type) echovirus 6 particles (lane 1), purified nonlytic virus particles recovered from PI cells (lane 2), and the cowpea strain of tobacco mosaic virus (lane 3) were electrophoresed through methyl mercury-agarose gels and stained with ethidium bromide.

I (100 $\mu\text{g/ml}$ for 1 h at 23°C) under conditions which excluded the activity of DNase degraded the RNA and abolished its infectivity. Plaque formation did not occur after inoculation of WISH cells with 19-fold-greater concentrations of RNA (30 ng) from the nonlytic particles produced by PI cells. Transfection of WISH monolayers in multiwells (4×10^5 cells) with 150 ng of viral RNA from lytic virus resulted in cytopathology and a yield of 5.5×10^4 PFU during a single growth cycle (8 to 12 h). However, no cytopathologic changes were observed after 7 days of incubation of monolayers transfected with 300 ng of viral RNA from particles produced by PI cells. The viral RNA was not degraded during manipulations before transfection, since genome-sized RNA was detected in samples used as inocula after electrophoresis in methyl mercury-agarose gels.

Viral structural polypeptide profiles of lytic and nonlytic particles. Several methods were used to detect and characterize viral structural polypeptides. Unlabeled or [^{35}S]methionine-labeled virus particles were recovered from linear CsCl and sucrose gradients after equilibrium centrifugation. The infectivity of echovirus 6 was associated with particles with a density of approximately 1.33 g/ml. After SDS-polyacrylamide gel electrophoresis, the polypeptides of disrupted, purified virus particles were visualized by silver staining or fluorography. As reported previously (6), the viral capsid consists of five polypeptides with apparent molecular masses of 31.5, 27.0, 25.8, 21.2, and 9.5 kilodaltons (kDa), and were initially designated in order of migration as VP0, VP1, VP2, VP3, and VP4. These polypeptides were shown to be viral in origin, since they migrated in SDS gels to the same positions before and after immunoprecipitation with polyclonal anti-echovirus 6 serum (6) (Fig. 3). The 21.2-kDa polypeptide band was not as intense as the larger polypeptides as detected by both silver staining (not shown) and fluorography of immunoprecipitates. Although the presence of the 9.5-kDa polypeptide was occluded by the

FIG. 3. Fluorograph of polypeptides of wild-type echovirus 6. Purified virus was recovered from linear CsCl gradients, and the capsid polypeptides of untreated lytic virus (lane A) and immunoprecipitated lytic virus (lane B) were resolved by electrophoresis in SDS-15% polyacrylamide gels. Molecular weights of the viral proteins were estimated from the positions of prestained marker proteins that were resolved on the same gel slab.

background in the film, this polypeptide was demonstrable in another photograph of the same virus preparation (see Fig. 5A). The same polypeptide profile was observed in 10, 12.5, and 15% polyacrylamide gel slabs and in gradient gels (10 to 20%). The patterns were not altered by the absence or presence of urea (2 to 8 M) in either the resolving gel buffer or the sample buffer or by pretreatment of crude virus preparations with trypsin (100 $\mu\text{g/ml}$, 23°C, 1 h), phenylmethylsulfonyl fluoride, or CHAPS (3-[3-cholamidopropyl-dimethylammonio]-1-propane sulfonate; Calbiochem-Behring, La Jolla, Calif.) before purification. However, the presence and the intensity of the 31.5-kDa polypeptide band were variable. A decrease in the concentration of the largest structural polypeptide coincided with increased intensity of the 21.2-kDa protein band (Fig. 4, lane A). This latter polypeptide was stable in sucrose but labile after extended exposure to CsCl. It was recoverable from purified virus after one but not two cycles of equilibrium centrifugation in CsCl. The band between the 21.2- and 9-kDa polypeptides (Fig. 4, lane A) is a cellular polypeptide that migrated with the virus in CsCl linear gradients and was not immunoprecipitated.

