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Evolution of Virus and Defective-Interfering RNAs in BHK Cells Persistently Infected with Sindbis Virus

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We analyzed a BHK cell line persistently infected with Sindbis virus for 16 months and a virus (Sin-16) cloned from these cells. Sin-16 virus was resistant to the defective interfering particles present in the original infection. We found that (i) cells infected with Sin-16 were impaired in the processing of a viral precursor glycoprotein, (ii) high-multiplicity passaging of Sin-16 gave rise to a variant that was able to generate and be inhibited by defective-interfering particles to which the original Sin-16 virus was resistant, and (iii) the persistently infected culture contained a heterogeneous mixture of defective Sindbis virus RNAs which were not packaged into extracellular particles. To determine whether these intracellular RNAs could interfere with the replication of Sin-16, we analyzed cells that were cloned from the persistently infected culture. One clone (A3) synthesized a single defective viral RNA which was lost with continued passaging in culture. Infection of A3 cells with Sin-16 showed that the presence of the defective RNA greatly enhanced cell survival and led to enrichment of this RNA. In contrast, cured cells were highly susceptible to killing by Sin-16, and survivors did not synthesize this RNA. Thus, A3 cells were not genetically altered in their response to Sin-16, but were protected from the cytopathic effects of infection by an RNA with the characteristics of a defective-interfering RNA.

In many RNA virus carrier cell systems defective-interfering (DI) particles have been pivotal in the establishment of the persistent state and continue to play a role in the long-term survival of these cultures (5, 7, 8, 17, 26). Despite the outward stability of such cultures, the infectious and defective viruses which they harbor are continuously changing, as evidenced by the appearance of new viral mutants and new deleted RNAs with time in culture. This evolution can be attributed to the high mutation frequency associated with RNA viruses (6, 19) and to the selective pressures that are unique to persistent infections, in which debilitated, slowly replicating mutants can accumulate as they cannot during lytic infections.

We have analyzed a BHK cell line persistently infected with Sindbis virus. This cell line was established with a stock of wild-type Sindbis virus heavily enriched with DI particles. Infectious virus isolated from this carrier culture 1 month after infection was temperature sensitive and noncytopathic to BHK cells (26). This virus and subsequent isolates were increasingly less sensitive to interference by wild-type DI particles (27). Similar decreases in sensitivity have also been observed in persistent infections established with vesicular stomatitis virus, rabies virus, and lymphocytic choriomeningitis virus (9–11). Escape from interference may be an important mechanism for sustaining viruses in cultures and preventing cell curing.

In this report we describe further studies on both a virus (Sin-16) with resistance to DI particle-mediated interference and cells obtained from the carrier culture after 16 months of infection. One major objective was to determine whether Sin-16 virus generated DI particles which interfered with its own replication. Two direct approaches to obtain DI particles gave unexpected results. First, high-multiplicity passaging of Sin-16 to produce DI particles led to the selection of a variant that was sensitive to the newly generated DI particles, but the original Sin-16 virus was resistant to these DI particles. Thus, the replacement of Sin-16 in the virus population during passaging precluded the isolation of a specific DI particle to which Sin-16 was sensitive. Second, defective RNAs that were observed in the persistently infected cells at the time of isolation of Sin-16 virus were not packaged and therefore could not be amplified and tested directly for inteference against Sin-16 virus.

Cloning of cells from the carrier culture provided a population of cells that synthesized a

single deleted species of viral RNA. This defective RNA was gradually lost from the cell population. Our studies of the interaction of Sin-16 virus with these cells as they evolved showed that the presence of the deleted RNA enhanced cell survival after infection with Sin-16. Our data suggest that this deleted RNA, which remained cell associated, is a DI RNA with the ability to interfere with the replication of Sin-16 virus and be rescued by this virus.

MATERIALS AND METHODS

Cells. BHK-21 cells and persistently infected BHK cells, as well as clones derived from the latter cells, were maintained at 37°C in Eagle minimal essential medium supplemented with 6% calf serum. During the first week after infection with Sindbis virus or its mutants, infected cells were maintained at 38°C in medium containing 6% fetal calf serum. This variation considerably improved survival after acute infection.

Cell cloning. Monolayers of persistently infected BHK cells were rinsed three times with medium and twice with trypsin and EDTA (26) and incubated at 37°C to detach all cells. The cells were resuspended in medium, pelleted, and washed three times. These washed cells were diluted to a density of approximately 1 cell per ml, and a 1-ml portion was placed in each well of 48-well dishes. After several days those wells which contained single colonies were noted. These wells were observed until the colonies were sufficiently large to be trypsinized and replated. At this time the clones were examined for the presence of plaque-forming virus and sensitivity to infection by wild-type Sindbis virus. Cells were frozen in liquid nitrogen as soon as possible after cloning (within 3 weeks).

