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Suppression of Multiplication of Avian Sarcoma Virus by Rapid Spread of Transformation-Defective Virus of the Same Subgroup

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We have tested the hypothesis that some transformation-defective (*td*) viruses grow faster than the avian sarcoma viruses (ASV) from which they are derived, resulting in establishment of interference by the *td* virus and suppression of the ASV multiplication. Using an ASV of subgroup A (ASV-A) that does not contain *td* virus and an independently isolated *td*ASV-A, we performed separate and mixed infections to test this hypothesis. At multiplicities of 1 or less, *td*ASV alone grew to higher titers and more rapidly than ASV alone. In mixed infections at low multiplicities that allowed spread of progeny virus, when as little as 10% of the virus inoculum was *td* virus, there was an excess of *td* virus by 2 days after infection and a decrease in the titer of ASV relative to a control infection with no *td* virus. In mixed infections at high multiplicities which minimized spread of progeny virus, there was no excess of *td* virus and the titer of ASV was not decreased relative to the control infection with no *td* virus. These data support the hypothesis that we proposed and indicate that deletions in the ASV *src* gene may not be a high-frequency event. We also present data concerning the amounts of unintegrated viral DNA found after the separate and mixed infections. There was no simple correlation between the amounts of unintegrated viral DNA early after infection and the titers of virus produced, indicating perhaps that virus production was determined by integrated viral DNA.

Stocks of avian sarcoma virus (ASV) often contain a second kind of virus (transformation-defective or *td* virus) which lacks the capacity to transform fibroblasts (7, 11, 16, 17, 22, 23). The RNA of *td* viruses is typically 10 to 15% smaller than that of the wild-type viruses (23). Moreover, the amount of *td* virus often exceeds the amount of nondefective virus by a factor of 10 or more.

Attempts to free the transforming virus of the *td* virus by isolating single foci of transformed chicken embryo fibroblasts after infection at low multiplicities (less than 0.001 focus-forming unit [FFU] per cell) sometimes yield foci which retain a high ratio of *td* virus. In addition, sometimes foci initially have low or undetectable levels of *td* virus, but nevertheless the *td* virus reappears in high ratios when the virus is passaged further through cells (7, 11, 16, 17, 22). Data of this type have been interpreted to indicate a high rate of spontaneous deletions in the ASV *src* gene. However, this hypothesis fails to account for the fact that virus stocks from other foci, if passaged at multiplicities of infection (MOIs) of one or less, remain free of *td* virus, as does virus cloned by more rigorous methods such as plating transformed cells in soft agar (16) or transfecting cells with unintegrated electrophoretically purified ASV DNA (our unpublished observations).

We propose an alternate explanation for these data. Our hypothesis is that some *td* viruses grow at a faster rate than the focus-forming viruses from which they are derived. The faster growth rate results in: (i) rapid spread of the *td* virus in cultures of chicken embryo fibroblasts and (ii) decrease in the titer of focus-forming virus produced by cells infected at low multiplicities with both *td* virus and focus-forming virus of the same subgroup. This decrease is the result of interference (15, 24) established by the more rapidly spreading *td* virus. Under this hypothesis, even a very small amount of *td* virus "contamination" of either a single focus picked under agar or a population of cells infected with ASV "only," can lead to an excess of *td* virus and suppression of ASV multiplication.

This paper tests this hypothesis by determining, at various MOIs, the relative multiplication

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of one pair of *td* and focus-forming viruses of the same subgroup after separate and mixed infections.

Unintegrated viral DNA accumulates early after infection of avian cells by retroviruses (1–3, 9). To determine whether there is any correlation between the amounts of unintegrated viral DNA early after infection and the titers of virus observed in these experiments, we also determined the amounts of unintegrated viral DNA produced in cells separately or mixedly infected with *td* and focus-forming viruses of the same subgroup.

MATERIALS AND METHODS

Cell cultures. Cells were propagated in Temin-modified Eagle minimal essential medium (Schwarz/Mann, Orangeburg, N.Y.) containing 20% tryptose phosphate broth and supplemented with 2% fetal bovine and 2% calf sera (referred to as complete medium). Fertile chicken eggs were obtained from SPAFAS, Storrs, Conn., and primary cultures of fibroblasts were prepared from 12-day-old embryos by standard techniques (19, 20). The chicken embryos were C/E and negative for avian leukosis virus, chicken helper factor (4, 24), and group-specific antigen of avian leukosis virus (12).

Viruses. The strain of virus used in this study is a descendant of the B77 virus used in previous studies from this laboratory (6, 26). At some time, recombination with a subgroup A avian leukosis-sarcoma virus probably occurred, yielding an ASV of subgroup A as determined by assay on C/A chicken cells and interference with Rous-associated virus-1. Therefore, this virus is now of subgroup A and is called ASV-A.

Rous-associated virus-61 (RAV-61) virus was originally obtained from H. Hanafusa.

