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Establishment and Maintenance of Persistent Infection by Sindbis Virus in BHK Cells

BARBARA WEISS, RICHARD ROSENTHAL, AND SONDRA SCHLESINGER*

Department of Microbiology and Immunology, Washington University School of Medicine, St. Louis, Missouri 63110

We have established a persistent infection of BHK cells with a preparation of Sindbis virus heavily enriched in defective interfering (DI) particles. The small fraction of cells that survived the initial infection grew out to form a stable population of cells [BHK(Sin-1) cells], most of which synthesized viral RNA and viral antigens. The presence of DI particles in this virus stock was required to establish this persistent state. BHK(Sin-1) cells released a small-plaque, temperature-sensitive virus (Sin-1 virus) as well as DI particles containing DI RNAs larger than those present in the original stock used to establish the persistent state. A cloned stock of Sin-1 virus, free of detectable DI particles, was able to initiate a persistent infection more quickly and with greater cell survival than the original stock of Sindbis virus containing DI particles. About 2 weeks after the Sin-1 virus-infected cells were cultured, DI RNAs arose and soon became the dominant viral RNA species produced by these cells.

Many highly cytopathic RNA viruses are capable of long-term persistence and replication in cultured mammalian cells. In the most extensively studied system involving vesicular stomatitis virus (VSV), three major factors were implicated in the establishment and maintenance of such persistently infected cultures: (i) defective interfering (DI) particles, (ii) mutations in the standard virus (particularly *ts* mutations), and (iii) interferon production. Huang and Baltimore (7) were the first to suggest that DI particles might have an important role in viral persistency, and Holland et al. (6) have provided strong evidence supporting this idea in a model system involving VSV grown in BHK cells. DI particles have also been shown to be a major factor in persistency involving Sendai virus (19), lymphocytic choriomeningitis virus (9, 27), and reovirus (4, 23). Temperature-sensitive mutants are frequently recovered from persistently infected cultures and are often required to establish persistency even in the presence of DI particles (16, 28-30). Recently Holland et al. demonstrated that numerous mutations in the genome of VSV accumulate with time in long-term carrier cultures (5). This selection for a highly mutated virus contrasts with the genetic stability observed during successive lytic cycles of infection with VSV and suggests that these mutations enhance the survival of infected cells. Ramseur and Friedman (17) have shown that interferon can facilitate the establishment of persistency with VSV in L cells. In addition, Sekellick and Marcus (21) have shown that certain *ts* mutants and DI particles of VSV are particularly good inducers of interferon. In further support of a role for interferon in persistent infection, Nishiyama et al. (13, 14) have isolated from a persistently infected L-cell culture a mutant of VSV which is a better inducer of and more sensitive to interferon than wild-type VSV.

Although the mechanisms of viral persistency are becoming increasingly clear for rhabdoviruses and other negative-strand viruses, the factors mediating viral persistency with the positive-strand alphaviruses are less well understood. Infection of invertebrate cell cultures with alphaviruses is noncytopathic, and persistent infections are readily established. During the maintenance of these infected cultures, temperature-sensitive mutants (22), antiviral factors (18), and DI particles (2, 11) can be demonstrated, although the relative importance of each of these factors in persistency has not been determined. Infection of vertebrate cells with alphaviruses is highly cytopathic, but it has also been possible to establish persistent infections in these cultures. Several investigators have suggested that DI particles mediate persistent infections in vertebrate cells, but the carrier cultures were not well characterized (1, 8, 20). In addition, Inglot et al. (8) have presented evidence implicating interferon in the regulation of the persistent state with Sindbis virus in mouse cells.

We have initiated experiments to examine the relative importance of DI particles, mutations in the standard virus, and interferon production in

the establishment and maintenance of persistently infected cultures involving the alphavirus Sindbis virus, grown in BHK cells. Our studies show that DI particles were required to establish a persistent infection with wild-type standard virus. The resulting infected cell cultures synthesized and released DI particles as well as a mutated standard virus. Both the mixed population of virus released from infected cells and the cloned mutant standard virus reestablished persistent infection with much greater facility than the original DI-enriched virus stock. DI particles were not involved in establishing the persistent infection initiated by the mutant virus, but during subsequent long-term maintenance of these cultures DI particles invariably became the dominant viral species detected in these cells.

MATERIALS AND METHODS

Cells. BHK-21 cells and persistently infected cultures derived from them were maintained at 37°C in Eagle minimal essential medium supplemented with 6% fetal calf serum. Primary chicken embryo fibroblasts grown in minimal essential medium containing 3% fetal calf serum were used in plaque assays as described previously (26).

Viruses. The Sindbis virus stock, containing DI particles, that was used to establish persistency was obtained by high-multiplicity passaging of wild-type Sindbis virus in BHK cells. The properties of this particular population of DI particles have been extensively described (10, 25, 26). The stocks of wild-type Sindbis virus, Semliki Forest virus, and VSV (San Juan) were prepared by passage at low multiplicity of infection (MOI) on BHK cells. The *ts* mutants (*ts*6 and *ts*24) were derived from the HR strain of Sindbis virus (14).

Plaque purification of virus released from BHK(Sin-1) cells. Cloning of the small-plaque virus released by BHK(Sin-1) cells was carried out on chicken embryo fibroblast monolayers. To obtain large plaques of this mutant, assays were carried out in 0.5% agar containing DEAE-dextran (100 μg/ml). Plaques were picked after growth at 30°C for 3 days. Three consecutive plaque purifications were performed with no intermediate amplification of the virus obtained from a single plaque. The thrice-cloned virus was then passaged in chicken embryo fibroblasts at 30°C at an MOI of 0.1. All experiments with the cloned virus were done with this virus stock.