The nonlytic virus particles produced by PI cells were purified by equilibrium centrifugation in sucrose and CsCl linear gradients and recovered in bands with a density of 1.33 g/ml. The presence of virus particles in fractions recovered from equilibrium gradients of extracts of PI cells was established by UV light absorption, density (1.333 g/ml), and electron microscopy (not shown). These virus particles contained the three larger polypeptides but did not contain the 21.2- and 9.5-kDa capsid polypeptides (Fig. 4, lane B). The smaller polypeptides were not visualized after disruption of nonlytic virus particles and electrophoresis in 10,

FIG. 4. Silver stain of the polypeptides of virus particles recovered from WISH (A) and PI (B) cells. Virus particles recovered from a CsCl linear gradient at a density of 1.33 g/ml were resolved by electrophoresis in an SDS-10% polyacrylamide gel slab. Prestained protein markers were used for estimation of the molecular masses (kilodaltons) of the viral polypeptides.

12.5, and 15% polyacrylamide gels and were not detected in either immunoprecipitates of PI cells (see Fig. 8B) or immunoblots of virus particles from PI cells (not shown) with the same polyclonal anti-echovirus 6 serum which immunoprecipitated lytic virus polypeptides (Fig. 3) (6).

Identification of viral structural polypeptides. In picornaviruses, VP0 is cleaved into VP2 and VP4 during the maturation of the virus particle (7). Our observation that reduction in the concentration of the largest polypeptide (31.5 kDa) was accompanied by increased concentrations of the 21.2- and 9.5-kDa proteins (Fig. 3, lane A, versus Fig. 4, lane A) suggested a precursor relationship between the largest protein and the two smaller polypeptides. Thus, the possibility was examined that the original designation of the polypeptides was incorrect. Fractions recovered from linear gradients after equilibrium centrifugation were assayed for infectivity and electrophoresed for presence of viral polypeptides (Fig. 5). Fraction 5A (also shown in Fig. 3A after a shorter exposure time) and fraction B of lytic virus had high infectivities (4.5×10^9 and 2.2×10^9 PFU/ml, respectively) and approximate densities for virions (1.34 and 1.32 g/ml, respectively). The five polypeptides were confirmed as viral proteins by immunoprecipitation with polyclonal anti-virus serum (Fig. 3B). Fraction C, recovered at a density of 1.29 g/ml, had low infectivity (1.0×10^7 PFU/ml), and the two smaller capsid proteins were not detectable. These results indicated that fraction C consisted primarily of natural empty capsids (natural top component). Since VP0 in natural top component does not undergo cleavage (17), our results suggested that the two missing polypeptides were VP2 and VP4.

Confirmation of the precursor relationship between the largest protein, VP0, and the two smaller polypeptides was obtained by immunoblots (Fig. 6). Purified lytic virus particles obtained from linear sucrose gradients were dissociated with urea, and the polypeptides were separated by SDS-polyacrylamide electrophoresis. Western (immuno-) blots of the polypeptides were reacted with both polyclonal anti-echovirus 6 antiserum and echovirus 6 antiserum that reacted with the 31.5-kDa polypeptide but not with the 27.0- or 25.8-kDa polypeptides. The immunocomplexes were visualized by fluorography. As demonstrated in Fig. 6A, the five polypeptides were detected by polyclonal anti-virus serum, whereas only the largest and two smallest polypeptides

FIG. 5. Fluorograph of echovirus 6 fractions recovered from a linear CsCl gradient. Wild-type echovirus 6 was centrifuged to equilibrium in an SW50.1 rotor ($100,000 \times g$, 48 h, 5°C). The density and infectivity of each fraction were determined. Fractions A and B contained lytic virus (4.5×10^9 and 2×10^9 PFU/ml, respectively) at densities of 1.34 and 1.32 g/ml, respectively. Fraction C contained 10^7 PFU/ml at a density of 1.29 g/ml. Molecular masses (in kilodaltons) of the viral polypeptides were estimated from the mobilities of prestained marker proteins resolved on the same gel slab after electrophoresis in SDS-15% polyacrylamide.