Virus stocks. A stock of the ASV-A was obtained in the following manner. Cultures of chicken embryo fibroblasts were inoculated with ASV-A at a very low MOI (less than 0.001 FFU/cell). After a 40-min incubation at 37°C, the cells were overlaid with complete medium containing 0.36% agarose. At 4 days after infection, cultures were fed with 2 ml of complete medium containing agarose. At 7 to 10 days after infection, foci of morphologically transformed chicken embryo fibroblasts were picked, using small-bore Pasteur pipettes. Foci were frozen and thawed three times and sonicated briefly to disrupt viral aggregates.

The virus designated ASV-S1 originated from a culture containing a single focus. The initial stock of ASV-S1 contained no excess of *td* virus. Even after many passages at MOIs of less than 1, *td* virus has never been found. However, once after passage at an MOI of 5 FFU/cell, *td* viral DNA was detected. However, there was no excess of *td* virus.

A second focus (among 20 that were picked), designated S4, was titrated and found to have *td* virus in excess of the focus-forming virus. An initial stock of this *td* virus, designated *td*ASV-S4, was obtained from a single culture beyond the endpoint of ASV-S4 focus formation.

Virus assays. Focus-forming virus was titrated in chicken embryo fibroblasts by making serial 10-fold dilutions and counting foci on replicate culture plates (usually three or four per dilution). Each culture dish containing 3×10^5 chicken embryo fibroblasts received 0.2 ml of inoculum containing virus in Eagle medium and 15 μg of Polybrene (Aldrich Chemical Co., Milwaukee, Wis.) per ml. The standard error of an individual titration is about 10%.

td virus was titrated in chicken embryo fibroblasts in the same manner as was focus-forming virus except that the *td* virus was scored on the basis of the presence of DNA polymerase activity (21). A minimum of 1 DNA polymerase-forming unit (DPFU) per 0.2 ml is assumed at the last dilution giving a positive response. The sensitivity of the FFU and DPFU assays are the same for an ASV (data not shown).

DNA extraction and purification. For analysis of unintegrated viral DNA, infected cells were subjected to the Hirt fractionation procedure (5). DNA was purified from the Hirt supernatant fractions in the following manner. Pronase (self-digested for 2 h at 37°C) was added to a final concentration of 250 μg/ml, and each lysate (from three cultures) was incubated at 37°C for 30 min and extracted twice with redistilled phenol saturated with 0.01 M Tris-hydrochloride (pH 7.3). The final aqueous phase was precipitated with 2 volumes of ice-cold 100% ethanol. The precipitate was dissolved in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and RNase A was added to a final concentration of 100 μg/ml. The extract was incubated for 30 min at 37°C, and pronase treatment, phenol extraction, and ethanol precipitation were repeated as before. The DNA precipitate was again dissolved in 0.1× SSC and concentrated by lyophilization.

Agarose gel electrophoresis and blotting. DNAs were electrophoresed through 6-mm-thick, horizontal 0.7% agarose slab gels containing 0.04 M Tris (pH 7.5), 0.05 M sodium acetate, 0.001 M disodium EDTA, and 5 μg ethidium bromide per ml.

Fragments of bacteriophage λ DNA after digestion with restriction endonuclease *Eco*RI (a gift from S. Mizutani) served as molecular mass markers. Also included as a molecular mass marker was *Eco*RI-digested plasmid pBR313, obtained from F. Blattner. *Eco*RI cuts pBR313 once, yielding a double-stranded linear molecule of about 6.0×10^6 daltons.

Blotting of DNA onto nitrocellulose filter paper was performed as described by Southern (14).

Hybridization probe. RAV-61 RNA was extracted from concentrated virions, and 60 to 70S RNA was isolated by centrifugation through 15 to 30% glycerol gradients (25). Complementary DNA (cDNA) made from a RAV-61 RNA template recognizes only the *gag* and *pol* gene nucleotide sequences of ASV-S1 and *td*ASV-S4 DNAs (that is, the 5′ half of the genome). This cDNA has no bias in detecting ASV-S1 and *td*ASV-S4 viral DNAs.

[^{32}P]cDNA (calculated specific activity of approximately 4×10^8 cpm/μg) was synthesized by avian myeloblastosis virus reverse transcriptase with oligomers of calf-thymus DNA (18) as primers and 70S RAV-61 RNA as template. Reactions were carried out at 37°C for 2 h in a final volume of 300 μl containing the following: 40 mM Tris-hydrochloride (pH 8.3); 10

mM $MgCl_2$; 50 mM KCl; 5 mM dithiothreitol; 0.3 mM each dATP, dGTP, and dTTP; 200 μCi of [α-^{32}P]dCTP (Amersham Corp., Arlington Heights, Ill.; 300 Ci/mmol); 25 μg of actinomycin D (a gift from Merck Sharp & Dohme, West Point, Pa.) per ml; 1 mg of primers; 3 to 5 μg of viral RNA heated to 85°C for 2 min; and 90 U of avian myeloblastosis virus DNA polymerase. Reaction mixtures were extracted with phenol saturated with 10 mM Tris-hydrochloride (pH 7.3) and with chloroform-isoamyl alcohol (24:1). Unreacted labeled dCTP was removed by repeated ethanol precipitations, and the RNA template was hydrolyzed with 0.3 N NaOH for 18 h at room temperature.

