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Establishment of Infection by Spleen Necrosis Virus: Inhibition in Stationary Cells and the Role of Secondary Infection

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The relationship of two early events in the establishment of infection by avian retroviruses, the inhibition of viral DNA synthesis in stationary avian cells and the secondary infection which occurs after infection of replicating cells, was investigated. When neutralizing antibody to spleen necrosis virus was used to prevent secondary infection, the amount of unintegrated linear spleen necrosis virus DNA detected was much lower in infected stationary cells than in infected replicating cells. The amount of unintegrated linear spleen necrosis virus DNA in stationary cells was less than one copy per cell even at high multiplicities of infection. Viral DNA synthesis resumed after stimulation of the cells to replicate. The time of this viral DNA synthesis was closely correlated with renewed cellular DNA synthesis. In addition, blocking secondary infection of replicating cells prevented the rate of virus production from reaching the high levels usually associated with a normal productive infection by SNV. Virus production increased if secondary infection was allowed. However, this rise in virus production was not proportional to the amounts of viral DNA integrated after secondary infection.

Retrovirus replication is dependent upon events associated with the cell cycle. In particular, virus production is inhibited in stationary fibroblasts infected with retroviruses (2, 9, 17–19, 21). Viral antigens and viral RNA synthesis are lower in infected stationary cells (8, 9), and less viral DNA is present in infected stationary cells compared with infected replicating cells (4, 5, 10, 23, 24). It was concluded from these studies that the synthesis of viral DNA is inhibited in stationary cells and that this prevents subsequent steps in the establishment of a productive infection.

Recent studies on the early stages of avian retrovirus infection demonstrated that, during the first few days of infection of replicating chicken embryo fibroblasts, secondary rounds of infection occur, resulting in an accumulation of viral DNA, especially unintegrated linear viral DNA (11, 25, 26). In light of these results, the possibility arose that interpretations of amounts of viral DNA in previous experiments were complicated by secondary rounds of infection occurring in replicating cells, but not in stationary cells. Our initial results with spleen necrosis virus (SNV) demonstrated that the large increase in the amount of viral DNA upon stimulation of infected stationary chicken cells to replicate was abolished by neutralizing antibody to SNV (I. S. Y. Chen, Ph.D. thesis, University of Wisconsin, Madison, 1981). This neutralizing antibody had no deleterious effect on the cells or their ability to replicate (Chen, Ph.D. thesis, 1981). The lack of increase in amount of viral DNA, therefore, was the result of an inhibition by the antibody of virus spread and secondary infection. We reinvestigated whether viral DNA synthesis was inhibited in infected stationary cells by using neutralizing antibody to limit the infection to the primary infection.

Our results show that for the avian retrovirus SNV, under conditions where secondary infection is blocked, complete unintegrated viral DNA is not synthesized in infected stationary cells. However, viral DNA is synthesized in replicating cells and in infected stationary cells when replication is allowed to resume. These studies, therefore, confirm the conclusions of previous studies.

The effect of secondary infection on the rate of virus production from replicating cells was investigated. Our results show that secondary infection is necessary to achieve the high rates of virus production usually associated with a normal productive infection by SNV. The mechanism of the increased rate of virus infection was investigated by studying the kinetics of synthesis of viral DNA after secondary infection.

MATERIALS AND METHODS

Cells and viruses. The general sources and procedures for obtaining and propagating avian fibroblast cells have been previously described (21).

Cultures of stationary cells were prepared as follows. Chicken and duck embryo fibroblast cells were plated in 100-mm culture dishes at a density of 2×10^6 and 3×10^6 cells per plate, respectively. The cells were grown in Eagle medium with 3.0% fetal bovine serum for 2 to 4 days until the density was about 4×10^6 to 5×10^6 cells per culture. The medium was then changed to Eagle medium containing 0.1% fetal bovine serum. (Cells maintained in the complete absence of serum died or detached from the culture dish, or both.) The cells were then incubated for 2 to 3 days more before use. Longer periods of serum depletion did not further decrease the fraction of replicating cells and resulted in increasing cell death.

Stationary cells were stimulated to replicate by changing the medium to Eagle medium containing 6% calf serum and 4% fetal bovine serum.

