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Temperature-Sensitive Mouse Cell Factors for Strand-Specific Initiation of Poliovirus RNA Synthesis

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Two cell lines, TgSVA and TgSVB, were established from the kidneys of transgenic mice carrying the human gene encoding poliovirus receptor. The cells were highly susceptible to poliovirus infection, and a large amount of infectious particles was produced in the infected cells at 37°C. However, the virus yield was greatly reduced at 40°C. This phenomenon was common to all mouse cells tested. To identify the temperature-sensitive step(s) of the virus infection cycle, different steps of the infection cycle were examined for temperature sensitivity. The results strongly suggested that the growth restriction observed at 40°C was due to reduced efficiency of the initiation process of virus-specific RNA synthesis. Furthermore, this restriction appeared to occur only on the synthesis of positive-strand RNA. Virus-specific RNA synthesis in crude replication complexes was not affected by the nonpermissive temperature of 40°C. In vitro uridylylation of VPg seemed to be temperature sensitive only after prolonged incubation at 40°C. These results indicate that a specific host factor(s) is involved in the efficient initiation process of positive-strand RNA synthesis of poliovirus and that the host factor(s) is temperature sensitive in TgSVA and TgSVB cells.

The genome of poliovirus is a single-stranded, positive-sense RNA composed of approximately 7,500 nucleotides to which are attached a small protein VPg at the 5′ end (4, 11, 20, 24) and poly(A) at the 3′ end (40). This RNA functions as mRNA to produce a single large precursor polyprotein of 247 kDa after entry into the host cell cytoplasm. The polyprotein is cotranslationally cleaved by proteases to form viral capsid proteins (VP1, VP2, VP3, and VP4) and noncapsid proteins (2A, 2B, 2C, 3A, 3B, 3C, and 3D). Of the viral noncapsid proteins, 2A, 3C, and 3CD are viral proteases involved in the processing of poliovirus polyprotein (19), 3B is VPg (20, 24, 28, 29, 39), 3D is viral RNA-dependent RNA polymerase (26, 28, 39), 2B and 2C are considered to play important roles in viral RNA synthesis (3, 5, 21, 25, 28, 29, 39), and 3A could have an important role(s) in uridylylation of VPg in the initiation process of RNA synthesis (12, 28, 35). Thus, of biologically significant knowledge of many viral proteins has been accumulated.

In addition to viral proteins, poliovirus requires host cell factors for its replication cycle. Poliovirus strains, except for type 2 virulent strains, infect only primates. Recent success in molecular cloning of genomic and complementary DNAs encoding human poliovirus receptor (PVR) allowed us to render nonprimate cells permissiveness for poliovirus infection by introducing the human DNAs into the cells. Indeed, mouse L cells transformed with the PVR DNAs are very susceptible to poliovirus (17, 22). Furthermore, transgenic mice carrying the human PVR gene were found to be susceptible to poliovirus (18, 27). These observations indicate that any kind of cells can be permissive for viral adsorption and penetration by introduction of PVR DNAs. This, in turn, makes it possible to find cells of nonprimate origin that have defects in host cell factors other than PVR supporting poliovirus infection.

Poliovirus RNA replication occurs on membranes induced by virus infection (5, 6, 8, 9, 35, 36). Indeed, the membrane fraction, called the crude replication complex (CRC), isolated from poliovirus-infected cells can synthesize virus-specific RNA (34, 35). This in vitro reaction represents mostly chain elongation of the positive-strand RNA of poliovirus (10). Takegami et al. (35), however, demonstrated that the CRC is able to synthesize nucleotidyl proteins, VPg-pU and VPg-pUpU, that are the 5′-terminal structures of all virus-specific RNA molecules except for the viral mRNA (24). This nucleotidyl protein was shown to be incorporated into VPg-pUUAAAACAGp, an RNase T_1-resistant oligonucleotide derived from the 5′ end of the poliovirus genome (11, 24, 34). Although neither the uridylylation of VPg nor the subsequent chain initiation of the RNA is efficient, these results suggest that VPg-pU(pU) is utilized as a primer for poliovirus RNA synthesis.

The amount of positive-strand RNA synthesized in infected cells is much larger than that of negative-strand RNA. This fact suggests that there is a difference(s) in initiation mechanisms for positive- and negative-strand RNA syntheses if only the viral RNA-dependent RNA polymerase ($3D^{pol}$) is involved in chain elongation of both strands. In fact, a template-priming mechanism has been proposed for synthesis of a negative-strand RNA molecule by using a soluble, membrane-free system including $3D^{pol}$ and a host cell factor (2, 37). According to the model, VPg is not involved in the initiation step of negative-strand RNA synthesis (37).

Here we report that poliovirus replication is generally temperature sensitive in mouse cells. Using cell lines (TgSVA and TgSVB) established from transgenic mice susceptible to poliovirus (18), we show that the temperature-sensitive step resides in the initiation process of positive-

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strand RNA synthesis of poliovirus, indicating that a host cell factor(s) is involved in some step(s) of the initiation process.