Viruses. Stocks of wild-type Sindbis virus were prepared by infecting BHK cells at low multiplicities. Sin-16 virus stocks [formerly designated Sin-1(16) virus (27)] were prepared in chicken embryo fibroblasts at a low multiplicity at 30°C because the yields of virus were usually much higher in these cells than in BHK cells. The titers of Sin-16 virus and uncloned viruses released from the persistently infected culture were determined on chicken embryo fibroblasts at 30°C by using 0.25% LE agarose (Marine Colloids, Inc.) and a 3-day development period. All other viruses were incubated for 2 days at 37°C with 1.5% Noble agar.

Biological interference assay. The biological interference assay was used to test for the presence of DI particles in virus populations released into the medium by persistently infected cells. To increase the sensitivity of the assay, preparations to be tested for interference were either concentrated by pelleting before use or passaged undiluted in BHK or chicken embryo fibroblasts. The latter passaging procedure generally enriches the resulting virus population for DI particles at the expense of helper virus and has been used as an amplification step (26).

Labeling and purification of viral RNAs. Cells were labeled with [^{3}H]uridine in the presence of actinomycin D, and RNA was purified as previously described (26). In all cases infection and labeling were performed at 30°C. Oligodeoxythymidylic acid [oligo(dT)] chromatography to select for polyadenylated RNAs has been described elsewhere (12a). The RNA was always carried through two cycles of chromatography and was heated for 10 min at 65°C before each application to the column.

Agarose gel electrophoresis. All RNA samples were denatured with glyoxal before electrophoresis by the method of Carmichael and McMaster (2) and were electrophoresed as described previously (26).

cDNA clones. Sindbis virus 49S RNA was reverse transcribed by using either oligo(dT) $_{12-18}$ or fragmented calf thymus DNA as the primer. After second-strand synthesis using the Klenow fragment of DNA polymerase I and appropriate tailing, the DNA was inserted into plasmid PUC4, a pBR322 derivative (25), by using standard procedures. Plasmids were screened for Sindbis virus-specific sequences by using the colony hybridization method of Grunstein and Wallis (3) and ^{32}P-labeled 49S and 26S RNAs as probes. Three clones were used in this study. The positions of these clones within the 49S genome were determined by restriction enzyme mapping and comparison with the known restriction sites for 49S RNA (14, 15; E. Strauss and J. Strauss, personal communication). The 5′ clone used in this study contains about 2,600 base pairs, begins about 43 bases from the 5′ end of 49S RNA, and is colinear with 49S RNA. The 3′ clone used is 890 base pairs long and is colinear with both 49S RNA and 26S RNA; from restriction enzyme analysis this clone appears to be derived from the immediate 3′ terminus of 49S RNA, but its precise boundaries are not known. The junction clone contains approximately 2,200 base pairs, one-half of which are in the nonstructural domain of 49S RNA. The 847-base pair *Ava*II fragment used in this study contains six nucleotides from the nonstructural gene region of 49S RNA; the remainder of the nucleotides lie within the 5′-proximal domain of the 26S region of the genome (unpublished data).

Blot hybridization. Glyoxal-denatured RNAs were electrophoresed through 1% agarose gels. The gels were treated with 50 mM NaOH to reverse the glyoxal adducts and nick the RNAs. The RNAs were then transferred electrophoretically to activated paper (23), as described elsewhere (12a). High-specific-activity DNA probes (5×10^7 to 1×10^8 cpm/μg) were prepared by nick translation essentially as described by Rigby et al. (16). Nick translations were allowed to proceed for 2 h at 15°C. Prehybridization and hybridization were performed as described elsewhere (12a). After hybridization, the blots were washed twice at room temperature in 2× SSPE (1× SSPE is 0.18 mM NaCl, 0.01 M $NaPO_4$ [pH 7.7], 1 mM EDTA) containing 0.1% sodium dodecyl sulfate for 10 min each and twice at 50°C in 0.1× SSPE containing 0.1% sodium dodecyl sulfate for 15 min each.

Immunofluorescence and ^{3}H autoradiography. Immunofluorescence and ^{3}H autoradiography were performed as described previously (26). Cells to be labeled for autoradiography were pretreated with actinomycin D (2 μg/ml) for 2 h before labeling with [^{3}H]uridine (25.5 Ci/mmol; 10 μCi/ml) for 4 h at 30°C.

[^{35}S]methionine labeling conditions and protein gel electrophoresis. Cells were pulse-labeled with [^{35}S]methionine and processed for gel electrophoresis essentially as described by Schmidt and Schlesinger (22). The cells were incubated in methionine-free medium for 1.5 h before labeling with [^{35}S]methionine

(20 μCi/ml). Labeled proteins were analyzed by polyacrylamide gel electrophoresis (21).