Labeling and purification of viral RNA. Lytically infected and persistently infected cells were labeled with [^{3}H]uridine (20 μCi/ml, 28.5 Ci/mmol) in the presence of actinomycin D (1.0 μg/ml) for a period of 8 or 16 h. [^{3}H]uridine was added to the lytically infected cells 1 h postinfection. RNA was isolated from BHK cells essentially as described previously (25). Cell extracts were obtained by lysing cells in buffer (0.05 M Tris, 0.1 M NaCl, 0.001 M EDTA, pH 7.5) containing 0.5% Triton and 0.5% recrystallized naphthalene disulfonate. All reagents were sterilized when possible and redistilled or recrystallized when heat sterilization was not possible.

Agarose gel electrophoresis. All RNA samples were denatured with glyoxal before electrophoresis by the method of McMaster and Carmichael (12). Denaturation was carried out for 1 h at 50°C, usually 1 day prior to electrophoresis. Electrophoresis was carried out using horizontal, 1.1% agarose slab gels (5 mm in thickness), prepared in 0.01 M sodium phosphate buffer (pH 7.0) and run for 3.5 h at approximately 3 V/cm (measured across the gel itself). Continuous recirculation of buffer was essential to maintain a constant pH during the run.

Immunofluorescence assay. Cells were grown on cover slips and, where indicated, infected with Sindbis virus. Cover slips were washed with phosphate-buffered saline (three times for 5 min each), drained, and dried overnight. Acetone fixation was carried out at room temperature for 10 min. Dried cover slip cultures were then incubated with 0.2 ml of a 1:100 dilution of rabbit antibody to Sindbis virus. After 30 min of incubation in a humid chamber at room temperature, the cells were washed three times with phosphate-buffered saline and then incubated with 0.2 ml of a 1:100 dilution of fluorescein-conjugated goat anti-rabbit antibody (Gateway Immuno Sera Co., St. Louis, Mo.). Cells were mounted on slides with Permount and examined with a Zeiss microscope equipped with an IV F1 epi-fluorescence condenser.

Autoradiography. Approximately 6×10^5 cells grown on cover slips were pretreated with actinomycin D (1 μg/ml) for 3 h at 37°C and then labeled with [^{3}H]uridine (10 μCi/ml) for 3 h in the presence of actinomycin D (1 μg/ml). Cover slips were then washed with phosphate-buffered saline, fixed in two changes of 5% trichloroacetic acid for 10 min at 4°C, and dehydrated with 70% ethanol followed by ether. Cover slips were counted in a scintillation counter with a toluene-based scintillant, rinsed with fresh toluene and ether, and then air dried. Mounted cover slips were coated with NTB2 (Kodak) liquid emulsion and exposed for 3 to 5 days at 4°C. Cells were stained with Giemsa stain after development for better visualization.

Immune lysis assay. The complement-mediated lysis of ^{51}Cr-labeled cells treated with viral antibody was carried out using the procedure described by Fan and Sefton (3). The Sindbis-infected BHK cell cultures were labeled with ^{51}Cr at 5 h after infection at an MOI of 100. Both the VSV and Sindbis antibodies were used at a 1:100 dilution.

Superinfection assay. Carrier or normal BHK cells were infected with virus at an MOI between 10 and 100 as indicated in the tables. After a 1-h adsorption period, monolayers were washed three times with warm phosphate-buffered saline and incubated for 16 to 18 h in growth medium. At 1 h after infection, a sample was removed to determine the residual virus present after this washing procedure.

RESULTS

Persistent infection of BHK cells with Sindbis virus. Infections of BHK cells with Sindbis virus are usually very cytopathic, whereas infections with virus stocks enriched in

DI particles show decreased cytopathogenicity. We were unable to establish a persistent infection of BHK cells using our stocks of standard Sindbis virus; the observation that DI particles reduced cell killing provided a basis for their use in establishing persistently infected cultures.

The preparation of Sindbis virus used to establish a persistent infection was a late-passage virus stock with a high degree of interfering activity (see Table 4). BHK cells, infected with this virus at an MOI of 5, were maintained at 37°C. Cytopathogenicity developed slowly, but after 4 days most of the cells had detached from the plate. Dead cells were removed from the plate by several changes of medium. By 9 days after infection, nine colonies of cells were visible. By 22 days the colonies were large; cells were then dispersed using 3 mM EDTA and replated without dilution. From this time on cells were subcultured at a 1:10 dilution when confluency was reached, usually every 4 days. Cells were dispersed with EDTA until passage 5 (38 days in culture). Thereafter trypsin was used in all subculturing. These cells have been designated BHK(Sin-1).

BHK(Sin-1) cells could not be distinguished from the original uninfected BHK cells by either morphology or growth rate. They produced low levels of virus at a rate of less than 1 PFU/cell per day and were resistant to infection by Sindbis virus (Table 1). These properties are common characteristics associated with persistently infected cultures. The experiments described below were undertaken to examine the extent of Sindbis virus expression in these cells approximately 3 months after they had been in culture, and to determine the factors that contributed to the stability of the virus-infected culture.

Absence of interferon in BHK(Sin-1) cells. We tested acid-treated medium from confluent BHK(Sin-1) cells for exogenous interferon using a plaque reduction assay with VSV; we were unable to detect interferon. Youngner et al. (29) and Ramseur and Friedman (17) reported similar results in L cells persistently infected with VSV, but demonstrated a role for endogenous interferon in maintaining the persistent state. In these latter examples, the infected cells were resistant to challenge by both homologous and heterologous viruses. As shown in Table 1, infection of BHK(Sin-1) cells with VSV produced normal yields of virus and total cell killing, providing further evidence that interferon was not a major factor in the maintenance of this persistent infection.