MATERIALS AND METHODS

Cells and viruses. HeLa S3 cell monolayers and African green monkey kidney cells were maintained in Dulbecco modified Eagle medium (DME) supplemented with 5% newborn calf serum (NCS) and used for virus preparation and plaque assay. Suspension-cultured HeLa S3 cells were maintained in RPMI medium supplemented with 5% NCS. Lα cells are L cells expressing cDNA encoding human PVRα and maintained in DME supplemented with 10% fetal calf serum (FCS) (17). TgSVA and TgSVB cells were established from kidney tissues of transgenic mice (ICR-PVRTg1) carrying the human gene for PVR (18), taking advantage of immortalization with simian virus 40 (SV40) T antigen as described in Results, and maintained in DME supplemented with 5% FCS. Poliovirus type 1 Mahoney strain was propagated in suspension-cultured HeLa S3 cells as previously described (15). SV40 (strain 777), herpes simplex virus type 1 (strain Mt), and Sindbis virus were also used.

Virus infection and growth. To examine virus growth, cells in 6- or 12-well plastic culture plate were infected with light-sensitive poliovirus prepared as previously described (17) at a multiplicity of infection (MOI) of 20. After incubation for 1 h at indicated temperatures, the cell cultures were exposed to light for 1 h in order to inactivate residual virus. Virus yields at 24 h postinfection were measured by virus titration on African green monkey kidney cells.

Analysis of proteins by PAGE. HeLa S3, TgSVA, and TgSVB cells in 12-well plates were infected with poliovirus at an MOI of 50. The cells were incubated in methionine-free DME at 37 and 40°C and labeled with 2 μCi of [^{35}S]methionine (1.45 Ci/mmol; Amersham) per ml for 30 min prior to harvest. At intervals of 1 h, the radiolabeled cell monolayers were collected and dissolved in sample buffer (62.5 mM Tris-HCl [pH 6.8], 2.3% sodium dodecyl sulfate [SDS], 100 mM dithiothreitol, 10% glycerol, with 0.024% bromophenol blue as an indicator). Samples were heated at 100°C for 5 min, applied to a 12.5% polyacrylamide gel (SDS-polyacrylamide gel electrophoresis [PAGE] plate 12.5; Daiichi Pure Chemicals Co., Ltd.), and run at 60 mA. After treatment with Amplify (Amersham), the gels were dried and exposed to Fuji X-ray films.

Labeling of infected cells with [^{3}H]uridine. Cells in 24-well plates were infected with poliovirus at an MOI of 20. After a 30-min adsorption period, DME supplemented with 5% NCS or FCS was added, and the cells were incubated at 37 and 40°C. At 2 and 2.5 h postinfection, actinomycin D (5 μg/ml, final concentration) and [^{3}H]uridine (1 μCi/ml, final concentration), respectively, were added. At indicated times, the medium was discarded and the cells were collected in cold phosphate-buffered saline and treated with trichloroacetic acid (TCA). TCA-insoluble radioactivities were measured in a universal scintillater (DOTITE; Scintisol EX-H) by a scintillation counter.

RNase protection assay. An RNase protection assay was performed as described by Kato et al. (16), with the modification described below. RNAs were prepared from HeLa S3, TgSVA, and TgSVB cells (4 × 10^7 each) that had been infected with poliovirus at an MOI of 20 and incubated for 5 h. The sense [^{32}P]RNA probe was synthesized in the presence of [α-^{32}P]CTP with T7 polymerase (Promega) from *Spe*I-linearized pBL-PVKB. The antisense [^{32}P]RNA probe was synthesized in the presence of [α-^{32}P]CTP with T3 polymerase (Promega) from *Bgl*I-linearized pBL-PVKB (see Fig. 5C). To reduce the specific activities of RNA probes, the reaction was performed with 12.5 μCi of [α-^{32}P]CTP per mmol. Plasmid pBL-PVKB is a derivative of plasmid vector pBluescript SK that has an insert of poliovirus cDNA corresponding to the genome region from nucleotides 3665 (*Kpn*I site) to 5600 (*Bgl*II site) at *Kpn*I and *Bam*HI sites of the vector (see Fig. 5C). The cytoplasmic RNAs (1 μg), after heating at 100°C for 5 min, were hybridized with excess amounts of the [^{32}P]RNA probes (5 × 10^4 cpm [Cerenkov]) at 37°C for 3 h in 20 μl of 40 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES; pH 6.7)–1 mM EDTA–0.4 M NaCl–80% formamide. The samples were then diluted with 15 volumes (300 μl) of an ice-cold buffer containing 10 mM Tris-HCl (pH 7.4), 5 mM EDTA, 300 mM NaCl, 6 μg of RNase A per ml, and 0.3 μg of RNase T_1 per ml and incubated for 30 min at 37°C. The reaction mixtures were further incubated after addition of 0.5% SDS and 1 mg of proteinase K per ml. The products, after extractions with phenol-chloroform (1:1) and chloroform, were recovered by ethanol precipitation and analyzed by electrophoresis on 5% polyacrylamide gels in the presence of 8 M urea.

Uridylylation of VPg and RNA synthesis in vitro. CRC was prepared from poliovirus-infected HeLa S3, TgSVA, or TgSVB cells as described by Takeda et al. (34), with the modifications described below. Cells (8 × 10^8) infected with poliovirus were incubated at 37°C and harvested at 5 h postinfection. Each CRC was suspended in 1.2 ml of buffer (10 mM Tris-HCl [pH 8.0], 10 mM NaCl, 15% glycerol, 1 mM dithiothreitol, 1% Trasylol [aprotinin, Bayer; 50,000 U/5 ml]), divided into 20-μl portions, and stored at −70°C.