RESULTS

Virus growth, RNA synthesis, and protein processing in cells infected with Sin-16 virus. Sin-16 virus is temperature sensitive in its ability to synthesize viral RNA in infected cells (27), but even at 30°C the rate of release and the final yield of virus were significantly lower than the values obtained with the wild-type virus (Fig. 1A). To determine whether these differences were due to an inability of Sin-16 virus-infected cells to produce substantial amounts of viral RNA even at the permissive temperature, we compared the rate of viral RNA synthesis in cells infected with Sin-16 and the rate in cells infected with wild-type virus. As shown in Fig. 1B, these rates were essentially the same.

Although cells infected with Sin-16 synthesized viral RNA at normal rates, they were impaired in their ability to carry out a step in viral glycoprotein processing. The cleavage of Sindbis glycoprotein PE2 to E2 is essential for virus formation (20). This step was clearly observed in cells infected with Sin-1, a virus isolated from an earlier stage in the persistent infection (Fig. 2). In contrast, this conversion was almost undetectable in cells infected with Sin-16 at either 30 or 39°C. This defect could explain the slow release and low yield of Sin-16 virus.

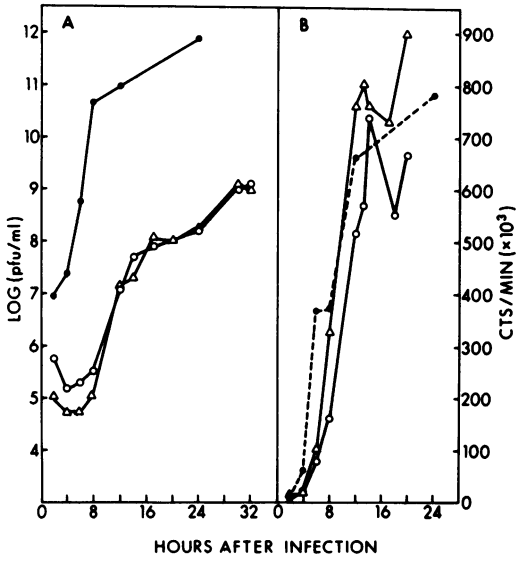

FIG. 1. Sin-16 virus replication. Symbols: ●, wild-type Sindbis virus; ○, Sin-16 early-passage virus; △, Sin-16 late-passage virus. (A) Virus growth as measured by the release of plaque-forming virus at 30°C. (B) Viral RNA replication as measured by the incorporation of [^{3}H]uridine into acid-precipitable material in cells treated with actinomycin D. Chicken embryo fibroblasts grown in 35-mm dishes were infected with virus at a multiplicity of 60 at 30°C in the presence of actinomycin D (1 μg/ml). At 1 h after infection [^{3}H]uridine (20 μCi/ml) was added. At different times virus-containing medium was removed for virus titration, the cells were washed and lysed with 0.5% Triton X-100–0.5% naphthalene disulfonate, samples were acid precipitated, and radioactivity was determined.

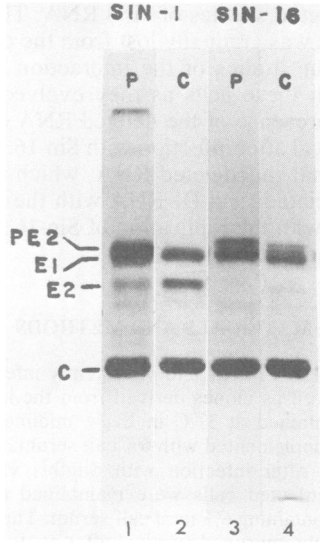

FIG. 2. Autoradiogram of [^{35}S]methionine-labeled intracellular viral proteins synthesized in Sin-1 virus- and Sin-16 virus-infected chicken embryo fibroblasts. Monolayers were infected at a multiplicity of 20 at 30°C. At 13 h postinfection the cells were starved for methionine for 1.5 h and then pulse-labeled with [^{35}S]methionine (20 μCi/ml). Lanes 1 and 3, Preparations pulsed for 30 min; lanes 2 and 4, preparations pulsed for 30 min and then chased for 1 h with 0.1 mM unlabeled methionine.

Generation of DI particles of Sin-16 virus by high-multiplicity passaging in BHK cells. Sin-16 virus is resistant to interference by those DI particles used to establish the persistent infection (27), as well as to interference by independently isolated DI particles generated from cloned Sindbis virus passaged on chicken embryo fibroblasts (unpublished data). This phenotype may be crucial to the survival of such a mutant virus in carrier cultures. However, since Sin-16 is highly cytopathic for BHK cells in standard infections, its replication in carrier cultures must be suppressed if the cells are to remain viable. We passaged Sin-16 at high multiplicity in BHK cells to determine whether Sin-16 can generate DI particles which inhibit its replication. Passaging was carried out at 30°C because of the temperature-sensitive phenotype of Sin-16 with respect to RNA synthesis (27). DI