Hybridization conditions. Presoaking of baked nitrocellulose filters and hybridization were carried out in sealed polyethylene bags at 41°C. Presoaking was for 16 to 20 h in a solution containing 50% formamide, 3× SSC, 0.02% polyvinylpyrollidone, 0.02% Ficoll, 0.02% bovine serum albumin, 50 μg of torula yeast RNA per ml, and 20 μg of denatured salmon sperm DNA per ml (13).

Hybridization was for 60 to 72 h at 41°C in the presoak buffer with the addition of 1×10^6 to 5×10^6 cpm of the cDNA per ml.

Filters were washed in the manner described by Shank et al. (13).

Autoradiofluorography and densitometry scanning. Autoradiofluorography was carried out at −70°C with Kodak X-omat X-ray film with the use of Ilford fast tungstate enhancing screens (10). The exposure time varied from 6 h to 7 days in different experiments.

Autoradiofluorograms were scanned with a Joyce-Loebel microdensitometer. (The DNA from *td*ASV-S4, 5.6×10^6 daltons, migrates faster than the DNA from ASV-S1, 6.1×10^6 daltons.) Under hybridization conditions similar to those used throughout this paper, a linear relationship was obtained between densitometry scannings of autoradiofluorograms and amounts of input, hybridizable viral DNA (8; data not shown).

RESULTS

Multiplication of ASV-S1 and *td*ASV-S4. To determine how quickly each virus multiplies after separate infection under conditions allowing cell-to-cell spread of progeny virus, separate infections were performed with ASV-S1 and *td*ASV-S4 at three different MOIs: 0.01, 0.1, and 1 FFU or DPFU/cell.

The titers of supernatant virus, expressed as FFU or DPFU/cell, are shown in Fig. 1A. (The data shown in Fig. 1B concerning the amounts of viral DNA in this experiment will be discussed later.) By 24 h after infection, the *td*ASV-S4 titers were higher than those of ASV-S1. At 66 h after infection, there was a 100-fold or greater difference. In addition, at 24, 48, and 66 h after infection, the titers of *td*ASV-S4 at all 3 MOIs were about the same. In contrast, ASV-S1 titers were not equivalent until 66 h after infection.

These data demonstrate that, at MOIs of 1 and less, *td*ASV-S4 alone grows to higher titers and more rapidly than does ASV-S1.

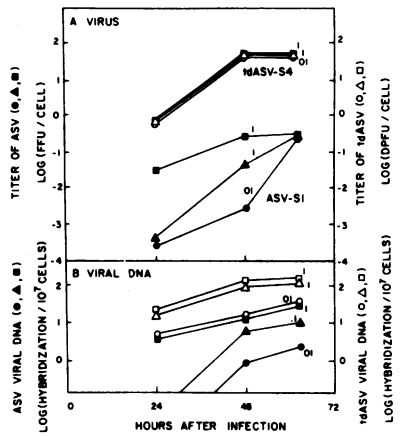

FIG. 1. *Multiplication of ASV-S1 and tdASV-S4. (A) Virus. Three sets of 12 cultures containing 2×10^6 chicken embryo fibroblasts were exposed to ASV-S1 at MOIs of 0.01, 0.1, and 1 FFU/cell, respectively. Three additional sets of 12 cultures of chicken embryo fibroblasts were exposed to tdASV-S4 at MOIs of 0.01, 0.1, and 1 DPFU/cell, respectively. In all experiments, supernatant virus was harvested at 24, 48, and 66 h after infection, and the cells from one culture were counted with the aid of a Coulter Counter. The medium was changed at 24 and 48 h after infection. Frozen virus samples were thawed and titrated simultaneously at a later time. Focus-forming titer designations in the figure are: ASV-S1 MOIs = 0.01 (●), 0.1 (▲) and 1 (■). DNA polymerase-forming titer designations are: tdASV-S4 MOIs = 0.01 (○), 0.1 (△), and 1 (□). (B) Unintegrated linear viral DNA. In all of the experiments described in (A), three cultures at each time were processed as described in the text, and purified Hirt supernatant DNA from 10^7 cells was electrophoresed in 0.7% (wt/vol) agarose slab gels and transferred to nitrocellulose filters by the method of Southern (14). Viral nucleotide sequences were detected by molecular hybridization with RAV-61 cDNA. Autoradiofluorograms were scanned, and the areas within the recorded peaks at positions corresponding to 6.1×10^6 daltons (ASV-S1 linear DNA) and 5.6×10^6 daltons (tdASV-S4 linear DNA) were measured in arbitrary units. EcoRI restriction fragments of λ DNA and of plasmid pBR313 served as molecular mass markers. The symbols used are as in (A) and indicate the amount of viral DNA of 6.1×10^6 daltons for ASV-S1 and the amount of viral DNA of 5.6×10^6 daltons for tdASV-S4.*

Decrease in the focus-forming titer of ASV-S1 at 90 h after infection with increasing MOI of *td*ASV-S4. We next determined whether mixed infection of chicken embryo fibroblasts with *td* and focus-forming viruses of the same subgroup resulted in a decrease in the titer of the focus-forming virus and an excess of the *td* virus. The MOI in these experiments was

again low to allow both viruses a chance to spread to uninfected cells.