Presence of virus-specific RNA in BHK-(Sin-1) cells. The synthesis of virus-specific RNA was detected in BHK(Sin-1) cells by the incorporation of [^{3}H]uridine into actinomycin D-treated cells (Table 2). The incorporation of [^{3}H]uridine in BHK(Sin-1) cells was increased over that obtained in uninfected cells, but it was only about one-fourth the amount obtained in a culture infected with standard virus. To determine whether the decreased rate of [^{3}H]uridine incorporation was due to the synthesis of viral RNA by only a small fraction of the BHK(Sin-1) cells or to the synthesis of less RNA per cell, we exposed the [^{3}H]uridine-labeled cultures to autoradiography. The micrographs shown in Fig. 1 demonstrate that most, if not all, of the BHK(Sin-1) cells were synthesizing viral RNA. The grains over the BHK(Sin-1) cells and the cells infected with standard virus were distributed mainly over the cytoplasm, whereas in the uninfected cells the grains were localized over the nucleus. The grain densities over the BHK(Sin-1) cells were uniformly lower than those over cells infected with Sindbis virus, demonstrating that the former synthesize less viral RNA per cell than the latter.

Expression of virus-specific protein in

TABLE 1. *Infection of BHK(Sin-1) cells with homologous and heterologous virus*

Cell line	Virus	MOI	Cytopathogenicity[a]	Virus yield[b] (PFU/ml)	
				1 h	16 h
BHK	Sindbis	135	+	ND[c]	5.5×10^8
BHK(Sin-1)	Sindbis	135	0	1.3×10^5	1.2×10^5
BHK	VSV	10	+	ND	7.5×10^8
BHK(Sin-1)	VSV	10	+	ND	6.0×10^8
BHK(Sin-1)			0	ND	3.5×10^5[d]

[a] Cytopathogenicity was scored at 16 h postinfection. +, Severe signs of cell damage; 0, no difference from an uninfected control cell population.

[b] Cells (10^6 per 35-mm dish) were infected with virus for 1 h at 37°C. Virus was removed, and the cells were washed three times with phosphate-buffered saline containing 1% fetal calf serum. Fresh medium was then added, and a sample was removed for plaquing at the indicated times.

[c] ND, Not done.

[d] In this case, all the plaques were small and characteristic of the virus released from these cells.

BHK(Sin-1) cells. To determine whether BHK(Sin-1) cells synthesized virus-specific proteins as well as viral RNA, we analyzed acetone-fixed BHK(Sin-1) cells for the presence of viral antigens using indirect immunofluorescent staining. These data (Fig. 2) showed that about 60% of the population expressed viral antigens.

Lysis of infected cells by virus-specific antibody and complement, as measured by ^{51}Cr release, is a sensitive method to detect viral antigens on cell surfaces (3). We have used this procedure to compare the expression of viral antigens on the surface of BHK(Sin-1) and cells infected with standard Sindbis virus. Our results (Table 3) show that the amount of ^{51}Cr released by the persistently infected cells was similar to, although reproducibly lower than, that released by cells infected for 6 h with Sindbis virus. These data provide additional evidence that most of the cells in the BHK(Sin-1) population were synthesizing viral antigens. In addition, some of these antigens were expressed on the cell surface.

Characterization of virus-specific RNA in BHK(Sin-1) cells. The particles released by BHK(Sin-1) cells interfered with the replication of standard Sindbis virus, suggesting that DI particles were also being released by these cells (Table 4). More definitive proof for the presence of DI particles was obtained by an analysis of the viral RNAs synthesized by these cells. A sample of the persistently infected culture was labeled with [^{3}H]uridine in the presence of actinomycin D for 16 h at 80 and 100 days after infection. The RNA was purified, denatured with glyoxal, and subjected to high-resolution gel electrophoresis. [^{3}H]uridine-labeled RNA was simultaneously isolated from cells infected with standard Sindbis virus and from cells infected with the late-passage stock of virus containing DI particles, which was used to initiate the persistent infection. As shown in Fig. 3, BHK(Sin-1) cells synthesized a large number of RNA species that were distinct from the standard viral 42S and 26S RNAs and from the original DI RNAs. The largest major band of RNA synthesized by BHK(Sin-1) cells (Fig. 3, lanes 4 and 5) had a calculated molecular weight of 1.50×10^6, compared to that of 1.65×10^6 for 26S RNA. The difference between the mobility of 26S RNA and that of the largest DI RNA band was more easily discerned when the largest DI RNA band was not as overexposed as it is in lanes 4 and 5. The molecular weights of the DI RNAs detected in these cells ranged from 1.5×10^6 to 0.8×10^6. The two major species of DI RNA present in BHK cells infected with the original DI particle-containing viral stock had molecular weights of 0.75×10^6 and 0.68×10^6 (lane 3 of Fig. 3), which were lower than the smallest major DI RNA observed in BHK(Sin-1) cells.

TABLE 2. *Labeling of BHK and BHK(Sin-1) cells with [^{3}H]uridine*[a]

Cell type	Virus	cpm of [^{3}H]uridine incorporated[b]
BHK	None	7,400 ± 723[c]
BHK	Sindbis	83,515 ± 885
BHK(Sin-1)	None	19,475 ± 3,120

[a] BHK or BHK(Sin-1) cells (3×10^5 cells per dish) were grown on glass cover slips in 35-mm dishes. At 24 h after plating, actinomycin D (1 μg/ml) was added to each plate, and, where indicated, cells were infected with Sindbis virus (MOI = 50). One hour later, [^{3}H]-uridine (10 μCi/ml) was introduced, and 4 h later the cover slips were processed as described in the text.