In vitro RNA syntheses were carried out by incubating 50 μl of the standard reaction mixture containing 20 μl of CRC (10). At indicated times, 0.2 μCi of [α-^{32}P]UTP was added to each in vitro reaction mixture. After indicated periods of incubation at either 36 or 40°C, the reactions were stopped by addition of 10% TCA. TCA-insoluble counts were determined in a scintillation counter.

In vitro formation of VPg-pU(pU) was carried out in 50 μl of reaction mixture containing 20 μl of CRC as described by Takeda et al. (34). At indicated times, 10 μCi of [α-^{32}P]UTP was added to the in vitro reaction mixtures. After incubation at 37 or 40°C, the reactions were stopped by addition of an equal volume of the sample buffer. The samples were heated for 5 min and diluted by the addition of 0.5 volume of H_2O and 5 volumes of radioimmunoprecipitation assay buffer. The VPg-pU(pU) formed was reacted against a complex of protein A-Sepharose and anti-VPg antibody (C1 and N10) (30). The immunoprecipitates were then subjected to electrophoresis on an SDS–12.5% polyacrylamide minigel (Daiichi Pure Chemicals), using a Laemmli gel buffer system.

RESULTS

Establishment of cell lines susceptible to poliovirus. To establish mouse cell lines susceptible to poliovirus infection, kidneys, spleens, brains, bone marrow, and whole embryos of transgenic mice (ICR-PVRTg1) expressing the human gene for PVR (17) were minced, dispersed by trypsinization, and cultured. A portion of these cultured cells was infected with SV40 or transfected with adenovirus E1A DNA (32). The cells expressing SV40 T antigen or E1A protein were tested for sensitivity to poliovirus infection. Although many cell lines were susceptible to infection, most sensitive were those expressing the SV40 T antigen derived from the kidney

TABLE 1. Sensitivity to poliovirus infection and production of poliovirus

Cells	Virus titer required for cytopathic effect ($\log_{10}$ PFU)		Virus yield ($\log_{10}$ PFU/ml)	
	37°C	40°C	37°C	40°C
Mouse				
TgSVA	1.0[a]	5.0	8.0[a]	5.0
TgSVB	1.0	4.5	8.5	6.0
TgN-5	3.0	5.5		
Tg-emb-7	2.5	5.5		
Lα	2.5	5.0		
Human				
HeLa	1.5	1.5	8.5	8.0
TIG-7	1.5	1.0		

[a] Measured in HeLa S3 monolayer cells.

(18). Among them, two clones were selected and named TgSVA and TgSVB.

Temperature-sensitive growth of poliovirus in mouse cells. In TgSVA and TgSVB cells, the plaque assay of poliovirus was carried out at either 37 or 40°C. Plaques were efficiently formed in both cell lines at 37°C but not at 40°C, whereas plaque numbers on HeLa cells at 40°C were the same as those at 37°C. A similar observation was obtained when the cytopathic effect of poliovirus was investigated at 37 or 40°C (Table 1). Virus yields in TgSVA and TgSVB cells in a single cycle of infection were examined at 37 and 40°C (Table 1). The amounts of infectious particles produced in these cells at 40°C were reduced to 1/1,000 (TgSVA cells) or 1/300 (TgSVB cells) of those at 37°C. The results indicate that the inefficient plaque formation observed at 40°C is a result of reduced efficiency in some replication step(s) of poliovirus.

To examine whether the inefficient plaque formation at 40°C is common to all mouse cells, Lα (L fibroblast cells, derived from mouse strain C3H/He, that express PVRα) (17), Tg-emb-7 (secondary cultures from an embryo of a transgenic mouse), and TgN-5 (a cell line established without the immortalization process from the kidney of a transgenic mouse) were infected with poliovirus and incubated at either 37 or 40°C (Table 1). These mouse cells also were temperature sensitive for the development of plaques and cytopathic effects induced by poliovirus. These results strongly suggest that the restricted growth of poliovirus in TgSVA and TgSVB cells at 40°C is not due to expression of SV40 T antigen, specific tissues, or specific strains of mice. As to human cells, TIG-7 cells (human fibroblasts), in addition to HeLa cells, were susceptible to poliovirus infection at both 37 and 40°C (Table 1). Thus, temperature-sensitive replication of poliovirus seems to be common to all mouse cells, suggesting that a cellular factor(s) for poliovirus replication is temperature sensitive in murine cells.

Herpes simplex virus (strain Mt) and Sindbis virus grew well in TgSVA and TgSVB cells at both 37 and 40°C (data not shown). Therefore, temperature-sensitive host factors are not utilized in replication of these viruses.

Temperature-sensitive step of poliovirus replication. To identify the temperature-sensitive steps in the poliovirus infection cycle in TgSVA and TgSVB cells, temperature shift experiments were carried out on the infected cells (Fig. 1). Virus yields were determined in HeLa, TgSVA, and TgSVB cells which had been shifted from the permissive temperature of 37°C to the nonpermissive temperature of 40°C or shifted from 40 to 37°C at intervals of 1 h during an infection cycle. As shown in Fig. 1A, the temperature shiftdown carried out up to 5 h postinfection appeared not to affect the virus yield. A longer incubation at 40°C (6 h or longer postinfection), however, resulted in a reduction of virus yield. This finding may indicate that the nonpermissive temperature leads to abortive infection of poliovirus by 6 h postinfection. When the temperature was shifted from 37 to 40°C up to 3 h postinfection, the virus yield was reduced (Fig. 1B). However, the temperature shiftup at 4 h (or later) postinfection provided the normal levels of virus yield. These results indicate that the temperature-sensitive step of poliovirus replication is at some point between 3 and 4 h postinfection in TgSVA and TgSVB cells.