SNV, a member of the avian reticuloendotheliosis virus species of retroviruses, has been previously described (21). Virus used in these experiments was prepared as follows. Chicken embryo fibroblast cells in 100-mm culture dishes were infected with SNV at a multiplicity of infection of about 0.1 PFU per cell and passaged twice so that all cells were infected. The cells were then rinsed twice with Eagle medium, and 5 ml of Eagle medium was replaced per culture. The medium was harvested 24 h later and stored at −70°C. The titers of virus obtained in this manner were about 10^8 PFU per ml.

SNV infectious titers were assayed as previously described by endpoint dilution of cytopathic effects or DNA polymerase activity (21).

Quantitative assay of amounts of SNV reverse transcriptase activity in culture medium was performed by a modification of a previous procedure (21). Debris was removed from culture medium by centrifugation at 1,500 rpm for 15 min in the model PR-2 International centrifuge. Culture medium was then centrifuged for 0.5 h at 18,000 rpm in a Beckman SW27 rotor or at 25,000 rpm in the SW41 or SW50.1 rotor. (All virus samples analyzed at one time were centrifuged in the same rotor.) After centrifugation, the virus pellet was suspended in 30 μl of a buffer containing 0.2% Nonidet P-40, 5 mM dithiothreitol, 1 mM EDTA, and 10 mM Tris-hydrochloride (pH 8.0); 25 μl of this sample was then added to 100 μl of the DNA polymerase reaction mixture, which consisted of 0.2 mM dATP, dTTP, and dGTP, 12.5 mM $MgCl_2$, 15 mM Tris-hydrochloride (pH 8.0), 0.2 mg of activated calf thymus DNA per ml, and 5 μCi of [α-^{32}P]dCTP (400 Ci/mmol). The reaction was incubated for 15 min at 37°C, and 30-μl portions were assayed for acid-precipitable radioactivity. All manipulations were performed at 4°C. Under these conditions, the amount of acid-precipitable counts was proportional to the amount of virus for amounts of infectious virus ranging from about 10^7 to about 10^9 PFU (data not shown).

Preparation of neutralizing antibody to SNV. SNV was purified by banding in sucrose density gradients (3). Four- to six-week-old New Zealand white male rabbits were injected subcutaneously with 1 mg of SNV. Starting 2 weeks later, the rabbits were injected with 250 μg of SNV at weekly intervals for a total of 4 weeks. Rabbits were bled and antibody was prepared. All antibody was incubated for 30 min at 56°C.

Preabsorption of the antibody with chicken cells was necessary to remove completely cytotoxic activity present in the antibody. The antibody was diluted 1:10 with Eagle medium and incubated on confluent monolayers of chicken embryo fibroblast cells, 3 ml per 100-mm culture dish. The cultures were rocked gently at 4°C for 1 h. This procedure was repeated three additional times.

The antibody was depleted of multiplication-stimulating activity (20) after dilution in Eagle medium to a final concentration of 1:50. Chicken embryo fibroblasts (3×10^6 per 100-mm culture) were grown in the presence of this diluted antibody, 5 ml per culture, for 3 days. This procedure was then repeated with fresh cells for 2 to 3 days until no mitotic cells were detected. The antibody was tested for multiplication-stimulating activity on stationary cells by autoradiography (see below).

Antibody was used in culture medium at a final concentration of 1:100. This concentration of antibody was sufficient to neutralize about 10^7 PFU of SNV per ml to less than 5 PFU per ml, after incubation for 0.5 h at 37°C.

Labeling and autoradiography of cells. Cells were labeled with 0.4 μCi of [*methyl*-^{3}H]thymidine per ml (50 to 80 Ci/mmol). For labeling periods longer than 24 h, the medium was changed, and fresh medium with label was added. After labeling, the cells were washed twice with cold phosphate-buffered saline solution and fixed with ethanol-acetic acid (3:1) for 0.5 h. The cells were rinsed twice with water and air dried.

Autoradiography of the cells was performed with Kodak Nuclear Track Emulsion (NTB3).

DNA extraction and purification. Unintegrated and integrated viral DNAs were prepared by the Hirt fractionation procedure (6) as previously described (3). Unintegrated viral DNAs to be analyzed by gel electrophoresis under denaturing conditions were treated with RNase A in 1 M NaCl to prevent hydrolysis of ribonucleotide linkages in the DNA (3).

Integrated viral DNA was further purified from unintegrated viral DNA by velocity sedimentation through a 5 to 20% NaCl gradient in an SW27.1 rotor for 11.5 h at 26,000 rpm.