Each of five cultures of chicken embryo fibroblasts was exposed to 0.2 ml of ASV-S1 at an MOI of 0.1 FFU/cell. The cultures were simultaneously exposed to *td*ASV-S4 at different MOIs so that the proportion of ASV-S1 in the total inoculum varied from 0.9 to 0.09. In addition, two control infections were made. In the first, cells were infected solely with ASV-S1 at an MOI of 0.1 FFU/cell. In the second, cells were infected solely with *td*ASV-S4 at an MOI of 1 DPFU/cell.

At 90 h after infection, supernatant media were harvested and virus titers were determined (Fig. 2A). (The data for Fig. 2B concerning the amounts of viral DNA in this experiment will be discussed later.) The titer of the focus-forming virus in the presence of *td* virus was decreased fivefold relative to the control with no *td* virus. In addition, a 3- to 10-fold excess of *td* virus was found in all mixed infections.

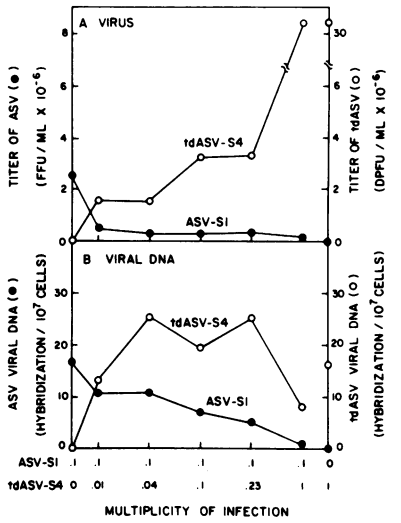

FIG. 2. *Mixed infection of chicken embryo fibroblasts with ASV-S1 and tdASV-S4. (A) Virus. Cultures of 2 × 10⁶ chicken embryo fibroblasts were inoculated with 0.2 ml of ASV-S1 at a titer of 10⁶ FFU/ml. The same cultures were also infected with 0.2 ml of different dilutions of a tdASV-S4 stock with a titer of 5 × 10⁷ DPFU/ml. In this way, the MOI of ASV-S1 was 0.1 FFU/cell in all of the mixed infections, whereas the MOI of tdASV-S4 varied from 0.01 to 1 DPFU/cell. In the control infections, parallel cultures of 2 × 10⁶ chicken embryo fibroblasts were infected with either ASV-S1 at an MOI of 0.1 FFU/cell or tdASV-S4 at an MOI of 1 DPFU/cell. After a 40-min incubation, 6 ml of Eagle medium with 20% tryptose phosphate broth, 2% calf serum, and 2% fetal calf serum was added. A subsequent change to the same medium was performed at 66 h after infection. Supernatant virus from each culture was harvested at 90 h after infection and frozen for later assay. Symbols: ●, ASV-S1 FFU/ml; ○, tdASV-S4 DPFU/ml. (B) Unintegrated viral DNA. For each culture described in (A), unintegrated viral DNA was prepared and quantified as described in the legend to Fig. 1. Symbols: ●, ASV-S1 linear DNA; ○, tdASV-S4 linear DNA.*

This experiment demonstrates a decrease in the titer of ASV-S1 after mixed infection with as little as 10% *td* virus. Since these mixed infections were done at a low MOI, spread of virus to uninfected cells occurred. Rapid spread of the *td* virus and establishment of interference (that is, *td* virus-infected cells become immune to superinfection by ASV or *td* viruses of the same subgroup [15, 24]) would result in fewer cells becoming infected with ASV-S1 than in the absence of *td* virus and, therefore, in the lower focus-forming titer of ASV-S1.

In other experiments at low MOI in which ASV-S1 was initially in large excess relative to the *td* virus (200- and 2,000-fold), there was no excess of *td* virus observed at 92 h after infection (Table 1). In addition, when cells were infected solely with ASV-S1 at an MOI of 0.1 FFU/cell and 24 h later with *td*ASV-S4 at an MOI of 0.05 DPFU/cell, there was no excess of *td* virus at 92 h after infection (Table 1).

These data demonstrate that *td* virus was not able to grow to excess by 92 h after infection under conditions that favored the spread of ASV-S1, that is, a large initial excess of ASV-S1 or a 24-h delay of *td*ASV-S4 infection. Under these circumstances, ASV-S1 apparently spread to all uninfected cells before the *td* virus.