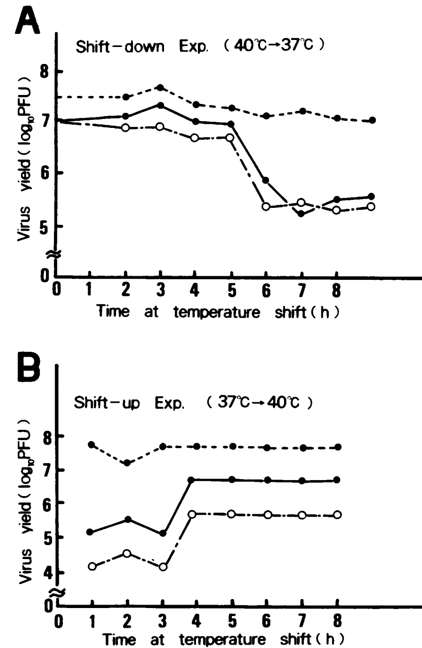

FIG. 1. Growth of poliovirus at 37 and 40°C. HeLa (●----●), TgSVA (●——●), and TgSVB (○) cells in 12-well plates were infected with light-sensitive poliovirus Mahoney strain at an MOI of 20 and cultured at 40 or 37°C as described in Materials and Methods. At 1-h intervals postinfection, portions of cells were shifted from 40 to 37° (A) or 37 to 40°C (B), and all cultures were frozen at 24 h after infection. Virus yields were measured by plaque assay and plotted at the indicated times of temperature shifts.

Adsorption and penetration of poliovirus. Poliovirus adsorption and penetration in TgSVA and TgSVB cells at 40°C were further examined by using light-sensitive poliovirus. TgSVA and TgSVB cells were incubated with light-sensitive poliovirus at 37 or 40°C in the dark as described in Materials and Methods. These cultures were exposed to light 1 h postinfection and further incubated at 37 or 40°C. The virus titers obtained at 24 h postinfection in cultures of HeLa and TgSVA cells are shown in Table 2. Incubation at 40°C for the first 1 h did not affect virus yields in either poliovirus-infected HeLa or TgSVA cells. Further incubation at 40°C caused a reduction of the virus yield only in TgSVA cells, not in HeLa cells (Table 2 and Fig. 1). A similar reduction of virus yield was obtained in the case of TgSVB cells (data not shown and Fig. 1). These results indicate that human poliovirus receptors on TgSVA and TgSVB cells are functional at the nonpermissive temperature of 40°C and that very early steps of virus infection, including adsorption and penetration

TABLE 2. Virus titers obtained at 24 h postinfection

Temp (°C)		Virus titer at 24 h postinfection	
0–1 h postinfection	1–24 h postinfection	HeLa	TgSVA
37		1.2×10^2	1.2×10^2
37	37	1.8×10^6	3.6×10^5
40	37	3.9×10^6	2.3×10^6
40	40	2.1×10^6	7.5×10^3

of virus, are not temperature sensitive in TgSVA and TgSVB cells.

Translation of poliovirus RNA in TgSVA and TgSVB cells. Translation of infected virion RNA was analyzed. A sensitive assay used for poliovirus proteins translated early in the infection cycle is to monitor the inhibition of host protein synthesis (31, 33). HeLa and TgSVA cells were infected with poliovirus at an MOI of 50 and incubated at 37 and 40°C in the absence or presence of 1 mM guanidine hydrochloride (Gua-HCl), an inhibitor for the initiation of virus-specific RNA synthesis (7). At 1-h intervals, a portion of the cells was pulse-labeled with [^{35}S]methionine for 30 min prior to harvest in order to examine the relative amount of protein synthesis. Poliovirus infection at either 37 or 40°C resulted in the accumulation of labeled poliovirus proteins in HeLa cells and the inhibition of host cell protein synthesis (Fig. 2A and B, lanes 1 to 5). When poliovirus-infected cells were incubated in the presence of Gua-HCl, the virus proteins were not detected by autoradiography because virus-specific RNA synthesis was blocked by the inhibitor (Fig. 2A and B, lanes 6 to 10). However, a gradual shutoff of host cell protein synthesis was observed, since a small amount of viral products was synthesized from the introduced virion RNA (8).