Gel electrophoresis and DNA transfer to nitrocellulose. The details of gel electrophoresis were as previously described (3). Prior to gel electrophoresis, some samples were denatured with glyoxal as previously described (3). Molecular sizes were determined using restriction enzyme-digested lambda DNA and pBR322 DNA as markers.

Transfer of DNA from agarose gels onto nitrocellulose filter paper was performed as described by Southern (16).

Molecular hybridization. Hybridization probes were prepared by nick translation of pBR60BSa1 DNA (14) (specific activity, approximately 10^8 cpm/μg) essentially as previously described (15), but with the modification that DNase I treatment and DNA polymerase I treatment were performed separately for 1 h each at 14°C.

Presoaking and hybridization of DNA immobilized on nitrocellulose filters were performed as previously described (3). Filters were washed and exposed as previously described (3).

Quantification of viral DNA was performed by comparing the area of densitometry tracings of viral DNA samples with that of serial dilutions of the SNV molecular clone, 14-44 (14), digested with *Sac*I. Only

samples subjected to electrophoresis on the same gel were compared. The efficiency of transfer from the gel to nitrocellulose filter paper was comparable for genome-size SNV DNA (8.3 kilobase pairs) and SNV DNA digested with *Sac*I (7.0 kilobase pairs). Integrated viral DNA was first digested with *Sac*I before electrophoresis.

RESULTS

Quantitative studies of viral DNA synthesis in stationary cells compared with replicating cells require that two conditions be controlled. The first of these is secondary infection by the virus. To limit the virus infection to the primary infection, we used neutralizing antiserum in the medium at a dilution such that no virus was detected in the medium by infectious assays. (Although virus spread by cell to cell contact cannot be prevented, the majority of secondary infections was prevented by the antibody [Chen, Ph.D. thesis, 1981].)

The second condition is to use cells which remain stationary when deprived of serum, yet have the ability to replicate when serum is replenished. We screened fibroblast cells of several different species for their ability to remain stationary when deprived of serum (see Materials and Methods). Chicken embryo, turkey embryo, quail embryo, and rat (Rat-1) fibroblasts were not suitable, since a large fraction of the cells (5 to 30% within 24 h) still divided when starved for serum (Chen, Ph.D. thesis, University of Wisconsin, Madison, 1981). However, in duck embryo fibroblast cultures less than 1.0% of the cells divided in 24 h when deprived of serum. After the addition of serum, a large proportion of the cells (20 to 50%) started DNA synthesis within 24 h. (All cells started DNA synthesis by 48 to 60 h after the addition of serum [Chen, Ph.D. thesis, 1981].) The kinetics of SNV replication in duck embryo fibroblast cells are similar to those in chicken embryo fibroblast cells (21, 22; Chen, Ph.D. thesis, 1981). Therefore, duck embryo fibroblast cells were used to study the kinetics of formation of viral DNA in stationary and replicating cells.

Kinetics of viral DNA synthesis in serum-starved duck embryo fibroblasts. Stationary duck embryo fibroblast cells were infected at a high multiplicity of infection (20 PFU/cell) to facilitate detection of small amounts of unintegrated linear viral DNA. Secondary infection was blocked by neutralizing antibody. When the cells were stimulated to replicate after infection, unintegrated linear viral DNA was made within 33 h in an amount consistent with the multiplicity of infection of about 20 PFU/cell (Fig. 1; +MSA, +AB). On the other hand, in cells deprived of serum, less than 0.5 copy of unintegrated linear viral DNA per cell was made (Fig.