TABLE 1. *Mixed infections with ASV-S1 in large excess of tdASV-S4 or after a 24-h delay of tdASV-S4 infection*

Virus MOI[a]		FFU/ml	DPFU/ml	Excess *td* virus
ASV-S1	*td*ASV-S4			
0.1	0.00005	5×10^6	$\leq 5 \times 10^6$	No
1	0.005	5×10^6	$\leq 5 \times 10^6$	No
0.1	0.05	3×10^6	3×10^7	Yes
0.1	0.05 (24 h after infection)[b]	5×10^6	$\leq 5 \times 10^6$	No

[a] Chicken embryo fibroblasts were infected at the indicated multiplicities. At 48 h after infection, culture media were replaced. At 92 h after infection, culture media were harvested and frozen for assay at a later time.

[b] Chicken embryo fibroblasts were initially infected solely with ASV-S1 at an MOI of 0.1 FFU/cell. After 24 h, the culture medium was removed, and the cells were infected with *td*ASV-S4 at an MOI of 0.05 DPFU/cell. At 48 h after ASV-S1 infection, the culture medium was replaced. At 92 h after infection, the culture medium was harvested and frozen for later assay.

Amounts of *td* and focus-forming viruses at early times after mixed infection at low MOIs. To determine how quickly *td*ASV-S4 overgrows ASV-S1 in mixed infections where virus spread can occur, two experiments were performed. In the first, chicken embryo fibroblasts were infected simultaneously with ASV-S1 at an MOI of 0.1 FFU/cell and with *td*ASV-S4 at an MOI of 0.01 DPFU/cell. In the second, the MOI of ASV-S1 was again 0.1 FFU/cell, but the MOI of *td*ASV-S4 was increased 10-fold to 0.1 DPFU/cell.

The virus titers at 1, 2, 3, and 4 days after infection are shown in Fig. 3A. (The data for Fig. 3B concerning the amounts of viral DNA in this experiment will be discussed later.) In both experiments, there was an excess of *td* virus by 2 days after infection.

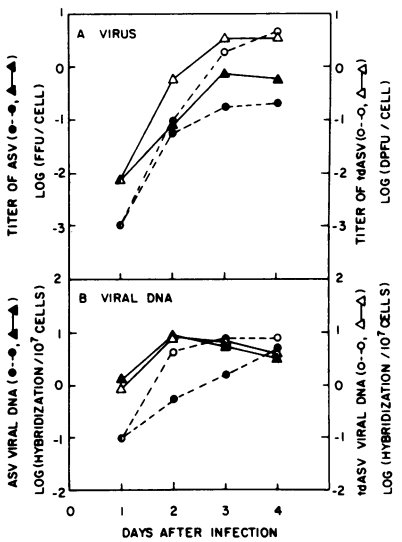

FIG. 3. *Virus titers and amounts of unintegrated linear viral DNAs at early times after mixed infection at low multiplicity with ASV-S1 and tdASV-S4. (A) Virus. Twenty cultures, each containing 2 × 10⁶ chicken embryo fibroblasts, were exposed to 0.4 ml of ASV-S1 at an MOI of 0.1 FFU/cell and tdASV-S4 at an MOI of 0.1 DPFU/cell (circles, broken line). At 1, 2, 3, and 4 days after infection, supernatant media were harvested and cells from one culture were counted. Media were changed at 2 days after infection. A parallel experiment was done simultaneously. The MOI of ASV-S1 was 0.1 FFU/cell, and the MOI of tdASV-S4 was 0.01 DPFU/cell (triangles, continuous line). Frozen virus samples from both experiments were thawed and titrated simultaneously later. Symbols: ○, tdASV-S4 at an MOI of 0.1; △, tdASV-S4 at an MOI of 0.01. (B) Unintegrated linear viral DNA. In both of the experiments described in (A), four cultures at each time were processed as described in the legend to Fig. 1. The symbols used are as in (A) and indicate the amounts of linear DNA hybridizing at 6.1 × 10⁶ daltons for ASV-S1 and the amounts of linear DNA hybridizing at 5.6 × 10⁶ daltons for tdASV-S4.*

These data demonstrate that, after mixed infection at low multiplicity under conditions allowing virus spread, *td* virus is in excess of focus-forming virus by 2 days after infection.

High-multiplicity mixed infection with ASV-S1 and *td*ASV-S4. To determine whether *td* virus would overgrow and suppress the multiplication of focus-forming virus under conditions of mixed infection when little or no virus spread could occur, a mixed infection at high multiplicity was performed. In addition, two separate control infections at high multiplicity were performed with ASV-S1 alone and with *td*ASV-S4 alone.