In TgSVA cells incubated at 37°C, synthesis of poliovirus proteins as well as shutoff of host cell protein synthesis were observed as in HeLa cells (Fig. 2C, lanes 1 to 5). However, the synthesis of poliovirus proteins was suppressed at 40°C (Fig. 2D, lanes 1 to 5), and the pattern of labeled proteins was nearly identical to that observed in Gua-HCl-treated cells incubated at either 37 or 40°C (Fig. 2C and D, lanes 6 to 10). The data indicated that the input poliovirus RNA was translated in TgSVA cells at 40°C, resulting in a gradual shutoff of host protein synthesis as in cells treated with

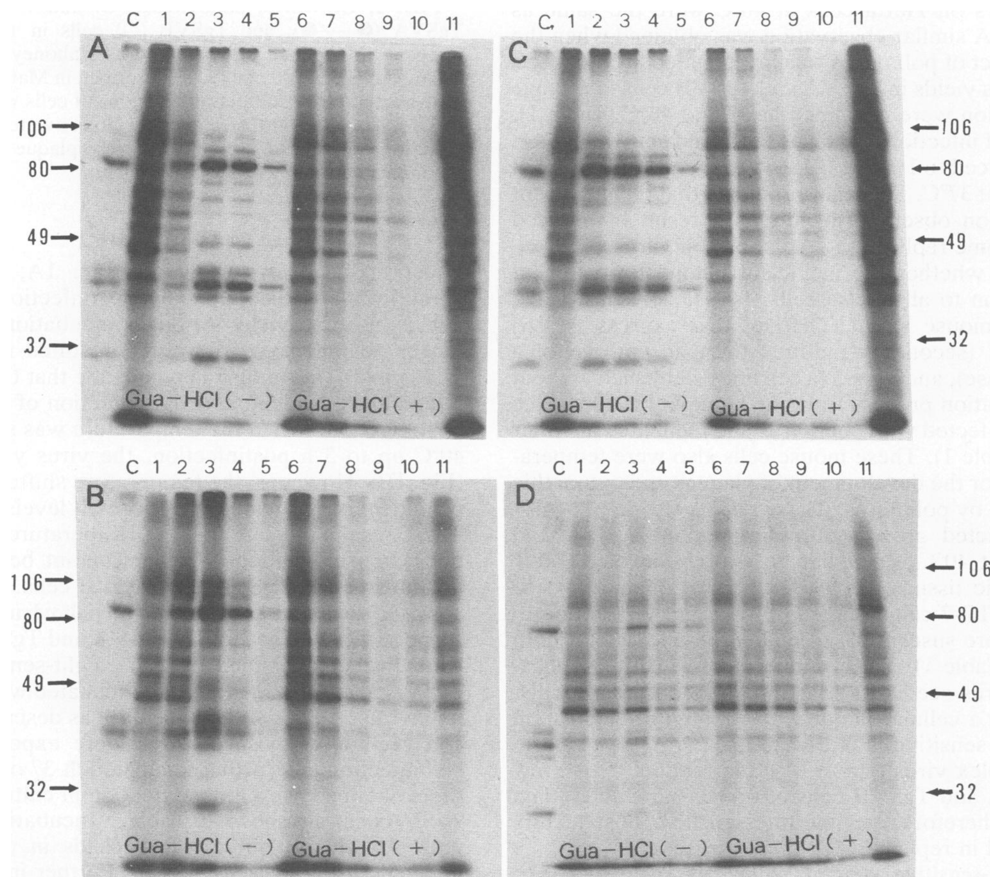

FIG. 2. Protein synthesis in HeLa and TgSVA cells infected with poliovirus. HeLa (A and B) and TgSVA (C and D) cells in 12-well plates were infected with poliovirus at an MOI of 50 and grown at 37°C (A and C) or 40°C (B and D) in the presence (lanes 6 to 10) or absence (lanes 1 to 5) of 1 mM Gua-HCl. The cells were labeled with [^{35}S]methionine by a 30-min pulse prior to harvest. At 1 (lanes 1 and 6), 2 (lanes 2 and 7), 3 (lanes 3 and 8), 4 (lanes 4 and 9), and 5 (lanes 5 and 10) h after infection, extracts were prepared and analyzed as described in Materials and Methods. Mock-infected cells were labeled in 30-min pulse at 5 h after mock infection (lane 11). For lanes 11 in panels B and D, 1/5 volume of the extract from mock-infected cells was loaded onto the gel. The samples for lanes C are extracts from infected HeLa cells and are the same as that for lane 5 in panel A. Sizes (kilodaltons) of protein markers are indicated on both sides.

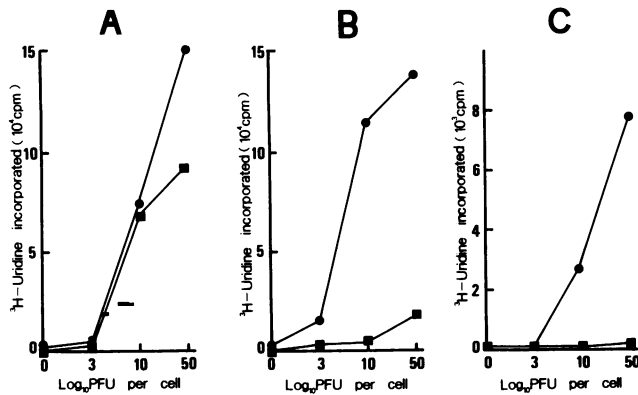

FIG. 3. Poliovirus RNA synthesis. HeLa (A), TgSVA (B), and TgSVB (C) cells in 12-well plates were infected with poliovirus at an MOI of 3, 10, or 50 and incubated at 37°C (closed circles) or 40°C (closed squares). At 2 and 2.5 h after infection, actinomycin D (final concentration of 5 μg/ml) and 0.1 μCi of [^{3}H]uridine, respectively, were added to the cultures. The cells were collected at 6 h after infection, and TCA-insoluble counts were measured by a scintillation counter.