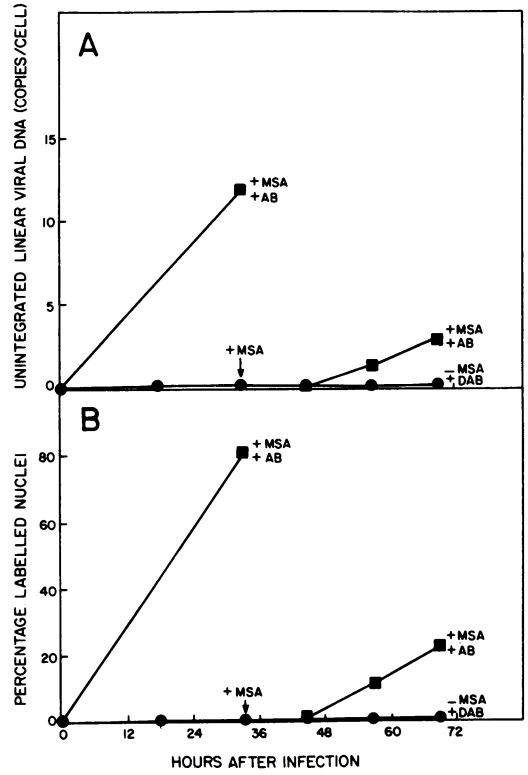

FIG. 1. Appearance in infected duck cells of unintegrated linear SNV DNA in the presence of neutralizing antibody to SNV. Serum-starved duck embryo fibroblast cells (5×10^6 cells per culture) were infected with SNV at a multiplicity of approximately 20 PFU/cell. After infection, the medium of five cultures was changed to medium containing fresh serum plus neutralizing antibody (+MSA, +AB). Cells were harvested by Hirt fractionation 33 h later. The medium of all other cultures was changed to medium containing neutralizing antibody which had previously been depleted of multiplication-stimulating activity (−MSA, +DAB) (see Materials and Methods). At 33 h after infection, the medium on some of these cultures was changed to medium containing fresh serum plus neutralizing antibody (+MSA). Five to ten cultures were harvested by Hirt fractionation at each of the points indicated (panel A). The Hirt fractions were prepared for quantification of unintegrated linear SNV DNA as described in Materials and Methods. The cells in some cultures were labeled with [^{3}H]thymidine, and autoradiography of these cells was performed as described in Materials and Methods (panel B). Prolonged serum deprivation resulted in a decrease of the number of cells per culture from 5×10^6 cells when infected to 2.5×10^6 cells at 72 h after infection. Prolonged serum deprivation also resulted in the longer average period of time for the initiation of cell DNA synthesis following serum stimulation at 33 h after infection compared with serum stimulation immediately after infection. Symbols: ●, cultures without serum; ■, cultures with serum.

1; −MSA, +DAB). This low amount of unintegrated linear viral DNA made in these serum-starved cells can be accounted for by viral DNA synthesis in the small number of cells which started DNA synthesis (equivalent to less than 1.0% of the cells per culture) (Fig. 1B; −MSA, +DAB). These results show that the synthesis of unintegrated linear viral DNA is inhibited in stationary duck cells.

Although stationary cells are not productively infected, renewed cell replication results in virus production (9, 19). Therefore, one prediction of the hypothesis that a productive infection is blocked because of an inhibition of viral DNA synthesis is that viral DNA synthesis should resume synchronously with renewed cell replication. This prediction was tested by stimulating infected serum-starved duck cells to replicate by the addition of serum 33 h after infection. The time course of appearance of unintegrated linear viral DNA paralleled the resumption of cellular DNA synthesis (Fig. 1). At 46 h after infection, 13 h following addition of serum, no cells had started to synthesize DNA and no increase in amounts of viral DNA was detected. By 71 h after infection, about 30% of the cells had undergone DNA synthesis, and significantly more viral DNA was detected in these cultures than in cultures of cells deprived of serum. Little or no secondary infection occurred in any of these cultures, since no infectious virus was detected in the medium (data not shown). (Without neutralizing antibody to block secondary infection, levels of viral DNA were about 5- to 10-fold higher [data not shown], consistent with our initial results with chicken cells.) These results confirm that viral DNA synthesis is inhibited in stationary cells, although some viral intermediate is maintained in these cells which is capable of synthesizing unintegrated linear viral DNA upon stimulation of the cells to replicate.

Since complete unintegrated linear viral DNA is not made in stationary cells, we determined whether any subgenomic species of viral DNA persisted in these cells. DNA samples from the above experiment (Fig. 1) were analyzed by gel electrophoresis under denaturing conditions followed by nucleic acid hybridization to viral DNA. Some genome-size viral DNA was detected at all times after infection of stationary cells (Fig. 2, −MSA). The amount was determined to be less than 0.2 copy per cell by comparison with standards of a molecular clone of viral DNA (Fig. 2, STANDARDS). This amount of DNA could be accounted for by the small number of cells undergoing replication (< 1.0%; see Fig. 1). Aside from this genome-size viral DNA, no other specific species of viral DNA was detected (Fig. 2). However, a heterogeneous distribution of hybridization to subgenomic viral DNA species was detected. Relative to the amount of genome-size viral DNA, more of the smaller subgenomic viral DNA was detected in serum-starved cells than in serum-fed cells (compare −MSA, 0–33 h and 0–58 h with +MSA, 0–33 h and 33–58 h, respectively). (Note that viral DNA from serum-fed cells which had not started to replicate [Fig. 2, +MSA, 33–46 h; see Fig. 1B] contained more small subgenomic viral DNA than viral DNA from cells 12 h later which had replicated [+MSA, 33–58 h].) The possible significance of this subgenomic viral DNA in stationary cells is discussed below.