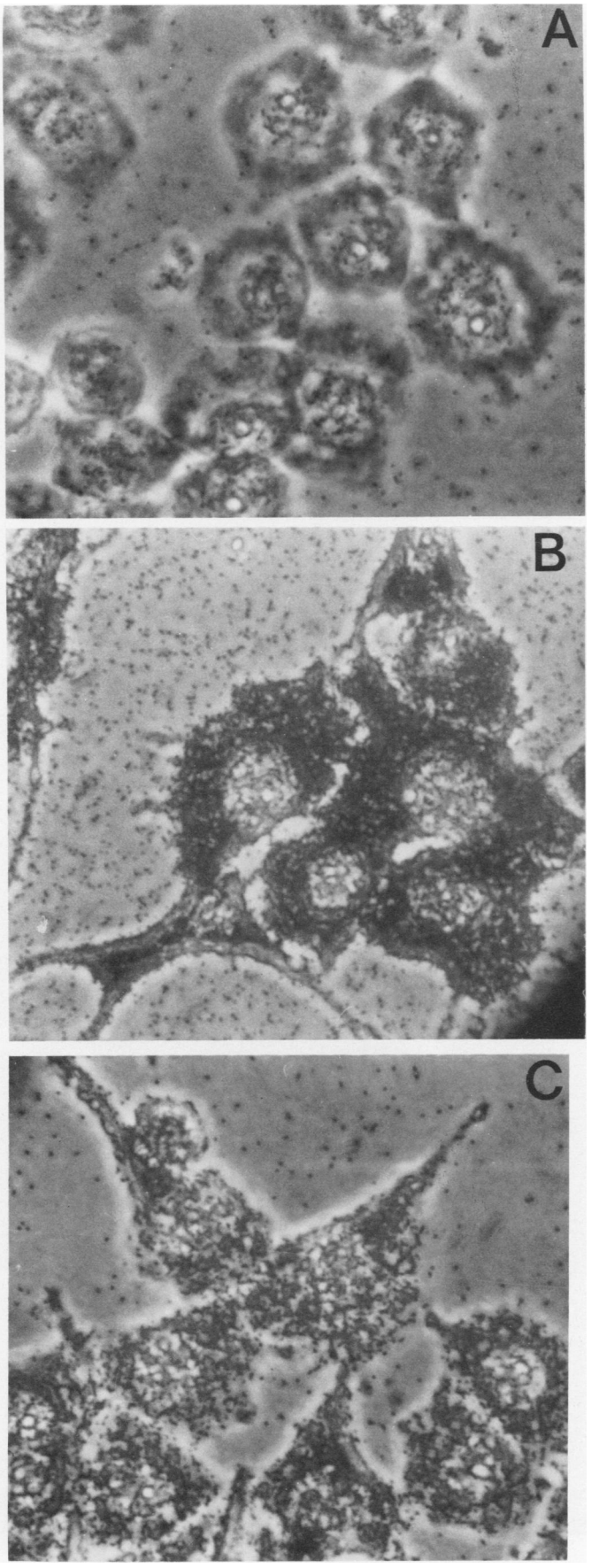
[b] Mean values ± standard error determined from three experiments. cpm, Counts per minute.

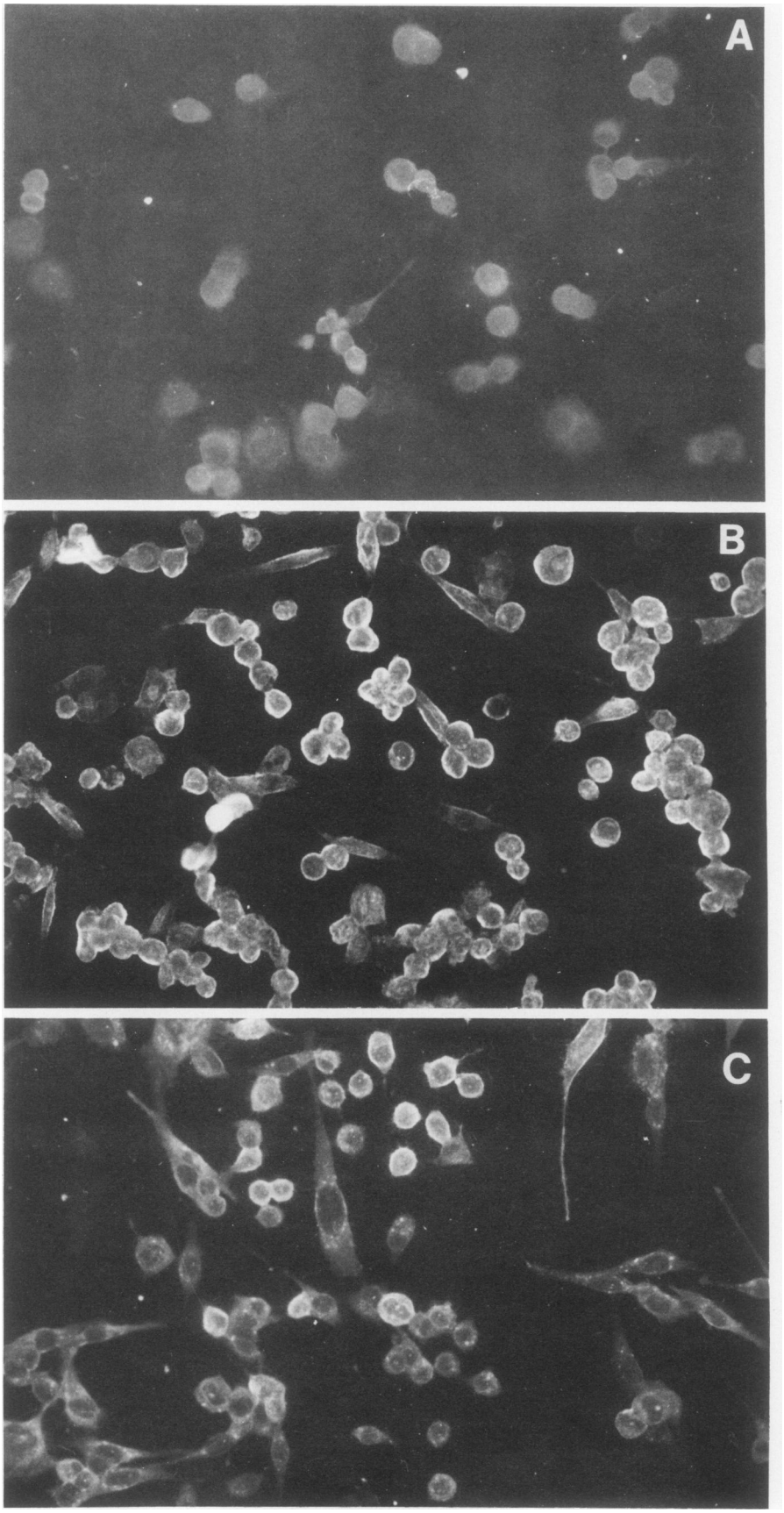
[c] The addition of higher concentrations of actinomycin D did not reduce this background.