interacted with the other antiserum (Fig. 6B). The slight discrepancy in mobility of the viral polypeptides was due to the use of different gels for the two immunoblots. Thus, the two smaller polypeptides are cleavage products of VP0. The fraction (1.33 g/ml) containing virus particles from PI cells also lacked the two smaller viral polypeptides (Fig. 4), suggesting that cleavage of VP0 did not occur in virus particles produced by PI cells. Further support for this conclusion was obtained by using partial proteolysis in combination with two-dimensional SDS-polyacrylamide gel electrophoresis (not shown). Hydrolysis products of the 31.5-kDa polypeptide included peptides that migrated to the same position as peptides contained in the lanes of the two smallest polypeptides. These peptides were not detected in the lanes containing hydrolysates of the 27- and 25.8-kDa polypeptides.

Presence of viral structural proteins in acutely infected WISH cells and PI cells. Previous immunofluorescent studies demonstrated the presence of viral proteins in PI cells (6). In order to detect the individual viral polypeptides, protein profiles of uninfected, acutely infected, and PI cells were examined after cells were labeled with [^{35}S]methionine for either 5 or 7 h (Fig. 7, fluorographs 1 and 2, respectively). Freshly passaged WISH and PI cells were grown to confluence in 2 days. One of each set of WISH cell cultures was inoculated with echovirus 6 at multiplicities of infection of 53 and 14 PFU per cell (Fig. 7, fluorographs 1A and 2B, respectively). The other sets of WISH cells and the PI cells were mock infected with phosphate-buffered saline. [^{35}S]methionine was added at 3.75 h after inoculation. The cells were washed and chased for 5 min with cold medium before harvest. All of the structural viral polypeptides (VP1

FIG. 6. Identification of VP0 and its cleavage products, VP2 and VP4. Purified echovirus 6 was recovered from linear sucrose gradients, and the capsid polypeptides were resolved by electrophoresis in two different SDS-12.5% polyacrylamide gels and transferred to nitrocellulose. Western blots were treated with polyclonal anti-echovirus 6 serum (A) and antiserum directed against VP0 (B). The viral polypeptide-antibody complexes were detected by attachment of ^{125}I -labeled purified protein A. The corrected designations of the viral structural proteins are indicated. The same virus preparation was used for each immunoblot. Molecular weights were estimated from the positions of prestained molecular weight marker proteins that were resolved in each gel slab.

to VP4) as well as VP0 were detected in acutely infected cells at approximately equal intensities at 7 and 9 h after infection (Fig. 7, fluorographs 1A and 2B). The concentrations of these polypeptides were not altered by the presence or absence of phenylmethylsulfonyl fluoride (0.5 mM) during the harvesting of the cells. The viral proteins were not readily visible in the PI cells that were labeled for 3 h even after extended exposure time of the fluorograph from 2 to 22.5 h (Fig. 7, fluorographs 1C and 1D, respectively). It was necessary to increase the labeling time to 5 h to detect viral polypeptides in PI cells (Fig. 7, fluorograph 2C). Only the three larger viral polypeptides were detected in PI cells. Confirmation of the viral origin of these proteins was obtained by immunoprecipitation (Fig. 8). Cellular extracts of PI cells were prepared after a prolonged labeling period (54 h) in order to demonstrate viral proteins in the presence of other proteins in an extract of PI cells. A sample of the extract was immunoprecipitated with polyclonal mouse anti-echovirus 6 serum (Fig. 8, lane B). The same antiserum that reacted with polypeptides of lytic virus in immunoprecipitates (Fig. 6) was used for this experiment, and it did not bind well to VP0 of either lytic or nonlytic virus particles, but it did react well in immunoblots with VP2 and VP4.