Also, no integrated viral DNA (<0.5 copy per cell) was detected by the hybridization method of Southern after *Sac*I digestion of high-molecular-weight (>20 × 10^6) DNA from stationary cells in the experiment of Fig. 1 (data not shown). Thus, the viral intermediate in stationary cells appears not to be a unique species of viral DNA.

Virus production in replicating chicken embryo fibroblasts as a function of secondary infection. Secondary infection of replicating cells results in an accumulation of unintegrated linear viral DNA (12, 25; Chen, Ph.D. thesis, University of Wisconsin, Madison, 1981). The kinetics of virus production performed with cells synchronized by serum starvation indicated that large amounts of virus (>10^7 PFU/ml) were produced after the rise in amounts of unintegrated linear viral DNA (data not shown). This result indicated that the secondary rounds of infection might contribute to a further increase in virus titer. In that case, blocking secondary infection should prevent an increase in the rate of virus production. The following experiments were designed to test this possibility.

Since cell division is required for a productive virus infection (see above; 9, 17–19), we chose cells for these experiments which had a high rate of cell replication, allowing more rapid secondary infection to occur. Therefore, chicken embryo fibroblasts were used because they had a higher rate of cell replication than duck embryo fibroblasts (Chen, Ph.D. thesis, 1981). Serum-starved chicken embryo fibroblasts were infected with SNV at a multiplicity of 5 PFU per cell, so that every cell would be infected. The cells were then stimulated to replicate by the addition of fresh serum to the medium. Neutralizing antibody was included in the medium to inhibit secondary infection. The extent of secondary infection was monitored by the levels of unintegrated linear viral DNA in the infected cells. In the absence of secondary infection, the amount of viral DNA by 48 h after infection was about five molecules per cell (Fig. 3A; +MSA, +AB). Secondary rounds of infection were allowed to occur 48 h after infection by changing the medi-

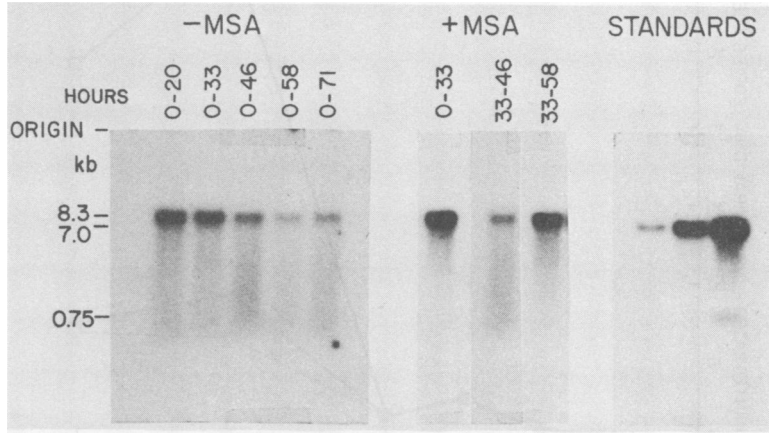

FIG. 2. Unintegrated viral DNA in infected duck embryo fibroblasts. Unintegrated viral DNA samples from infected serum-starved duck embryo fibroblasts were prepared from the cells described in the legend to Fig. 1 and were denatured with glyoxal. Depending on the sample, viral DNA from 0.5×10^7 to 2.0×10^7 cells was subjected to electrophoresis in 1.0% agarose gels. Serial threefold dilutions of the SNV molecular clone, 14–44 (14), digested with *Sac*I, were subjected to electrophoresis in parallel to serve as molecular size markers and to allow quantification of viral DNA species. Viral DNA was detected by the hybridization method of Southern (16). Autoradiograph exposures were adjusted to compare the structures of different viral DNAs and, therefore, do not reflect relative amounts of viral DNA per cell. −MSA: Viral DNA samples from infected stationary cells which were deprived of serum for the indicated period of time (see Fig. 1). +MSA: Viral DNA samples from infected stationary cells which were fed serum for the indicated period of time (see Fig. 1).

um of some cultures to medium with fresh serum, but without neutralizing antibody. Amounts of viral DNA per cell increased (Fig. 3A; +MSA, −AB), demonstrating that reinfection had occurred. (The decrease in the amount of viral DNA at 96 h after infection may be related to the cytopathic phenomenon described by Weller and Temin [26].)