The multiple DI RNAs observed in BHK(Sin-1) cells were also seen when BHK cells were infected with the uncloned virus preparation released from BHK(Sin-1) cells. This result demonstrated that the different size classes of DI RNAs present in BHK(Sin-1) cells could be packaged into particles.

Stability of BHK(Sin-1) cells to variations in temperature. The virus released from BHK(Sin-1) cells was a small-plaque, temperature-sensitive variant (Table 5). If the survival of the persistently infected cells were dependent on the temperature-sensitive phenotype of the virus, growing the cells at a lower temperature (30°C) might have led to increased viral replication and cell death, whereas growth at the restrictive temperature (40°C) might have led to curing of the cells or to selection of a non-temperature-sensitive revertant. We were unable to affect the stability of the persistently infected cells, however, by altering the temperature. BHK(Sin-1) cells grown at 30°C for 25 days did not show any increase in cell death. The cells grown at 40°C for 34 days showed no evidence of curing. They remained resistant to superin-

FIG. 1. *Autoradiography of cells pulse-labeled with [^{3}H]uridine for 3 h. (A) Uninfected BHK cells. (B) BHK cells infected with standard Sindbis virus (MOI = 100; pulse-labeled from 3 to 6 h postinfection). (C) BHK(Sin-1) cells. In all cases cells were exposed to actinomycin D (1 μg/ml) for 3 h before labeling.*


A
B
C


A
B
C

fection by Sindbis virus and continued to release a temperature-sensitive virus.

Curing of BHK(Sin-1) cells. The autoradiography and antibody studies established that a large fraction of BHK(Sin-1) cells were synthesizing Sindbis virus-specific products. Although persistent infections with RNA viruses may appear, as in this case, to be completely stable, it has been found that the association between virus and cell may be lost when individual cells are cloned from the population (6). To determine whether this would also be true for BHK(Sin-1) cells, we examined 11 clones derived from this culture. The cloning efficiency was 68%, providing evidence that we were analyzing a representative sample of the cells. Nine of the clones were considered to be cured of virus based on plaque assays of the medium, immunofluorescence, and complete susceptibility to infection by Sindbis virus. Two of the clones were still infected, based on these tests. The same percentage of cells stained positive for viral antigens in these clones as in the original persistently infected culture, indicating that the original BHK(Sin-1) cells did not represent a stable mixture of viral antigen-positive and antigen-negative cells.

Isolation and characterization of the small-plaque variant produced by BHK-Sin-1) cells. We have initiated a more detailed study of the virus isolated from BHK(Sin-1) cells. As a first step the virus was purified free of DI particles by three successive plaque isolations. It was considered to be free of DI particles based on its inability to interfere with the replication of standard virus (Table 4). A further criterion for the absence of DI particles was that BHK cells infected with this virus synthesized only the standard viral RNA species (Fig. 4, lane 3).

The purified variant (Sin-1) was temperature sensitive in the ability to synthesize RNA in infected cells (Table 6). Even at 30°C, cells infected with this newly isolated virus synthesized less viral RNA than cells infected with the standard wild-type virus.

Establishment of persistent infection with the cloned virus from BHK(Sin-1) cells. Our initial attempt to establish persistent infection of BHK cells with Sindbis virus required the presence of DI particles in the virus

TABLE 3. *Immune lysis of persistently infected cells*[a]

Cell type	Virus	Antisera	Percent ^{51}Cr released[b]
BHK	None	Sindbis	2.5 ± 0.61
BHK	None	VSV	2.2 ± 0.15
BHK	Sindbis	Sindbis	73 ± 2.4
BHK	Sindbis	VSV	4.3 ± 3.2
BHK(Sin-1)	None	Sindbis	53 ± 9.9
BHK(Sin-1)	None	VSV	3.5 ± 0.93

[a] Those cells infected with Sindbis virus received 100 PFU/cell 4.5 h before labeling with ^{51}Cr. Each cell culture containing 3×10^5 cells was labeled for 1 h with 13 μCi of ^{51}Cr per ml in minimal essential medium plus 1% fetal calf serum. The plates were then rinsed five times and incubated in 0.4 ml of this medium, containing a 1:80 dilution of antiserum and a 1:20 dilution of guinea pig complement, at 37°C for 45 min. The supernatant fluid was removed, and the culture was rinsed twice. The rinses were combined with the first supernatant fluid. The remaining cells were then lysed by freezing and thawing two times with water.

[b] The percentage of ^{51}Cr released was calculated as described by Fan and Sefton (3) and is based on a total incorporation (supernatant plus lysate from frozen and thawed cells) of approximately 40,000 cpm. The data are mean values ± standard error determined from four experiments.