TABLE 1. Sensitivity of early- and late-passage Sin-16 virus to DI particles generated from wild-type or Sin-16 virus

Virus[a]	DI particle source	Virus yield (PFU/ml)	
		Without DI particles	With DI particles
Wild type	Sin-16	7.5×10^9	1.9×10^8
Sin-16 (early passage)		1.9×10^8	1.7×10^8
Sin-16 (late passage)		1.3×10^9	1.1×10^8
Wild type	Wild type[b]	2.2×10^9	3.5×10^6[c]
Sin-16 (early passage)		3×10^8	3.8×10^8
Sin-16 (late passage)		1.7×10^9	1.4×10^9

[a] The helper viruses were used at a multiplicity of 5. Infections were carried out at 30°C. Wild-type Sindbis virus was grown for 17 h; the mutants were grown for 48 h.

[b] The wild-type stock used was purified on a sucrose gradient and UV irradiated to inactivate the infectious virus at a dose which did not affect the DI particle activity (27).

[c] The greater interference observed with wild-type DI particles compared with Sin-16 late-passage DI particles does not reflect a difference in DI particle inhibition properties between the two DI particle populations, but was the results of different amounts of DI particles being introduced in each case.

particles were generated based on decreased titers and the synthesis of defective RNA (data not shown). During passaging we observed an increase in plaque size for the standard Sin-16 virus. For this reason we cloned the virus from a DI-containing passage and compared its sensitivity to DI interference with that of a low-multiplicity-derived stock of early-passage Sin-16 (Table 1). The early-passage virus was resistant to Sin-16 virus-derived DI particles, whereas the late-passage virus and the wild-type Sindbis virus were sensitive to these DI particles. Both the original Sin-16 virus and the variant, however, were resistant to wild-type virus-derived DI particles. Thus, the late-passage Sin-16 virus had an interference pattern distinct from that of the wild-type virus. This virus was similar to early-passage virus with respect to RNA synthesis (Fig. 1B) and the defect in protein processing, even after 28 passages at high multiplicity.

Defective RNAs present in the persistently infected culture were not packaged. Since conventional high-multiplicity passaging did not yield a source of DI particles that were capable of interfering with the replication of early-passage Sin-16, we attempted to obtain such particles from the persistently infected culture from which Sin-16 was isolated. This culture synthesized a spectrum of high-molecular-weight defective RNAs (molecular weight range, 2×10^6 to 3×10^6). These RNAs were labeled with [^{3}H]uridine in the presence of actinomycin D, were polyadenylated (Fig. 3), and were Sindbis virus specific as determined by hybridization to cDNA probes derived from the 5′ and 3′ regions of the standard viral genome (Fig. 4, lane 2). A subset of these RNAs could be hybridized to a junction fragment cDNA probe (see above) spanning the region of 49S RNA containing the 5′ terminus of 26S RNA. None of these RNAs was detected in particles that were released into the culture medium by the persistently infected cells. Our standard criteria for detecting DI particles of Sindbis virus in the culture fluids of infected cells are (i) interfering activity and (ii) the ability of a preparation of virus presumed to contain DI particles to produce virus-specific RNAs distinct from the standard 49S and 26S species in infected cells. Using these criteria, we were not able to detect DI particles either by direct interference (Table 2) or by examination of the intracellular viral RNA pattern obtained in cells coinfected with standard helper virus and virus concentrated from the medium of the persistently infected culture (data not shown).

Cloning of cells from the persistently infected culture. Our rationale for cloning cells from the culture was twofold. First, a likely explanation for the heterogeneous pattern of viral RNAs (Fig. 3) was that individual cells produced different species of cell-associated defective RNAs; thus, each clone should produce a more homogeneous population of RNAs. Second, cloning should give rise to some cells which are cured, and these could be tested for alterations in susceptibility to virus infection.

We initially examined 16 independent clones which fell into three classes based on sensitivity to infection by Sindbis virus. The majority of these (11 clones) were completely resistant to

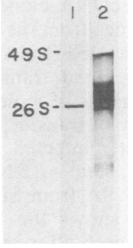

FIG. 3. Electrophoretic analysis of glyoxal-denatured RNAs obtained from BHK cells persistently infected with Sindbis virus for 16 months: polyadenylic acid-selected RNAs from wild-type Sindbis virus-infected BHK cells (lane 1) and uncloned persistently infected BHK cells (lane 2).

infection, as was the parent culture. Three of these clones were analyzed further and were found to produce relatively homogeneous species of defective viral RNA (Table 3). Two of the clones were completely susceptible to Sindbis virus and by this criterion were considered cured. Initially, three clones were partially resistant to infection; because these clones were different from the parent culture, one of them (clone A3) was analyzed in more detail.