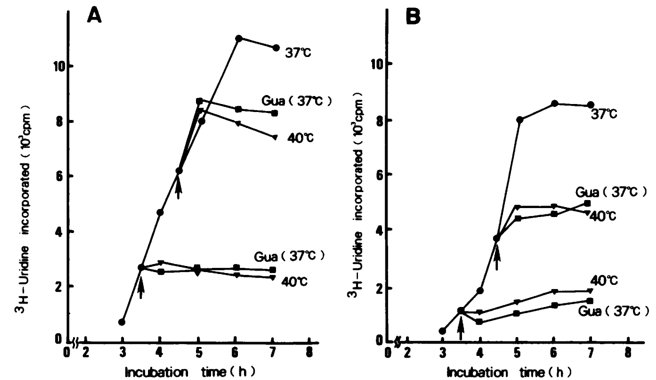

FIG. 4. Effects of incubation at 40°C and Gua-HCl on poliovirus RNA synthesis. TgSVA (A) and TgSVB (B) cells in 12-well plates were infected with poliovirus at an MOI of 20 and incubated at 37°C (closed circles). At 2 h and 2.5 h after infection, actinomycin D (final concentration of 5 μg/ml) and 0.1 μCi of [^{3}H]uridine, respectively, were added. At 3.5 and 4.5 h after infection, portions of cultures were shifted to 40°C (closed triangles) or supplemented with Gua-HCl (Gua; final concentration of 1 mM) (closed squares). At 3, 3.5, 4, 4.5, 5, 6, and 7 h postinfection, cells were collected, and TCA-insoluble counts were measured by a scintillation counter.

Gua-HCl. Thus, the temperature-sensitive steps of poliovirus replication in TgSVA cells appears not to reside in the virus-specific protein synthesis. A slight synthesis of poliovirus proteins observed in TgSVA cells at 40°C may be due to a high MOI of poliovirus (Fig. 2D, lanes 3 and 4).

RNA synthesis in TgSVA and TgSVB cells. To examine viral RNA synthesis at the nonpermissive temperature of 40°C, HeLa, TgSVA, and TgSVB cells were infected with poliovirus at MOIs of 3, 10, and 50. At 6 h after the infection, incorporation of [^{3}H]uridine in poliovirus RNAs was measured. Incorporation in TgSVA and TgSVB cells cultured at 40°C was markedly reduced compared with that at 37°C (Fig. 3B and C). However, the incorporation occurred at the same level in HeLa cells at both temperatures when the cells were infected with poliovirus at an MOI of 10 (Fig. 3A). When HeLa cells were infected with a high dose (MOI of 50) of virus, the cells were damaged, and many cells were detached from dishes at 6 h after the infection at 40°C, resulting in an apparent difference in radioactivity incorporated at 37 and 40°C. These results indicate that some process(es) of poliovirus RNA synthesis is defective in TgSVA and TgSVB cells at 40°C.

To examine whether the defect is in the initiation or in the elongation process of viral RNA synthesis, temperature shift experiments were carried out. TgSVA and TgSVB cells infected with poliovirus were incubated at the permissive temperature of 37°C. Portions of the infected TgSVA and TgSVB cells were then shifted to the nonpermissive temperature of 40°C at 3.5 and 4.5 h postinfection, while the remaining cells were kept at 37°C with or without 1 mM Gua-HCl. Incorporation of [^{3}H]uridine ceased immediately in cell cultures shifted to 40°C at 3.5 h, and the incorporation patterns were found to be similar to those in cell cultures treated with Gua-HCl at 37°C (Fig. 4). In TgSVA cells treated with Gua-HCl at 4.5 h postinfection, by which time a large number of viral replication complexes must be formed, normal incorporation of [^{3}H]uridine was observed temporally and ceased shortly thereafter (Fig. 4A). This delay in cessation of the incorporation may be due to continued elongation of viral RNA synthesis. A very similar incorporation pattern was observed in the TgSVB (Fig. 4B) cell cultures shifted to 40°C at 4.5 h postinfection. The incorporation patterns obtained after the temperature shiftup were always very similar to those after the addition of Gua-HCl. Thus, poliovirus RNA synthesis in TgSVA and TgSVB cells at 40°C may be blocked at a step(s) other than the chain elongation of RNA. These results may suggest that the step(s) detected in mouse cells has some relation to those affected by Gua-HCl, although the mechanism for the effect of Gua-HCl on the initiation of poliovirus RNA synthesis is not clear at present.

Quantitative RNase protection experiments were then carried out to determine the relative amounts of positive- and negative-strand RNAs present in the infected cells as described in Materials and Methods (Fig. 5). A large amount of positive-strand RNA was detected in both TgSVA and TgSVB cells incubated at 37°C (Fig. 5A, lanes 4 and 7), but the amount was greatly reduced at 40°C (Fig. 5A, lanes 5 and 8). On the other hand, similar levels of positive-strand RNA were detected in HeLa cells at 37 and 40°C (Fig. 5A, lanes 1 and 2), whereas the amount of negative-strand RNA appeared not to be reduced at 40°C in HeLa, TgSVA, and TgSVB cells (Fig. 5B). Slight differences in the intensities of radioactive bands are observed in Fig. 5B. This relative intensity pattern was not reproducible in repeated experiments. These results suggest that the positive-strand RNA synthesis of poliovirus was specifically blocked in TgSVA and TgSVB cells at the nonpermissive temperature.