In the mixed infection experiment, chicken embryo fibroblasts were infected simultaneously with ASV-S1 at an MOI of 5 FFU/cell and *td*ASV-S4 at an MOI of 5 DPFU/cell. In the two separate infections, cells were infected either with ASV-S1 at an MOI of 5 FFU/cell or with *td*ASV-S4 at an MOI of 5 DPFU/cell.

The titers at 1, 2, and 3 days after infection for each experiment are shown in Fig. 4A and B. (The data for Fig. 4C and D concerning the amounts of viral DNA in this experiment will be discussed later.) The titers of focus-forming virus at all three times were nearly the same after both the mixed infection (Fig. 4B) and the ASV-S1 only infection (Fig. 4A), and there was no excess of *td* virus after the mixed infection.

These data demonstrate that overgrowth of *td* virus and suppression of multiplication of focus-forming virus after mixed infections do not occur under conditions in which cell-to-cell spread of *td* virus is minimized.

Amounts of ASV-S1 and *td*ASV-S4 unintegrated linear viral DNA after separate infections. Unintegrated viral DNA accumulates early after infection of avian cells with retroviruses (1-3, 9). To determine whether there was any correlation between the rapid spread of *td* virus and the suppression of focus-forming virus and the amounts of unintegrated viral DNA of ASV-S1 and *td*ASV-S4, the amounts of unintegrated viral DNA in the experiments described above were measured. Viral DNA was extracted by the Hirt procedure, purified, and separated by gel electrophoresis. The DNA was transferred to nitrocellulose filters and hybridized to [^{32}P]cDNA. The amounts of DNA were determined from autoradiograms.

In the experiments shown in Fig. 1A, it was demonstrated that *td*ASV-S4 alone, at MOIs of 1 and less, grew to higher titers and more rapidly than did ASV-S1. The amounts of unintegrated linear viral DNA present in these experiments were measured to determine whether there were

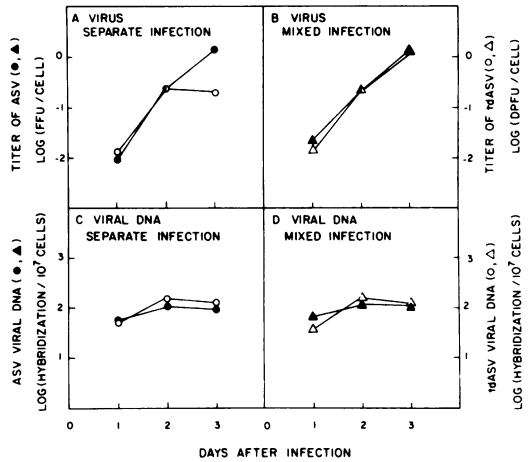

FIG. 4. *Mixed and separate high-multiplicity infections with ASV-S1 and tdASV-S4. (A) Virus (separate infection). Twelve cultures, each containing 2 × 10⁶ chicken embryo fibroblasts, were exposed to ASV-S1 at an MOI of 5 FFU/cell. Twelve additional cultures, each containing 2 × 10⁶ chicken embryo fibroblasts, were exposed to tdASV-S4 at an MOI of 5 DPFU/cell. In both experiments, supernatant virus was harvested at 1, 2, and 3 days after infection, and the cells from one culture were counted. Media were changed at 1 and 2 days after infection. Frozen virus samples were thawed and titrated simultaneously later. Symbols: ●, ASV-S1 FFU/cell; ○, tdASV-S4 DPFU/cell. (B) Virus (mixed infection). Twelve cultures, each containing 2 × 10⁶ chicken embryo fibroblasts, were simultaneously exposed to ASV-S1 at an MOI of 5 FFU/cell and tdASV-S4 at an MOI of 5 DPFU/cell. All cultures were processed in the same way as and simultaneously with those described in (A). Symbols: ▲, ASV-S1 FFU/cell; △, tdASV-S4 DPFU/cell. (C) Unintegrated linear viral DNA (separate infection). At 1, 2, and 3 days after infection in the experiments described in (A), three cultures were processed as described in the legend to Fig. 1. The symbols used are the same as in (A) and indicate the amount of viral DNA of 6.1 × 10⁶ daltons for ASV-S1 and the amount of viral DNA of 5.6 × 10⁶ daltons for tdASV-S4. (D) Unintegrated linear viral DNA (mixed infection). At 1, 2, and 3 days after infection in the experiments described in (B), three cultures were processed in the same way as and simultaneously with those described in (C). The symbols used are the same as in (B) and indicate the amount of viral DNA of 6.1 × 10⁶ daltons for ASV-S1 and the amount of viral DNA of 5.6 × 10⁶ daltons for tdASV-S4.*

also larger amounts of *td*ASV-S4 DNA than ASV-S1 DNA (Fig. 1B).

The amounts of *td*ASV-S4 DNA were higher at all MOIs and at all time points than the amounts of ASV-S1 DNA. When the same MOIs for each virus at 48 and 66 h after infection were compared, there was about a 10-fold difference in the amounts of viral DNA detected. At 66 h after infection, the amounts of unintegrated viral DNAs were different for each initial MOI, although the virus titers were the same (Fig. 1A).