Properties of clone A3. Clone A3 cells were studied as soon after cloning as possible. Upon initial analysis (within a few weeks of cloning), the cells were found to be semipermissive for Sindbis virus (the titers of virus obtained after infection with wild-type Sindbis virus were 20-fold lower than the titers obtained with BHK cells), and after labeling with [^{3}H]uridine in the presence of actinomycin D, A3 cells synthesized a single RNA species, which was approximately one-half the size of genomic RNA; this RNA was designated 0.5 RNA (Table 3 and Fig. 5A, lane 1). This RNA was polyadenylated and hybridized specifically to a 5′ cDNA probe derived from Sindbis virus 49S RNA (Fig. 4, lane 3). The specificity of the cDNA was demonstrated by comparing polyadenylic acid-containing RNAs from the following types of cells: (i) cells infected with wild-type Sindbis virus, (ii) cells persistently infected for 16 months, (iii) A3 cells, and (iv) uninfected BHK cells. The RNA samples were denatured, electrophoresed through a 1% agarose gel, and then transferred to activated paper (23). To monitor transfer, ^{32}P-labeled rRNA was transferred simultaneously (Fig. 4, lane 1). The cDNA probe detected only the defective RNAs (Fig. 4, lanes 2 and 3) and standard genomic RNA (lane 5); it did not hybridize to 26S RNA, the predominant viral RNA present in BHK cells infected with Sindbis virus. The relative amounts of 49S and 26S RNAs in this extract are shown in the fluorogram of Fig. 4, lane 5, after reversal of hybridization and impregnation of the activated paper with 2,5-diphenyloxazole to detect the [^{3}H]uridine-labeled bands (Fig. 4, lane 6). No nonspecific hybridization to the oligo(dT)-selected BHK RNA was found (Fig. 4, lane 4). The 0.5 RNA was also detected with a cDNA probe specific for the 3′-terminal sequences of the RNA, but not with a probe that recognized the junction region which spans the 5′ terminus of the 26S RNA. Thus, Sindbis virus-specific RNA synthesis in clone A3 was dominated by the replication of a deleted RNA species. Although neither 49S RNA nor 26S RNA was detected by intrinsic labeling with [^{3}H]uridine or with the cDNA probes, we assume that the 0.5 RNA was synthesized by using the viral replicase encoded by genome length RNA. The ratio of DI RNAs to standard RNAs was usually high, and we would not have detected RNA molecules that were present at concentrations severalfold less than the concentration of the 0.5 RNA. With time in culture this RNA was gradually lost from A3 cells, and the semipermissive phenotype gave way to complete permissiveness. By 1 month after cloning, detectable levels of viral RNA were found in only a small fraction of A3 cells. This fraction was determined by autoradiogra-

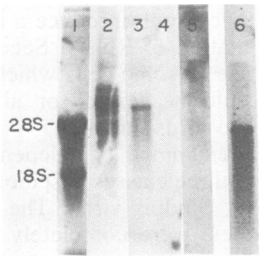

FIG. 4. Blot hybridization of polyadenylic acid-selected RNAs to a cDNA clone containing Sindbis virus-specific sequences from the 5′ end of 49S RNA. Lane 1, In vivo ^{32}P-labeled rRNAs that were included in the electrophoresis and transferred to activated paper as internal markers of molecular size and controls for the efficiency of transfer; lane 2, intracellular RNA from persistently infected BHK cells; lane 3, RNA from A3 cells; lane 4, RNA from uninfected BHK cells; lane 5, RNA from Sindbis virus-infected BHK cells. Lane 6 shows the same region of the activated paper as lane 5 after reversal of hybridization, impregnation with 2,5-diphenyloxazole, and fluorography, demonstrating that although 26S RNA was the predominant viral species present, it did not hybridize with the 5′ cDNA clone used as the probe. Approximately 1 μg of RNA was electrophoresed in lanes 3 through 5; 4 μg was used in lane 2.

TABLE 2. Interference assay to determine whether DI particles were released from persistently infected cells after varying periods of time in culture

Age of culture (mo)	Multiplicity of infection	% of control[a]
8[b]	0.03	0.05
9	0.05	1.4
16[c]	0.4	100

[a] BHK cells were coinfected with wild-type Sindbis virus at a multiplicity of 10 and virus obtained from persistently infected cells at the multiplicities indicated. Virus from these cells was concentrated by centrifugation (see text), and the titers were determined before use.

[b] In this experiment an interference titration assay gave a ratio of DI particles to standard virus particles of about 800:1.

[c] The virus was obtained after a one-cycle amplification of 16-month virus by passaging in BHK cells (see text).