TABLE 4. *Homologous interference as a measure of the presence of DI particles*

Virus added (MOI)	Virus yield (PFU/ml)
Standard Sindbis[a] (10)	2.3×10^{10}
Standard Sindbis (10) plus late-passage Sindbis (5)[b]	7.0×10^6
Standard Sindbis[c] (10)	6.0×10^9
Standard Sindbis (10) plus uncloned Sin-1 (5)[d]	6.0×10^7
Standard Sindbis (10) plus cloned Sin-1 (5)	5.3×10^9

[a] BHK-21 cells (10^6 cells) were infected with standard virus alone or simultaneously coinfected with standard virus and a late-passage stock of Sindbis virus. The virus yield was determined 16 h postinfection.

[b] This preparation is the one used to establish the BHK(Sin-1) culture.

[c] BHK-21 cells were infected with standard virus in the presence of actinomycin D (2 μg/ml) for 1 h before challenge with either the uncloned or cloned Sin-1 virus preparation. The virus yield was determined 16 h postinfection.

[d] The medium from BHK(Sin-1) cells was centrifuged (3 h at 102,000 × *g*), and a sample from the resuspended pellet was used to obtain an MOI of standard virus of 5.

FIG. 2. *Indirect immunofluorescence staining of BHK cells. (A) Uninfected cells. (B) Cells infected with standard Sindbis virus. (C) Persistently infected cells. Cells were prepared as described in the text.*

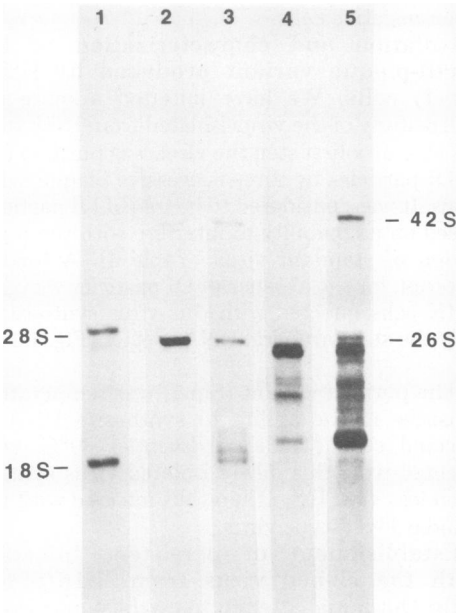


FIG. 3. *Viral RNA species synthesized by BHK(Sin-1) cells. [^{3}H]uridine-labeled intracellular viral RNAs denatured in glyoxal were separated by high-resolution horizontal agarose gel electrophoresis. Lane 1, BHK ribosomal 28S and 18S marker RNAs; lane 2, viral RNAs synthesized during a standard Sindbis virus infection; lane 3, viral RNAs synthesized in cells infected with the late-passage virus used to establish BHK(Sin-1) cell cultures. The viral RNAs present in BHK(Sin-1) cells labeled after 80 days and 100 days in culture, respectively, are shown in lanes 4 and 5.*

TABLE 5. *Temperature sensitivity of virus released by BHK(Sin-1) cells*

Temp[a] (°C)	Efficiency of plaquing[b]	
	Standard Sindbis virus	Sin-1 virus
30	1.0	1.0
37	0.9	0.88
38	0.95	0.29
40	0.85	0.00059

[a] Temperature of incubation for plaque titration. Plaquing was carried out on chicken embryo fibroblast monolayers.

[b] The efficiency of plaquing is the ratio of the number of plaques observed at the temperature tested over that obtained at 30°C.

preparation and considerable time to allow those few surviving cells to grow out. In contrast, we were able to establish persistent infections quite readily with Sin-1 whether or not DI particles were present in the preparation. When the infections with Sin-1 virus were carried out at 37 or 38°C, the level of cell death was greatly

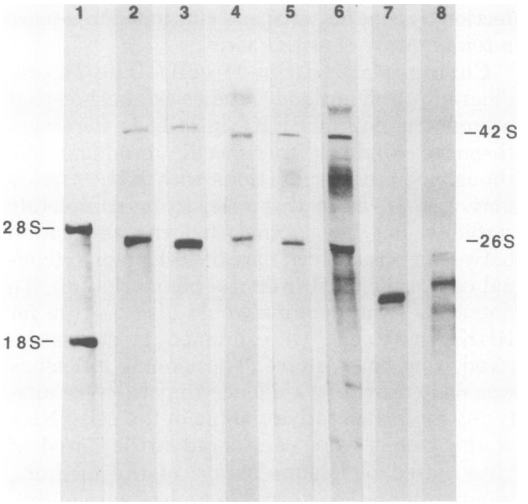


FIG. 4. *Agarose gel electrophoretic analysis of glyoxal-denatured viral RNAs synthesized by persistently infected BHK cells established with the cloned Sin-1 virus. Cells were labeled with [^{3}H]uridine at various times after infection, and the RNA was prepared for electrophoresis as described in the text. rRNA's from BHK cells and standard Sindbis viral RNAs are shown in lanes 1 and 2, respectively. Lanes 3 through 8 show the patterns of labeled viral RNAs obtained with cells at 1, 4, 9, 15, 26, and 18 days after infection with the cloned Sin-1 virus at an MOI of 50 or greater. Lanes 3, 4, and 8 are from different carrier cultures and represent cultures 1, 4, and 18 days after infection. Lanes 5, 6, and 7 are from a single culture at 9, 15, and 26 days after infection. The slight discrepancy in mobility of 42S and 26S RNA seen in certain lanes arises because all of the samples were not electrophoresed simultaneously and represents the minor mobility variation observed from one electrophoresis run to another.*