In vitro RNA synthesis. We further examined RNA synthesis in vitro by using CRCs isolated from HeLa, TgSVA, and TgSVB cells infected with poliovirus at the permissive temperature of 37°C. [α-^{32}P]UTP radioactivity was linearly incorporated in the acid-insoluble fraction up to 15 min at both 36°C (Fig. 6A) and 40°C (Fig. 6B). Since poliovirus RNA synthesis in the CRC represents mostly the elongation reaction (10, 28), these results indicate that the elongation reaction of poliovirus RNA synthesis in vitro is not affected by the nonpermissive temperature. This result is compatible with the observations in the temperature shift experiments shown in Fig. 4.

Poliovirus RNA synthesis on membranes is considered to

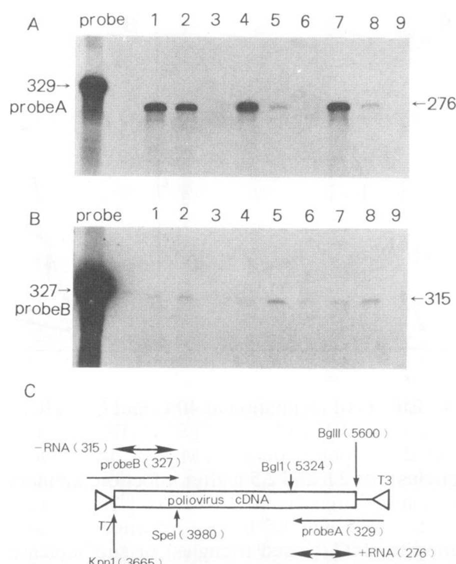

FIG. 5. (A and B) RNase protection analysis. HeLa (lanes 1 to 3), TgSVA (lanes 4 to 6), and TgSVB (lanes 7 to 9) cells were infected with poliovirus at an MOI of 20 and incubated at 37°C (lanes 1, 4, and 7) and 40°C (lanes 2, 5, and 8). At 5 h after infection, RNAs were extracted from cells and hybridized with ^{32}P-labeled RNA probes as described in Materials and Methods. RNAs extracted 1 h after infection (lanes 3, 6, and 9) were also examined. The RNase-resistant products were analyzed in 5% polyacrylamide gels in the presence of 8 M urea. Positive-strand (A) and negative-strand (B) RNAs were detected. Rabeled RNA probes (5×10^4 cpm [Cerenkov]) alone were used in the probe lanes. Exposure periods were 3.5 (A) and 14 (B) h. Sizes are indicated in nucleotides. (C) Structures of probes A and B.

be primed by a nucleotidyl protein, VPg-pU(pU), produced in the virus-specific replication complex that consists of a number of viral and cellular components (34, 35). Accordingly, relative amounts of the nucleotidyl protein produced in CRCs incubated at 36 and 40°C were compared, and the ratios of relative amounts of VPg-pU(pU) formed at 36°C to those formed at 40°C were then calculated (Fig. 7). Over successive experimental trials, formation of the nucleotidyl protein in the CRC prepared from TgSVA cells was consistently more effective at 36°C than at 40°C except for the first 30 min (Fig. 7B), while the CRC from HeLa cells was equivalent in synthesis of the nucleotidyl protein at both temperatures (Fig. 7A). Thus, the replication complex from mouse cells becomes inactive with respect to the formation of VPg-pU(pU) more rapidly at 40°C than at 36°C. The results suggest that the functional replication complexes already formed in the CRCs from mouse cells are active in uridylylation of VPg but the function is somehow less stable at 40°C than at 36°C in comparison with the CRC from HeLa cells.

DISCUSSION

We have shown that mouse cells are generally resistant to poliovirus infection at 40°C. A similar phenomenon was observed in our preliminary experiments involving Chinese hamster ovary cells. It may be possible that resistance to poliovirus infection at an elevated temperature is characteristic of rodent cells. It should be noted that these cells of mouse and Chinese hamster origins grow efficiently at 40°C.

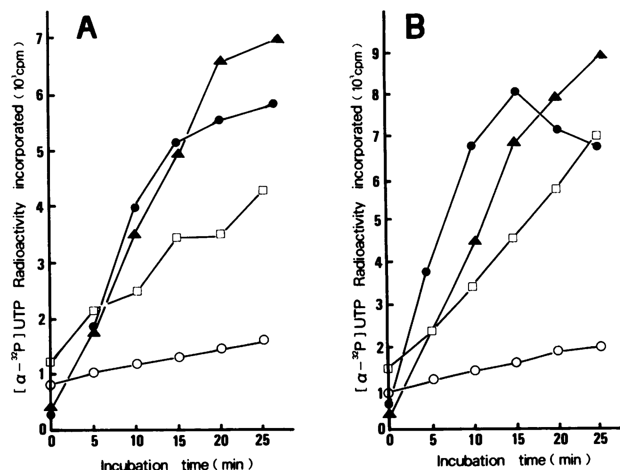

FIG. 6. Poliovirus RNA synthesis in vitro. HeLa (suspension culture) (closed circles), TgSVA (closed triangles), and TgSVB (open squares) cells were infected with poliovirus at an MOI of 50. Mock-infected HeLa suspension culture cells were also used (open circles). At 6 h after infection, CRCs were prepared from these cells as described in Materials and Methods. In vitro synthesis of poliovirus RNA was carried out at 36°C (A) or 40°C (B).