The effect of this secondary infection on the rate of virus production was determined. Since the presence of antibody precluded the use of infectious virus assays, the relative amount of virus produced was determined by measuring DNA polymerase activity (see Materials and Methods). Control experiments demonstrated that the presence of antibody had no effect on DNA polymerase activity (data not shown). In the presence of antibody, virus production remained relatively constant. However, a large increase in virus production was observed when the antibody was removed from the media and secondary rounds of infection occurred (Fig. 3C, compare +MSA, −AB, with +MSA, +AB). This level of virus production is comparable to that observed in a normal productive infection of chicken cells by SNV (10^8 PFU/ml). These results were not the result of a direct inhibition of virus production by antibody, since the antibody had no effect on preexisting virus production in chronically infected cells (data not shown). This result indicates that secondary infection is necessary to generate the levels of virus usually produced in a productive SNV infection of replicating cells.

The role of secondary rounds of infection in generating an increased rate of virus production was confirmed in a study using serum-starved cells to inhibit viral DNA synthesis. This inhibition of viral DNA synthesis effectively blocks productive infection by SNV (see Fig. 1; 9). In the experiment described above, 48 h after infection, the medium was removed from the infected replicating cells and changed to medium without neutralizing antibody and, also, without fresh serum. Secondary infection did not occur in these cultures as is shown by the lack of increase in amounts of viral DNA (Fig. 3A; −MSA, −AB). Virus production also did not increase to the levels of replicating cells in which secondary infection was allowed to occur (Fig. 3C; compare +MSA, −AB, with −MSA, −AB). The observed lack of increase of virus titers to these maximal levels was not due to a metabolic effect of depriving replicating cells of serum, since other studies demonstrated that preexisting virus production is unaffected in serum-starved cells (data not shown; 9, 22).

The mechanism by which virus production is increased after secondary infection was investigated by determining the amounts of integrated viral DNA in these cells. High-molecular-weight cell DNA was purified so it was free from unintegrated viral DNA, digested with *Sac*I, and subjected to electrophoresis. The amounts of