Effect of echovirus 6 infection on cellular protein synthesis. Significant turnoff of protein synthesis in PI cells did not

FIG. 7. Fluorographs of cellular protein profiles. (Panel 1) Lytically infected WISH cells (53 PFU per cell) (lane A), uninfected WISH cells (lane B), and PI cells (lanes C and D). (Panel 2) Uninfected WISH cells (lane A), lytically infected WISH cells (14 PFU per cell) (lane B) and PI cells (lane C). Monolayers were labeled with ^{35}S methionine at 3.75 h after inoculation with either virus or phosphate-buffered saline. Cellular proteins from each culture were harvested into sample buffer at 7 (panel 1) and 9 h (panel 2) after infection and submitted to electrophoresis in SDS-12.5% polyacrylamide slab gels. Fluorographs of PI cells (panel 1) were exposed for 2 (lane C) and 22.5 h (lane D). The viral structural proteins are indicated by small arrows. Molecular masses (in kilodaltons) were determined from the migration positions of prestained marker proteins in the same gel slab and are indicated on the left.

occur (Fig. 7, fluorographs 1C, 1D, and 2C). Comparison of the protein profiles of uninfected and acutely infected cells (Fig. 7, fluorographs 1B, 1A, 2A, and 2B) suggested that a decrease in cellular proteins accompanied the appearance of viral proteins. In order to examine this possibility, WISH cells were pulse-labeled for 10 min with ^{35}S methionine at 0, 1.5, 3, and 6 h after infection with echovirus 6 (3 PFU per cell) and chased with cold medium for 5 min before harvest. The fluorograph in Fig. 9 demonstrates that turnoff of cellular protein synthesis did progress with time during lytic infection. However, shutdown of cellular proteins was incomplete at 6 h, when mature virions can be recovered from cells (15).

DISCUSSION

We have developed a persistently infected cell line (PI cells) which contains RNA of echovirus 6. Genome-sized viral RNA can be recovered from cytoplasmic extracts of PI cells as well as from particles recovered from PI cells (Fig. 1 and 2). Transfection of uninfected, parental WISH cells by the RNA from purified particles did not lead to a turnoff of cellular protein synthesis, appearance of cytopathology, or production of lytic virus particles. These results suggest that the viral RNA in PI cells has undergone one or more alterations which allow it to persist in the absence of cellular destruction, to confer cellular resistance to superinfection by

FIG. 8. Fluorograph of proteins in extracts from PI cells. Cell monolayers were labeled with [35 S]methionine for 54 h, and cellular proteins, extracted in the presence of phenylmethylsulfonyl fluoride, were separated by electrophoresis in SDS-12.5% polyacrylamide gels. Lane A, Total radiolabeled cellular proteins in extract; lane B, profile of the same cellular preparation after immunoprecipitation with the anti-echovirus 6 polyclonal serum used in Fig. 6. Viral polypeptides are indicated on the right. Molecular masses (in kilodaltons [Kd]) on the left were determined from the migration of prestained marker proteins in the same gel slab.

wild-type virus, and to produce defective virus particles. It is possible that the RNA that is incorporated into particles differs from the viral RNA retained in PI cells. It would be interesting to determine whether the unincorporated intracellular viral RNA is infectious. This possibility remains to be investigated and is dependent upon the isolation of the relatively small amount of this viral RNA (approximately 0.01%) from extracts of cellular RNA.