The amounts of unintegrated linear viral DNA detected in cells infected with *td*ASV-S4 were greater than the amounts detected in ASV-S1-infected cells. This result parallels the difference in titers for the two viruses. However, these data also show that the amounts of unintegrated viral DNA measured are different even when the virus titers are the same.

Amounts of unintegrated linear viral DNA at 90 h after low-multiplicity mixed infection with ASV-S1 and *td*ASV-S4. The amounts of unintegrated linear viral DNA from the experiment shown in Fig. 2A were determined as described above and are shown in Fig. 2B. There was a progressive decrease in the amount of ASV-S1 DNA with an increasing initial MOI of *td*ASV-S4. There was about 10-fold more ASV-S1 DNA when there was no *td* virus present than when *td* virus constituted 90% of the initial virus inoculum. For each initial MOI, the amount of *td*ASV-S4 DNA after the mixed infection was greater than the amount of ASV-S1 DNA. However, the amounts of *td*ASV-S4 DNA found in these experiments did not follow any apparent pattern.

These data show that the amounts of ASV-S1 linear viral DNA decreased as the multiplication of ASV-S1 was inhibited by the rapid spread of *td*ASV-S4.

Amounts of unintegrated ASV-S1 and *td*ASV-S4 DNAs at early times after mixed infection at low multiplicities. *td*ASV-S4 was present in excess of ASV-S1 by 2 days after infection (Fig. 3A). To determine whether there was also an excess of *td*ASV-S4 viral DNA by 2 days after infection, the amounts of unintegrated linear viral DNA present in these experiments were measured (Fig. 3B).

In the experiment in which the ASV-S1 MOI was 0.1 FFU/cell and the *td*ASV-S4 MOI was 0.1 DPFU/cell, there was an excess of *td*ASV-S4 viral DNA by 2 days after infection. In this experiment, the excess of *td* viral DNA correlates with the excess *td* virus found at 2, 3, and 4 days after infection.

In the parallel experiment in which ASV-S1 was initially about 10-fold in excess (that is, the MOI of ASV-S1 was 0.1 FFU/cell and the MOI of *td*ASV-S4 was 0.01 DPFU/cell), there were about the same amounts of ASV-S1 and *td*ASV-S4 DNAs, although there was an excess of *td* virus (Fig. 3A). In this experiment, there was no correlation of excess *td* virus and excess *td* viral DNA.

Finally, the amounts of unintegrated closed

circular DNA corresponding to each virus in this experiment were determined. The relative amounts were similar to those of the unintegrated linear DNAs. However, the amounts detected were about 5- to 10-fold less (data not shown).

Amounts of ASV-S1 and *td*ASV-S4 unintegrated linear viral DNA in separate and mixed high-multiplicity infections. In Fig. 4A and B, it was shown that the multiplication of ASV-S1 was not suppressed and that *td*ASV-S4 did not grow to excess in a mixed infection at an MOI of greater than 1. The amounts of unintegrated linear viral DNA found in the mixed and separate high-multiplicity experiments are shown in Fig. 4C and D. The amounts of ASV-S1 and *td*ASV-S4 viral DNAs were about the same at 1, 2, and 3 days after both separate and mixed infections.

These data show that under apparent single-cycle growth conditions, that is, a high MOI resulting in fewer or no initially uninfected cells, the amounts of unintegrated ASV-S1 DNA early after infection were the same in the presence and in the absence of *td*ASV-S4. This result parallels that obtained for the virus titers (Fig. 4A and B).

DISCUSSION

In this paper we have presented data testing the hypothesis that some *td* viruses grow faster than the focus-forming viruses from which they are derived, resulting in the establishment of interference (15, 24) by the *td* virus and the suppression of multiplication of the focus-forming virus. Our data support this hypothesis.

Virus infections. We performed two types of infections with one pair of *td* and focus-forming viruses of subgroup A to test the hypothesis. In the first, cells were infected at MOIs of 1 or less. Since many cells were uninfected after the initial inoculation, progeny virus was able to spread to uninfected cells and, apparently, also to reinfect already infected cells before interference was established. Under these conditions, we observed how quickly *td* and focus-forming viruses multiplied (Fig. 1A). *td*ASV-S4 alone grew to higher titers and more rapidly than did ASV-S1 alone. In mixed infections at these MOIs, we tested the relative growth of each virus under conditions of competitive infection of uninfected and already infected cells. In the mixed infections shown in Fig. 2A, when as little as 10% *td* virus was present in the initial virus inoculum, the multiplication of ASV-S1 by 90 h after infection was suppressed fivefold relative to the control with no *td* virus. In the mixed infections shown in Fig. 3A, when *td*ASV-S4 constituted 10 or 50% of the initial virus inoculum, it was able to grow in excess of ASV-S1 by 2 days after infection. These experiments demonstrate that *td*ASV-S4, under conditions allowing competitive growth, is able to overgrow ASV-S1 and suppress its multiplication, presumably by establishing interference (15, 24).