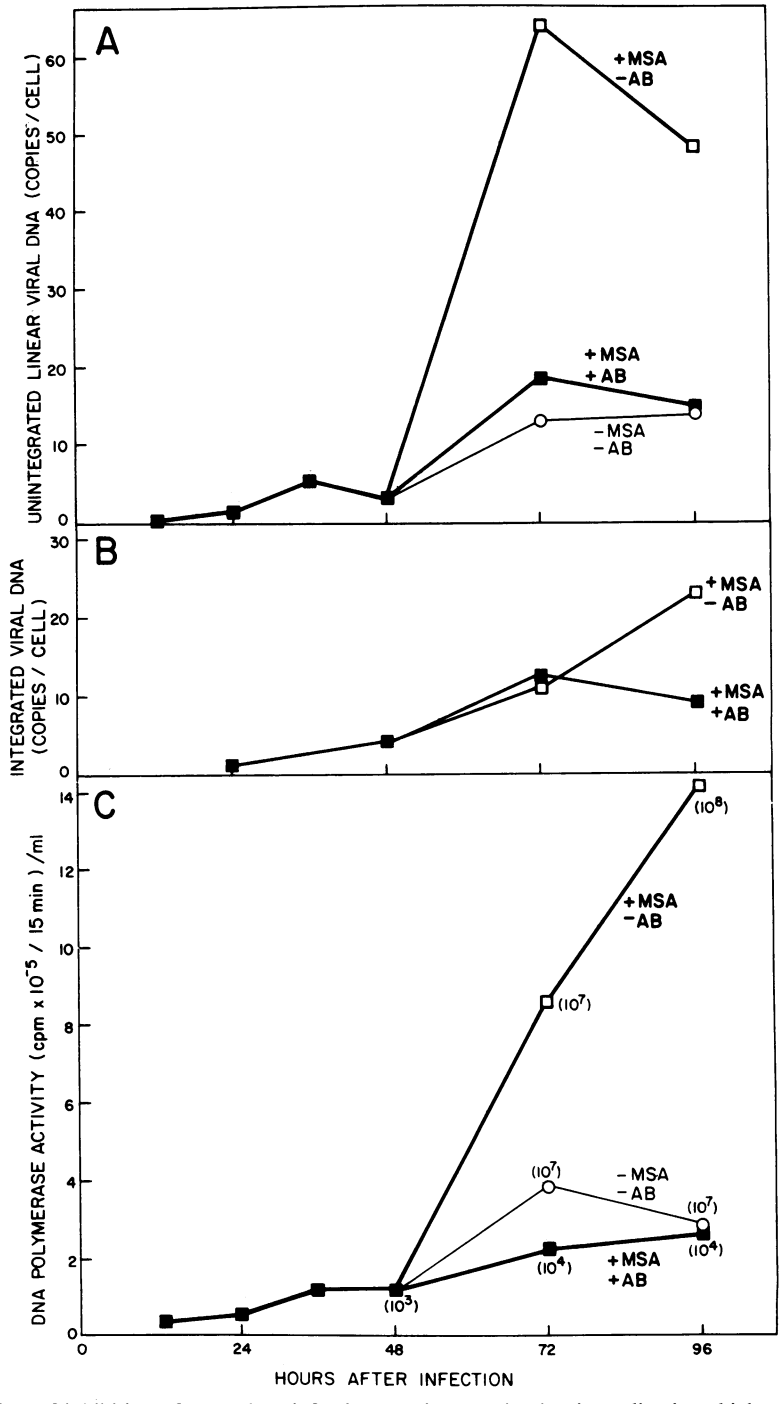

FIG. 3. Effect of inhibition of secondary infection on virus production in replicating chicken cells. Serum-starved chicken embryo fibroblasts (4×10^6 cells per plate) were infected with SNV at a multiplicity of approximately 5 PFU/cell. Immediately after infection, the medium was replaced with medium containing fresh serum plus neutralizing antibody (+MSA, +AB). At 48 h after infection, the medium on some cultures was removed. The cultures were washed several times with Eagle medium, and medium with fresh serum but without neutralizing antibody was added (+MSA, −AB). At the same time, medium on some other cultures was changed to medium without fresh serum (−MSA, −AB). Media on all cultures were replenished every 24 h. At different times after infection, five cultures were pooled for quantification of amounts of viral DNA and relative amounts of virus by Hirt fractionation of the cells (panels A and B) and harvesting of culture medium (panel C), respectively. Quantification of relative amounts of virus and preparation of Hirt fractions for quantification of relative amounts of linear unintegrated viral DNA and integrated viral DNA were as described in Materials and Methods. Symbols: ○, cultures without serum; □, cultures with serum; ■, cultures with serum and neutralizing antibody. Numbers in parentheses are approximate effective virus titers (PFU/ml). (Note that virus titers in the presence of antibody are not proportional to the relative amounts of virus present.)

viral DNA were determined by the nucleic acid hybridization method of Southern (16). In cells where secondary infection was prevented by antibody, the kinetics of appearance of integrated viral DNA were less than, but roughly parallel to, the kinetics of appearance of unintegrated linear viral DNA in the same cells (Fig. 3B; +MSA, +AB). When secondary infection was allowed to occur at 48 h after infection, the amount of integrated viral DNA did not increase to as great an amount as did the unintegrated linear viral DNA. Instead, it increased gradually to about 20 copies per cell by 96 h after infection (Fig. 3B; +MSA, −AB). This increase in DNA was not proportional to the increase in the rate of virus production (Fig. 3C). These results indicate that, during secondary infection, no simple relation exists between virus production and levels of integrated viral DNA.

The above results demonstrate that blocking secondary infection prevents virus production from increasing to the high levels observed in a productive infection of replicating cells. These results indicate that secondary infection is an event required for the generation of a normal productive SNV infection in replicating cells.