Thus, the nonpermissive temperature of 40°C may affect only the function of the host factor(s) in supporting replication.

Further analysis using cells from transgenic mice carrying the human PVR gene revealed that the defect resides only in the synthesis of virus-specific positive-strand RNA. Thus, the nonpermissive temperature may inactivate some cellular factor(s) involved in positive-strand RNA synthesis. Andino et al. (1) reported that the formation of a ribonucleoprotein complex at the 5′ end of poliovirus RNA includes a host cell factor in addition to viral proteins ($3C^{pro}$ and $3D^{pol}$) and that disruption of the formation of the ribonucleoprotein complex resulted in selective reduction of positive-strand RNA syn-

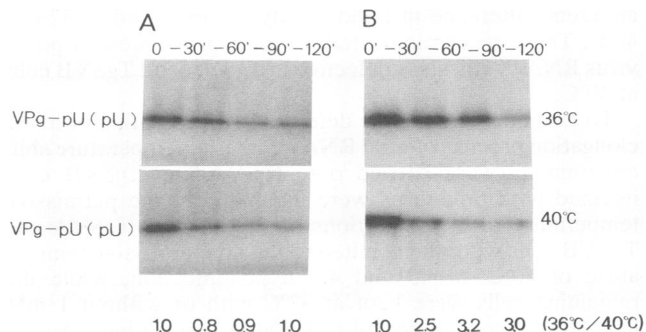

FIG. 7. In vitro uridylylation of VPg. CRCs were isolated from poliovirus-infected HeLa (A) and TgSVA (B) cells. Reaction mixtures including the CRCs were incubated at 36 or 40°C. At incubation times of 0, 30, 60, and 90 min, reaction mixtures were supplemented with [α-^{32}P]UTP and incubated for 30 min. VPg-pU(pU) synthesized at different incubation times was detected by immunoprecipitation followed by gel electrophoresis as described in Materials and Methods. Relative amounts of VPg-pU(pU) were monitored by an image analyzer; ratios of the amount VPg-pU(pU) synthesized at 36°C to that synthesized at 40°C are shown at the bottom.

thesis. It is therefore of interest to determine whether the host factor involved in the complex is a molecule equivalent to the mouse cell factor(s) proposed here. In any event, positive- and negative-strand RNA syntheses appear to differ in fundamental initiation mechanisms.

The effects of the nonpermissive temperature on poliovirus RNA synthesis in mouse cells resemble those of Gua-HCl (Fig. 4). This observation suggests that the temperature of 40°C inhibits some step(s) in the initiation process of RNA synthesis. Furthermore, poliovirus RNA synthesis continued to occur at the normal rate immediately after the temperature shift at 4.5 h postinfection (Fig. 4). Since in vivo synthesis of a complete length of poliovirus RNA during virus infection takes only about 1 min (13), incorporation of [^{3}H]uridine in TgSVA cells for approximately 30 min suggests that some replication complexes, if not all, are still active in initiating RNA synthesis at the nonpermissive temperature. Thus, the temperature-sensitive step(s) of poliovirus RNA synthesis may exist in some step(s) preceding RNA chain initiation, such as uridylylation of VPg and/or formation of complexes required for the uridylylation.

Uridylylation of VPg (Fig. 7B) in the CRC from infected mouse cells was examined for its temperature sensitivity. The reaction was not affected by the nonpermissive temperature of 40°C for the first 30 min. However, prolonged incubation of the CRC at 40°C appeared to result in inactivation of the function more rapidly than that at 36°C. The reason for this phenomenon is not clear at present. It is possible that formation of the membranous complex required for uridylylation is more unstable in CRC from mouse cells at an elevated temperature than at 36°C in comparison with that from HeLa cells. If so, stabilization of such complexes may be one function of the host factor(s) described here. It is also possible that formation of new membranous complexes for the uridylylation occurs to some extent and is blocked at an elevated temperature in the CRC from mouse cells. In that case, complex formation in vitro may be very inefficient, since the temperature effect was observed only after prolonged incubation at 40°C.

Guinea and Carrasco (14) reported that cerulenin, a selective inhibitor of lipid biosynthesis, inhibited positive-strand RNA synthesis of poliovirus, suggesting that poliovirus genome replication requires continuous synthesis of phospholipids (23). However, the incorporation of [^{3}H]glycerol into lipid fractions of TgSVA and TgSVB cells was not affected by the nonpermissive temperature of 40°C (unpublished results). Therefore, the defect in the mouse cells appears not to be in a step(s) of lipid biosynthesis.

Viral and cellular factors possibly involved in the initiation process of poliovirus RNA synthesis on membranes are 3AB (12), $3C^{pro}$ and $3D^{pol}$ (1, 28, 38), 2C and 2B (5, 28, 29, 39), and host factors (1, 14). Thus, the replication complex for poliovirus RNA synthesis appears to be fairly complicated (28, 35, 39). Therefore, it may not be easy to elucidate the precise interaction among these components involved in the initiation process. The mouse cell factor(s) described here may be functionally associated with a viral protein(s). To identify such a viral protein(s), a poliovirus mutant that is able to replicate efficiently in TgSVA and TgSVB cells at 40°C is currently being prepared.

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