TABLE 3. Properties of four cloned cell lines derived from a persistently infected culture

Cells	Superinfection sensitivity (cytopathic effect)	Virus release	Viral RNAs		Mol wt
			Standard	Defective	
BHK	+10	−	−	−	
A1	0	+	+	+	2.0×10^6 to 2.6×10^6
A2	0	−	+	+	3.0×10^6
A3	+2/+8[a]	−	−	+	2.0×10^6
A4	0	−	−	+	2.7×10^6

[a] Cytopathogenicity in A3 cells developed slowly over 48 h and was scored as +2 after 24 h and +8 after 48 h. The other cell clones did not change between 24 and 48 h.

phy of cells labeled with [^{3}H]uridine in the presence of actinomycin D for 4 h. The cells were coated with Kodak nuclear track emulsion (type NTB3), and after 11 days of exposure the population of cells which showed high cytoplasmic grain intensities was measured (Table 4). Only 5 to 6% of the A3 cells were scored as positive. This was a conservative estimate since high background values were a problem due to nuclear labeling (which could not be reduced by increasing the actinomycin D concentration due to toxicity to cells at concentrations above 2 μg/ml). By 3 months after cloning these cells appeared to be completely cured. The 0.5 RNA was no longer detectable by in vivo labeling even after polyadenylic acid selection (Fig. 5A, lane 2) or by hybridization with both 5′ and 3′ cDNA probes (data not shown).

Several lower-molecular-weight RNAs labeled with [^{3}H]uridine were observed in the oligo(dT)-selected fractions shown in Fig. 5A. These species were also present in uninfected BHK cells, as shown in Fig. 5B, in which the RNA from A3 cells (in culture from 2.5 months) is compared with RNA from uninfected, actinomycin D-treated BHK cells. The origin of these species in not known. Their molecular weights ranged from 1.2×10^6 to 3.4×10^5; the major species had molecular weights of 7×10^5, 4.5×10^5, and 3.8×10^5. They did not hybridize to the Sindbis virus-specific cDNA probes used in this study (Fig. 4, lanes 3 and 4). These species were much less prominent in lytically infected cells labeled in the same manner.

Infection of A3 cells with Sin-16 virus. Since A3 cells were susceptible to infection with Sin-16 virus, we determined whether they could be distinguished from normal BHK cells during the establishment of a persistent infection. Our specific goal was to ascertain whether there was a correlation between cell survival after infection with Sin-16 virus and the expression of the 0.5 RNA associated with A3 cells. We compared A3 cells which had been in culture for 1 month, the same cells after they had been in culture for 3 months, and BHK cells. Infection of each of these cultures with Sin-16 virus led to the establishment of a persistent infection. The length of time required for monolayers to return to confluence after infection was proportional to the extent of cell death. Thus, confluent monolayers of BHK cells infected with Sin-16 virus could be obtained approximately 17 days after infection. The A3 cells that had been in culture for 1 month grew into stable monolayers by 4 to 6 days after infection with Sin-16, whereas 3-month cultures took 16 to 18 days to reach confluency. All of these cultures produced virus and were resistant to superinfection, like the original carrier cell line.

The RNA species synthesized in the cultures persistently infected with Sin-16 in the presence

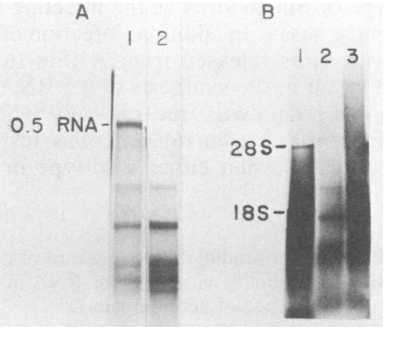

FIG. 5. Agarose gel electrophoresis of oligo(dT)-selected RNA from A3 cells. (A) One- and 3-month A3 cells (lanes 1 and 2, respectively) were labeled with [^{3}H]uridine in the presence of actinomycin D at 30°C for 17 h; the drug was added 1 h before the addition of label. After purification and polyadenylic acid selection, the RNAs were denatured in 1 M glyoxal and electrophoresed as described in the text (24,000 and 84,000 cpm were applied to lanes 1 and 2, respectively). (B) Comparison between the RNA pattern observed for 2.5-month A3 cells (lane 2) and the pattern observed for uninfected BHK cells (lane 3). In both cases the RNAs were labeled with [^{3}H]uridine in the presence of actinomycin D and polyadenylic acid selected by oligo(dT) chromatography. Lane 1 contained [^{3}H]uridine-labeled rRNAs from BHK cells, which were used as markers.

of actinomycin D are shown in Fig. 6. BHK(Sin-16) cells (Fig. 6, lane 2) synthesized predominantly 49S and 26S RNAs, as well as a class of large RNAs (molecular weights, 2.5×10^6 to 3×10^6). No band was observed in the region of 0.5 RNA (molecular weight, 2.0×10^6). The 1-month A3 cells which produced 0.5 RNA before infection (Fig. 5A) synthesized this RNA in higher amounts after infection and also synthesized 49S and 26S RNAs (Fig. 6, lane 3). In contrast, the 3-month A3 cells infected with Sin-16 closely resembled BHK(Sin-16) cells; no 0.5 RNA was made in either culture (Fig. 6, lane 4). Thus, there was a definite correlation between the fraction of cells that survived infection with Sin-16 and the expression of the 0.5 RNA species. The increased cell killing that occurred concomitant with the failure to detect the 0.5 RNA implies that this deleted RNA suppressed Sin-16 replication in a manner like that observed for DI particle RNAs; it further suggests that cured A3 cells were not mutant BHK cells with an enhanced ability to survive in the presence of Sin-16 virus.