DISCUSSION

Inhibition of viral DNA synthesis in stationary cells. Previous studies concluded that viral DNA synthesis is inhibited after infection of stationary cells (4, 24). Although this restriction of viral DNA synthesis in stationary cells turns out to be more complex than previously thought, the basic conclusion is confirmed in this study. The nature of the stable intermediate which is capable of initiating viral DNA synthesis when the infected cells are stimulated to replicate is still unknown. Analysis of viral DNA species in stationary cells indicates that the intermediate is not a discrete subgenomic species of DNA. However, subgenomic viral DNA was detected in a heterogeneous distribution of molecular sizes. It is possible that these species may account for the initial amount of infecting virus. The viral intermediate in stationary cells, thus, may be incomplete transcripts of viral DNA resulting from some unknown restriction in stationary cells. Such a hypothesis is supported by earlier observations on the effect of DNA synthesis inhibitors and bromodeoxyuridine plus light on the subsequent production of virus after stimulation of infected stationary cells to replicate (2).

The synthesis of viral DNA correlates closely with the renewed synthesis of cellular DNA. Because of the asynchronous recovery of the ability of the stationary cells to replicate, these experiments do not determine the exact stage of the cell cycle which is permissive for synthesis of viral DNA. However, it is likely that renewed viral DNA synthesis begins with the initiation of cell DNA synthesis. Viral DNA synthesis probably does not require mitosis. Although not measured here, few cells would have entered mitosis when cell DNA synthesis and viral DNA synthesis were first detected (see Fig. 1). (While this work was in progress, Humphries et al. reported that viral DNA synthesis and integration occurred in colchicine-treated cells [7].) The effect of colchicine in inhibiting virus production (9, 21) may be a result of the arrest of cells in mitosis, consequently preventing cells from initiating a second round of DNA synthesis. This effect would inhibit secondary virus infection, also required for production of high virus titers (see below).

Several other types of inhibitors, including cycloheximide (27) and interferon (1), have been used to prevent productive infection by retroviruses. The relation of these agents to the cell cycle has yet to be determined and may be relevant to the inhibition of viral DNA synthesis in stationary cells.

Role of secondary infection in retrovirus infection. The experiments presented here demonstrate that secondary infection is required for the production of high levels of SNV. High rates of virus production among different viruses of the avian leukosis-sarcoma virus species can be correlated with their ability to initiate secondary rounds of infection (25, 26). Such results are consistent with the results obtained here and indicate that the role of secondary infection in generating high rates of virus production is a common feature of avian retroviruses which are capable of initiating secondary rounds of infection.

The mechanism by which secondary infection causes a large increase in virus production is not clear. It is commonly assumed that transcription of viral DNA occurs with integrated viral DNA used as a template. However, the results presented here indicate that no linear relationship exists between amounts of integrated viral DNA and virus production. Although the increases in amounts of unintegrated linear viral DNA appear to correlate with increases in the rate of virus production, there is no evidence to support transcription of unintegrated viral DNA. Furthermore, in chronically infected cells, the levels of unintegrated viral DNA decrease to a low level (less than five copies per cell), whereas virus production remains at a high level (data not shown; 12, 25). Therefore, the increase in virus production appears not to be related in a simple way to amounts of viral DNA.

The results observed here may reflect a basic difference of viral DNA expression after a pri-

mary infection compared with that after a secondary infection. It is possible that, in secondary infection, the viral DNA integrated is a more efficient template for transcription. The use of genetically marked viruses (14) to experimentally distinguish virus expression of the primary infection from that of a secondary infection should help to clarify the role of secondary infection in increasing virus production.

Secondary infection may also be relevant to the establishment of interference by retroviruses. Continued secondary infection of some cells which have not established interference would enable these cells eventually to establish interference. On the other hand, those cells which are not able to establish interference may die from an "overdose" of secondary infection as described by Weller et al. (25, 26).

Summary of events in the establishment of a productive retrovirus infection. Multiple interactions between the virus and cell occur during the early stages of establishment of retrovirus infection. The cell must enter a particular stage of the cell cycle to allow viral DNA synthesis to occur. Establishment and expression of the provirus is followed by virus production. For viruses having particular envelope genes, secondary rounds of infection then occur (25). Virus production increases further, probably leading to further secondary infection. As a consequence of the secondary infection, cytopathic effects occur (25, 26). The cells which survive are resistant to secondary infection (25, 26), presumably because they have established interference to further infection by viruses of the same envelope type. This selection process results in chronically infected cells that produce large amounts of virus. In the infection of stationary cells, virus production is blocked by the inhibition of viral DNA synthesis and, consequently, inhibition of all subsequent events including the secondary infection process required for high levels of virus production.

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