If the conditions for mixed infection and competitive growth were altered in favor of ASV-S1, however, no excess of *td* virus was observed (Table 1). In two of the experiments shown in Table 1, the initial MOI of ASV-S1 (1 and 0.1 FFU/cell) was in large excess (200- and 2,000-fold) of the MOI of *td*ASV-S4 (0.005 and 0.00005 DPFU/cell); that is, *td*ASV-S4 constituted only 0.5 and 0.05% of the initial virus inoculum. In the presence of so little *td* virus initially, ASV-S1 was able to spread to the remaining uninfected cells and *td*ASV-S4 did not grow in excess by 92 h after infection. In another experiment shown in Table 1, infection with *td*ASV-S4 (at an MOI of 0.05 DPFU/cell) was delayed 24 h relative to infection with ASV-S1 (at an MOI of 0.1 FFU/cell). Again, there was no excess of *td* virus at 92 h after infection, whereas simultaneous infection at the same multiplicities resulted in a 10-fold excess of *td* virus. In the delayed infection, progeny ASV-S1 had a "head start" in spreading to uninfected cells. Therefore, there was no excess of *td* virus by 92 h after infection. These data also are consistent with the hypothesis in that we were able to prevent the appearance of an excess of *td*ASV-S4 by 92 h after infection by compensating for the slower growth of ASV-S1.

In the second type of virus infection, all cells were initially multiply infected. Under these conditions, spread of progeny virus is apparently minimized, since all cells quickly become immune to superinfection (15, 24). Under these conditions, the titer of ASV-S1 was the same in the presence (Fig. 4B) or in the absence (Fig. 4A) of *td*ASV-S4. In addition, after mixed infection, there was no excess of *td* virus (Fig. 4B). These data are consistent with our hypothesis in that there was no suppression of ASV-S1 and no excess of *td* virus under conditions that prevented spread of progeny *td*ASV-S4.

Cloning of ASV. The results in this paper lead to a method of testing stocks of ASV to determine whether they are free of *td* virus. Cloned ASV is passaged a few times at a low MOI and tested for an excess of *td* virus. In addition, infected cells are tested for the presence of unintegrated *td* viral DNA.

The results in this paper also provide a possible explanation for the high frequency of *src* deletions observed in studies of focal isolates of

ASV (7, 11, 16, 17, 22, 26). A small amount of *td* virus may have been present in "cloned" ASV, especially if the cloned virus was obtained by isolating a single focus of transformed chicken embryo fibroblasts. Such contamination was probably present in a previous study from this laboratory (26). The B77 virus-A used in this study was contaminated by *td* virus of subgroup C. Duck cells are resistant to B77 virus-A and sensitive to *td* virus of subgroup C. Pseudotypes of B77 virus-A with *td*B77 virus C envelopes were responsible for the high frequency of virus able to infect duck cells (unpublished observations).

Amounts of unintegrated viral DNA. In this paper we also presented data concerning the amounts of unintegrated ASV and *td*ASV DNA early after separate and mixed infections. These data show that there is no simple correlation between the amounts of supernatant virus and the amounts of unintegrated linear viral DNA. For example, in the separate growth experiments (Fig. 1A), the amounts of ASV-S1 DNA at 66 h after infection (Fig. 1B) were different for each initial MOI, despite the fact that virus titers (Fig. 1A) were the same. The amounts of *td*ASV-S4 DNA at 66 h after infection (Fig. 1B) were also different, but, again, the virus titers were the same (Fig. 1A). In the experiment shown in Fig. 2A, the titers of ASV-S1 in all of the mixed infections were about the same, but there was a 10-fold difference in the amounts of viral DNA (Fig. 2B). Moreover, the titers of *td*ASV-S4 steadily increased from the lowest MOI to the highest (Fig. 2A). However, the corresponding amounts of viral DNA did not follow a similar pattern (Fig. 2B). In both of the low-multiplicity infections shown in Fig. 3, there was an excess of *td* virus by 2 days after infection (Fig. 3A). In the experiment for which the MOI of ASV-S1 was 0.1 FFU/cell and that of *td*ASV-S4 was 0.1 DPFU/cell, there was an excess of *td* virus DNA by 2 days after infection. However, in the parallel experiment for which the MOI of ASV-S1 was 0.1 FFU/cell and that of *td*ASV-S4 was 0.01 DPFU/cell, there were equal amounts of viral DNA at all times, even though there was an excess of *td* virus by 2 days after infection.

We conclude from these data that although, in general, there are somewhat higher amounts of unintegrated *td*ASV-S4 DNA than of unintegrated ASV-S1 DNA, particularly under conditions allowing cell-to-cell spread of progeny virus, the relative amounts of unintegrated ASV-S1 and *td*ASV-S4 DNA are not reliable indicators of the amounts of each virus.

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