The 0.5 RNA synthesized in A3 cells infected with Sin-16 was not packaged. As discussed above, we were unable to detect DI particles in the extracellular fluids from persistently infected BHK cells (Table 2). Using similar techniques, we could not demonstrate any interference activity in virus preparations obtained from A3 cells persistently infected with Sin-16 by using wild-type or Sin-16 virus as the infecting helper virus in the assay. In addition, infection of BHK cells with virus released from A3(Sin-16) cells did not result in the synthesis of 0.5 RNA (data not shown). This was true not only when the virus from the A3(Sin-16) cells was tested by itself, but also when either wild-type or Sin-1 virus was included in the infecting inoculum as a helper virus.

TABLE 4. Autoradiographic measure of cells synthesizing Sindbis virus-specific RNA in the presence of actinomycin D[a]

Cells	Total no. of cells scored	% Positive[b]
BHK	838	<0.2
BHK infected with Sindbis virus[c]	860	86
A3 (1 month in culture)	2,938	5–6

[a] Cells were grown on cover slips in 35-mm dishes and were exposed to actinomycin D (2 μg/ml) for 2 h before labeling with [^{3}H]uridine (10 μCi/ml).

[b] Only cells showing a heavy concentration of cytoplasmic grains were scored as positive after an 11-day exposure with type NTB3 liquid emulsion.

[c] Cells were infected with Sindbis virus at a multiplicity of 100 in the presence of actinomycin D at 30°C; 2 h after infection [^{3}H]uridine was added, and incorporation was allowed to continue for 4 h.

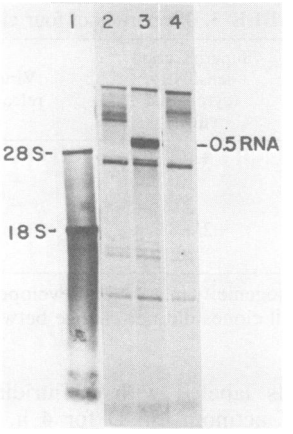

FIG. 6. Agarose gel electrophoresis of Sindbis viral RNAs: RNAs from A3 and BHK cells persistently infected with Sin-16 virus. Lane 1, rRNA; lane 2, BHK (Sin-16) cells; lane 3, 1-month A3(Sin-16) cells; lane 4, 3-month A3(Sin-16) cells. All cultures were treated with actinomycin D (1 μg/ml) for 1 h before labeling with [^{3}H]uridine (20 μCi/ml) for 17 h at 30°C.

We also infected A3 cells with Sin-1 virus, a virus obtained from BHK cells 1 month after the establishment of the persistent infection (26), and obtained persistently infected cells which had an RNA profile identical to that shown in Fig. 6 for A3(Sin-16) cells. The 0.5 RNA was not present in virus released from these cells.

DISCUSSION

DI RNAs can play an important role in establishing and maintaining a persistent viral infection in cultured cells. Several factors may intercede to permit the continued survival of both cells and virus. Thus, the suppression of virus replication by DI particles should favor the elimination of the virus, whereas the selection of mutants resistant to the original DI RNAs should enhance virus survival. Changes related to interference by DI RNAs are not the only changes that occur in persistently infected cultures. The standard virus may become less cytopathic (26), and, in the case of vesicular stomatitis virus, mutationally altered viruses that are able to interfere with the replication of the original virus appear in the population (24). Furthermore, as described by Ahmed et al. for reovirus-infected L cells (1), the host cells may also become less susceptible to the cytopathic effects of the virus. In these studies the selection of mutant cells and the selection of interfering infectious virus were not immediate events required for the establishment of persistence, but

occurred after extended cultivation of the infected cells.