TABLE 6. *Temperature sensitivity of Sin-1 virus for RNA synthesis in BHK cells*

Temp (°C)	[^{3}H]uridine incorporation[a] (cpm)	
	Standard Sindbis virus	Sin-1 virus
30	34,962	6,420
38	69,562	628
40	978	0

[a] The data have been corrected for the incorporation (counts per minute; cpm) in comparable uninfected cells treated with actinomycin D (2 μg/ml). Adsorption of each virus (MOI = 100) was at 30°C for 1 h. Cells were then shifted to the appropriate temperature. Labeling was from 5 to 7 h postinfection with 20 μCi of [^{3}H]uridine per ml.

reduced. As measured by trypan blue exclusion, about 15% of the cells died 24 h after infection with Sin-1 plus DI particles and about 30% after infection with cloned Sin-1. In each case, by 6 to

7 days after infection, a stable virus-producing cell population was obtained. The ability to survive after infection with cloned Sin-1 was dependent on temperature; at 30°C essentially all of the infected cells died. We considered the possibility that the enhanced ability of the cloned virus to establish persistency was directly related to its temperature-sensitive phenotype. If this were true, other RNA$^-$ temperature-sensitive mutants might also show this property. We examined parallel cultures of BHK cells infected either with *ts*6 or *ts*24, both RNA$^-$ temperature-sensitive mutants (15), or with the cloned Sin-1 virus. Infections were initiated at 30°C for 6 h at MOIs of 0.5, 5.0, and 50.0. Thereafter, cultures were maintained at 37°C. All infections with *ts*6 resulted in total cell killing. Infections with *ts*24 also virtually destroyed these cultures. A few cells, however, survived and slowly grew to form colonies. Repeated cycles of growth and cell death ensued for several months, until a more stable carrier state evolved. Thus, an RNA$^-$ phenotype per se is not sufficient to confer on Sindbis virus the ability to establish a stable persistent infection.

About 6 days after infection with Sin-1 at 37°C with or without DI particles, the cultures were tested for their sensitivity to superinfection by Sindbis virus and were found to be completely resistant. They were sensitive to heterologous viruses (Table 7), suggesting that interferon was not involved in the establishment of persistency. Immunofluorescent staining was carried out on cells infected with cloned Sin-1 or with Sin-1 plus DI particles. In both cases a large percentage of the cells were viral antigen positive.

Generation of DI RNA in BHK cells persistently infected with Sin-1. Carrier cultures initiated by infection with cloned Sin-1 virus did not contain detectable levels of DI RNA for at least 1 week after infection. As shown in Fig. 4, lane 3, the RNA pattern observed in BHK cells infected with the cloned virus at an MOI of 70 and labeled within the first 24 h after infection was indistinguishable from the early-passage wild-type pattern shown in lane 2. The remaining lanes in this figure show the viral RNA pattern seen in three independent persistently infected cultures established with the cloned virus and labeled at selected times during their growth. Lane 4 shows that in one of these cultures at 4 days postinfection no DI RNAs were detectable. Lane 5 shows another independent carrier culture, in which the dominant species made at 9 days postinfection were still 42S and 26S RNA. Lanes 6 and 7 show the viral RNAs detectable in this same culture at two subsequent times after infection, 15 days and 26 days. At 15 days postinfection, the standard viral RNAs still predominated; a number of new bands were visible, both larger and smaller than 26S RNA. Thus, by 15 days, DI RNAs were present but were not markedly enriched for in these cultures. The diffuse band between 42S and 26S RNA may represent a large set of DIs with small size differences. None of these RNA bands persisted for very long (Fig. 4, lane 7), and by 26 days there was one dominant species of DI RNA. Lane 8 represents another culture persistently infected with Sin-1 and illustrates that the major species of DI RNA appearing during the first month after infection may differ in size in independently established carrier cultures.

TABLE 7. *Infection of BHK cells carrying cloned Sin-1 with homologous and heterologous virus*

Days postinfection with Sin-1	Virus added	MOI	Virus yield (PFU/ml)	
			1 h	18 h
0	Sindbis	100	ND[a]	2.1×10^9
8	Sindbis	100	1.5×10^4	3.0×10^5
0	VSV	10	ND	3.3×10^8
8	VSV	10	$<10^2$	3.6×10^8
0	Semliki Forest	100	ND	2.4×10^9
8	Semliki Forest	100	ND	2.0×10^9

[a] ND, Not done.

We have attempted to increase the sensitivity of detection of DIs by using the virus released from early passages of Sin-1-infected cells to infect BHK cells. This type of infection would amplify any DI particles present in the population. Virus stocks were obtained from three independently established virus-producing cultures at 7, 13, or 16 days after infection. In each case the cells from which the virus was taken were considered to be DI negative based on the analysis of virus-specific intracellular RNA patterns. By amplification, as shown in Fig. 5, it was possible to detect minor species of [^{3}H]uridine-labeled RNA migrating between 42S and 26S RNA. These results suggested that DI RNAs were being generated at a low level in these cells, even though at these times they could not be detected directly in the persistently infected cultures. The data clearly demonstrate, however, that DI RNAs did not represent a significant fraction of the viral RNA found in BHK cells during the initial period establishing persistent infection with Sin-1 virus.