The viral capsid proteins of echovirus 6 were originally designated on the basis of their migration in SDS-polyacrylamide gels (6). The present study indicates that this designation was incorrect. Natural empty capsids (17) contain VP1, VP3, and VP0, whereas complete virions contain VP1, VP3, and the cleavage products of VP0 (VP2 and VP4). The absence of the two smaller polypeptides in natural empty capsids of echovirus 6 (Fig. 5C) indicated that these proteins are VP2 and VP4. Immunoblots and partial hydrolysis were used to identify VP0. As shown in Fig. 6B, neutralizing antiserum that reacted with the 31.5-kDa polypeptide of echovirus 6 reacted with Western blots of the largest and two smallest polypeptides. Additional support for the identity of the 31.5-kDa polypeptide as VP0 was obtained by the use of partial hydrolysis and two-dimensional electrophoresis of the polypeptides of virions. The observation that VP2 migrates ahead of VP3 during electrophoresis in SDS-polyacrylamide gels is not unique for echovirus 6, since a similar observation was reported for echovirus 9 (16). The identities of VP1 and VP3 remain to be confirmed by determination of the gene order of the 27- and 25.8-kDa polypeptides, and they are now arbitrarily designated in order of mobility.

Previously, we reported that the virus particles from PI cells lacked VP3 (6). VP4 was assumed to be present, since

FIG. 9. Effect of lytic echovirus 6 infection on cellular protein profiles. Cellular monolayers were labeled with [35 S]methionine at 0, 1.5, 3, and 6 h after virus inoculation (indicated below the lanes) (3 PFU per cell). Cells were harvested into sample buffer and electrophoresed in an SDS-15% polyacrylamide gel slab. Viral proteins in the fluorograph are indicated on the right.

it migrated with the dye front in most gels because of its small size (9.5 kDa). In this report, we demonstrate that the virus particles from PI cells actually lack VP2 and VP4. Our inability to detect VP2 and VP4 in extracts of PI cells in the presence of an inhibitor of proteolysis (Fig. 9) as well as in particles produced by PI cells (Fig. 4) suggests that the lack of these polypeptides is not due to instability of the cleaved products but rather to a defect in the maturation process (7). This defect coincides with the inability of particles from PI cells to attach to uninfected, parental WISH cells. In this respect, the defective particles are similar to immature particles (provirions), which contain all the necessary viral components without the final processing of VP0 into VP2 and VP4 and do not attach to susceptible cells (5). The particles from PI cells differ from provirions because they do not contain RNA that produces lytic infection. They also differ from defective interfering poliovirus particles (12), since they do not prevent the infectivity of lytic virus (6).

VP0 was detected in lytic as well as nonlytic virus preparations. The relatively high concentration of VP0 in purified virus preparations and immunoprecipitates of lytic virus (Fig. 3 and 5) probably represents the presence of immature virus particles. The cells were not in synchrony at the time of inoculation (0.1 PFU per cell), and maturation (i.e., cleavage of VP0 to VP2 and VP4) was most likely incomplete at the time of harvest for virus after several growth cycles. In other preparations obtained after a high multiplicity of infection (5

to 20 PFU per cell) and harvested after one growth cycle at 8 to 9 h after inoculation (Fig. 4A), VP0 was present in relatively low concentrations compared with the concentrations of the other capsid proteins.

Persistence of the viral genome does not appear to be dependent upon the production of defective particles by PI cells. The yield of particles from PI cells (6) during nonlytic infection is high and similar to the yield of virions from lytic infection ($\geq 1,500$ particles per cell). Yet the defective particles cannot attach and thus are unable to impart their genome into other cells via receptor-mediated endocytosis. Since essentially all of the PI cells carry viral RNAs which are synthesized continuously (6) (Fig. 1), the viral RNA is probably transferred to daughter cells during division. Thus, the particles produced by PI cells may be nonfunctional by-products of the persistent infection.

The mechanism(s) for retention and transfer of viral RNA remain to be determined. Synthesis of cellular proteins was not inhibited substantially during the steady-state infection by echovirus 6 (Fig. 8). Also, incomplete turnoff of protein synthesis occurs during lytic infection (Fig. 8 and 9). It is likely that coevolution of viruses and cells occurs, as has been reported for persistent infections by other picornaviruses (3, 4, 9), and that cellular alterations contribute to the maintenance of the viral genome and the resistance of PI cells to superinfection by echovirus 6. Our steady-state infection provides a model for the study of the cellular and viral changes that may occur during chronic infections.

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