In the persistent infection which we followed, DI particles were particularly prominent in the regulation of virus expression and in the evolution of viruses with altered sensitivity to interference by these particles (26, 27). Mutant virus Sin-16, which was isolated from this cell line after 16 months of culture, is resistant to interference by DI particles and is highly cytopathic for normal BHK cells. The stability of the persistently infected cell population from which this virus was derived and the abundance of defective RNAs observed within these cells suggested that these RNAs were capable of suppressing Sin-16 replication. However, major changes in the DI population occurred during prolonged cultivation of these cells. The DI RNAs shifted in size from being smaller than 26S RNA earlier in the history of the culture to being significantly larger than 26S RNA. In addition, these RNAs, although expressed at high levels intracellularly, were not packaged. Therefore, a direct demonstration of the ability of these molecules to interfere with Sin-16 virus replication was not possible. The large size of these RNAs cannot account for the inefficient packaging since DI RNAs of this size have been observed in virus populations after lytic passaging (4). Thus, some structural property of these DI RNAs must be different.

Our attempts to generate de novo DI particles to which Sin-16 was sensitive (by high-multiplicity passaging of the mutant) led to the selection of a variant of Sin-16 before the accumulation of DI particles. These DI particles were not able to interfere with the initiating stock of Sin-16 but did inhibit replication of the variant. To determine whether there were DI RNAs to which Sin-16 was susceptible, we turned to the persistently infected cells from which this virus was derived. Cloning of these cells provided a cell culture in which we could examine the interaction of Sin-16 with a strongly cell-associated defective RNA.

Cells cloned from persistently infected cultures may be cured of the infection (1, 8). This was true for a small percentage (12%) of the clones that we analyzed, but several clones showed responses to challenge with wild-type Sindbis virus that were intermediate between the responses of cured cells and the responses of persistently infected cells. These clones could be infected with Sindbis virus with the induction of mild cytopathogenicity, in marked contrast to the parent carrier cells, which were resistant to superinfection. An analysis of one such clone, clone A3, showed that a single deleted Sindbis virus-specific RNA species, 0.5 RNA, was made in these cells. Autoradiography of individual cells showed that within 1 month after cloning only a fraction (5 to 6%) of the cells was synthesizing viral RNA. After more extended growth of the cells, the 0.5 RNA characteristic of this clone was no longer detectable either by in vivo labeling or by blot hybridization. Thus, the A3 cell clone appeared to be gradually cured of Sindbis virus-related RNA. These cured A3 cells were as susceptible as BHK cells to cell killing by Sin-16 virus. In both cases the survivors were few, and repopulation of the cultures was slow. In contrast, A3 cells that expressed the 0.5 RNA were more resistant to cell killing. A larger fraction of cells survived infection, and repopulation occurred rapidly. The additional observation that the increase in cell survival was correlated with enhanced synthesis of the 0.5 RNA in A3 cells persistently infected with Sin-16 indicated that Sin-16 was able to rescue this defective RNA. These survival studies provided strong, albeit indirect, evidence that the 0.5 RNA was a DI RNA which was able to protect cells from cytopathogenicity otherwise induced by Sin-16 virus. In addition, the results of these studies suggested that cured A3 cells were not genetically altered in susceptibility to Sin-16 virus.

The cell cultures resulting from infection with Sin-16 all produced virus and were resistant to superinfection, like the original parent cell culture. The A3 cells infected with Sin-16 further resembled the uncloned culture in that the defective 0.5 RNA could not be demonstrated in released virions under conditions that have been used successfully to demonstrate DI particles released from earlier persistently infected cultures (26). Infection of A3 cells which had been in culture for 1 month with another mutant of Sindbis virus, Sin-1 (26), also led to intracellular enrichment of the 0.5 RNA. In this case as well, the 0.5 RNA was not detected in extracellular particles although significant amounts of infectious virions were released.

Our interest in Sin-16 virus has been focused on its resistance to DI particles. This virus has at least one other distinct phenotypic characteristic; cells infected with Sin-16 virus are impaired in their ability to cleave the precursor of viral glycoprotein E2. It is difficult to know whether this phenotype confers any advantage on a virus in a persistently infected culture. Mutations in genes coding for membrane proteins appear to be common in viruses present in persistently infected cultures (13, 18, 19), suggesting that they are not selected against with the same rapidity as would be expected in standard cytopathic infections. The survival of a virus, such as Sindbis virus, in a persistent infection depends on its ability to be transmitted to most of the cells in the culture. The inefficiency with

which a virus could be released into the extracellular medium may have been a contributing factor in the curing of the cloned A3 cells with continued passaging. At the time that we first analyzed the A3 cells, the 0.5 RNA was the only detectable Sindbis virus-specific RNA in the cells. This RNA was extensively deleted; thus, its replication had to depend on viral enzymes provided by a helper virus present in the culture. The existence in A3 cells of a slow-growing, poorly expressed virus susceptible to interference by the 0.5 RNA could explain the eventual curing of cells through suppression of replication and dilution upon repetitive passaging. Infection of these cells with Sin-16 (or Sin-1) virus rescued cells that still retained the DI RNA. In the original uncloned population of persistently infected cells, reinfection of cells which may have lost the standard viral genome and complementation among virus mutants may have taken place continually to ensure the survival of virus during long-term cultivation.

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