DISCUSSION

We initiated a study of persistent infection with Sindbis virus to identify the factors involved in establishing this type of infection and in maintaining this virus in cultured cells. Sev-

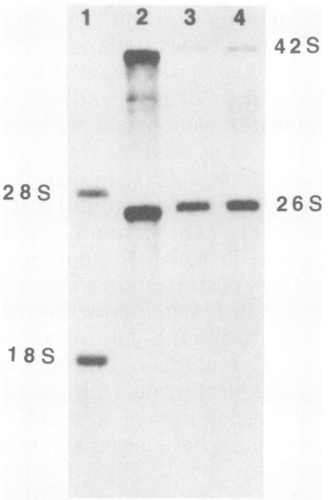


FIG. 5. *Viral RNA species synthesized in BHK cells infected with virus released from DI-negative persistently infected cultures. Lane 1, 18S and 28S rRNA's. Lanes 2, 3, and 4, [^{3}H]uridine-labeled intracellular viral RNAs synthesized in actinomycin D-treated cells after infection with virus from 7-, 13-, and 16-day cultures, respectively. Each virus was obtained from an independently initiated Sin-1-infected cell culture. The virus was concentrated by pelleting and resuspended to give an MOI of 3. RNAs were denatured with glyoxal and electrophoresed through a 1.1% agarose gel as described in the text. Lane 2 shows a particularly high ratio of 42S to 26S RNA. This anomalous result was not obtained with any of our other preparations of viral RNA.*

eral conclusions can be drawn from our initial results. First, with our strain of standard Sindbis virus, the presence of DI particles in the stock was essential to establish a persistent infection in BHK cells. The cells that grew out after infection remained viable and have never undergone a period of crisis during the more than 200 days they have now been in culture. Second, based on the synthesis of RNA in the cytoplasm of actinomycin D-treated cells, most of the cells were infected. Third, interferon did not appear to be a critical factor in establishing or maintaining this infection in BHK cells. Fourth, most of the cells cloned from the BHK(Sin-1) culture no longer retained any of the characteristics of persistently infected cells, demonstrating that the expression of viral genes was not a permanent genetic property of the cells. The loss of virus from these cells after cloning indicated that a significant number of uninfected cells can arise in the population. The appearance of uninfected cells could be explained if the replication of standard virus were suppressed to such an extent by DI RNA that the level of virion genomes would be too low to be passed on to progeny cells. In a mass culture of BHK(Sin-1) cells, uninfected cells would be rapidly reinfected by virus and DI particles released by neighboring cells. This type of reinfection would not occur during the growth of a colony from a single cell.

The standard virus obtained from BHK(Sin-1) cells was a small-plaque, temperature-sensitive variant. Our initial characterization of this virus showed that it had a temperature-sensitive defect in RNA synthesis. An important distinction between Sin-1 and the original standard Sindbis virus was that infection with the former was much less cytopathic. BHK cells infected with Sin-1 at 37°C established a persistent infection in the absence of DI particles. Since this decrease in cytopathogenicity may be a crucial factor in the initial establishment of persistent infection, it would be important to know what alterations in the viral genome caused the phenotype of decreased cytopathogenicity. We were not able to isolate either ts^+ or large-plaque revertants of Sin-1, and it is probable that this variant has multiple mutations. Although we are presently examining the properties of this mutant, it may be difficult to identify the particular changes associated with decreased cytopathogenicity.

The major viral RNA species in BHK(Sin-1) cells were DI RNAs, but these RNAs were larger and more heterogeneous than those present in the original stock used to establish the carrier culture. This observation suggests that DI RNAs were being generated continually from virion RNA and that those present originally did not have a strong selective advantage over newly generated ones. The multiple species of DI RNA synthesized in BHK(Sin-1) cells may result from the synthesis of different DI RNAs in different cells. There are at least two reasons to believe that the population of BHK(Sin-1) cells is heterogeneous: the heterogeneity of the DI RNA pattern, and the discrepancy between the percent of cells scored as infected by autoradiography and the percent scored as infected by immunofluorescence. One possible explanation for this discrepancy is that the level of viral antigen produced in a cell reflected the extent to which viral mRNA synthesis could be suppressed in that cell by a particular DI RNA.

One goal of our present study was to investigate the role of DI particles of Sindbis virus in persistent infections. When BHK cells were infected with the cloned stock of Sin-1 virus at 37°C, a large fraction of the cells survived the infection and grew out to form a stable infected culture in which most of the cells contained viral antigens and interferon was not detectable. During this period little or no DI RNA synthesis was detected, demonstrating that DI particles

were not required during the initial events leading to the establishment of persistent infection.

To establish a persistently infected culture it is essential to obtain a balance between sufficient viral replication to maintain the virus in the culture and cell survival. DI particles can provide one means of protecting cells from the cytopathic effects of infection. They, or some other agent (such as interferon) which would be capable of dampening an infection, would be required to initiate a persistent infection when the standard virus is particularly virulent. Thus, DI particles were required in establishing persistent infection with our original standard Sindbis virus, and both DI particles and interferon have been important in establishing persistent infections with VSV (6, 17, 21, 29). Infection of BHK cells by Sin-1 virus at 37°C or by rabies virus, as shown by Holland et al. (6), was sufficiently noncytopathic so that a persistent infection could be established in the absence of DI particles or interferon. In both these cases, DI particles arose in the culture soon after the infected cells were stabilized and had begun to grow.

In discussions of persistent infections a distinction has been made between persistency at the population level, in which only a small fraction of the cells are infected, and a persistent state in which most of the cells contain viral antigens (24; J. J. Holland, S. I. T. Kennedy, B. L. Semler, C. L. Jones, L. Roux, and E. A. Grabau, Compr. Virol., in press). In the former case, most of the cells are resistant to infection due to the presence of interferon or some other means of preventing viral replication. Holland et al. have proposed that the latter case is one in which DI particles play an important role. Our finding that the majority of BHK(Sin-1) cells were stably infected and did not undergo crisis even at 30°C suggests that suppression of viral replication by DI RNA is an important factor in protecting these cells from environmental changes that could enhance viral replication and lead to cell death. Our future studies of persistent infection by Sindbis virus in different cell lines will address the question of whether DI particles are always associated with stable persistent infections caused by this virus.

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