Independent Expression of Avian Sarcoma Virus in Doubly Infected Chicken Embryo Fibroblasts

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Infection of a chicken cell with avian sarcoma virus requires division of the infected cell before synthesis of infectious progeny is initiated. This requirement for a cell division for the complete expression of avian sarcoma virus has been examined further with chicken embryo fibroblasts infected with two distinct viruses. Chicken cells infected with and producing a mutant of Rous sarcoma virus temperature sensitive for transformation (*ts*LA24PR-A) were arrested in G₀ by depletion of serum factors from growth medium. These stationary cells continued to produce infectious progeny in the absence of further cell division. Superinfection of the stationary cells with the wild-type Prague strain of Rous sarcoma virus (PR-RSV-C) produced a stable double infection in these cells. Progeny of the superinfecting PR-RSV-C, however, were not detected until these cells underwent division after stimulation with fresh serum-containing medium. The addition of colchicine to these serum-stimulated cells, although not affecting production of the *ts*LA24PR-A, inhibited the appearance of progeny of the superinfecting PR-RSV-C. These experiments indicate that each avian sarcoma virus infection of a chicken embryo fibroblast requires division of the infected cell for production of that virus regardless of whether or not the cell is already producing a similar virus. The results suggest, therefore, that the requirement for a cell division represents a requirement for an event that controls virus expression in a "cis-acting" fashion specific for the provirus.

The replication cycle of viruses belonging to the avian leukosis-sarcoma virus complex involves a DNA intermediate, the provirus (1, 12). Full expression of these viruses requires the complete synthesis of this DNA intermediate and its transcription into viral RNA. It has previously been demonstrated, using chicken embryo fibroblasts (CEF) synchronized by the depletion of specific serum factors from growth medium, that both the initiation of viral RNA synthesis and the production of viral progeny require division of the newly infected cells (8, 9). The addition of colchicine to infected cells after the addition of serum-containing medium inhibits the appearance of new progeny virus. The requirement for division of the infected cell for production of avian leukosis-sarcoma virus progeny has been called activation (13).

Recent studies have demonstrated that, whereas proviral DNA synthesis is initiated in stationary cells (2), the newly synthesized DNA is incomplete (6, 15). Analysis of minus- and plus-strand viral DNA shows that both the amount of DNA synthesis and the length of the DNA strand synthesized are reduced in stationary cells when compared with dividing cells. It has also been demonstrated that synthesis and integration of the provirus require host cell func-

tions associated with serum stimulation of the infected cell (15). Recent experiments using synchronized cells have shown that both synthesis and integration of proviral DNA occur during S-phase cellular DNA synthesis (E. H. Humphries, C. Glover, and M. E. Reichmann, *Proc. Natl. Acad. Sci. U.S.A.*, in press). This study further demonstrated that, whereas colchicine inhibits the synthesis of progeny virus, synthesis and integration of the provirus are unaffected. It appears, therefore, that avian leukosis-sarcoma virus infection can be inhibited in two ways. First, the proviral DNA is not completely synthesized in stationary cells. Second, although serum stimulation is required for the complete synthesis and integration of the provirus, the integrated provirus is not expressed until after the first cell division.

The present experiments were carried out to determine whether the control on the expression of the provirus that requires cellular division, that is, the requirement for an event that is blocked by treatment of cells with colchicine, is specific for each provirus. The results demonstrate that, although a cell is producing one virus, the requirement for a subsequent cell division to activate the provirus formed by a superinfecting virus is not eliminated. These ex-

periments indicate that the cell division that controls the expression of the provirus operates on the integrated provirus in a "cis-acting" fashion.

MATERIALS AND METHODS

Cells and viruses. CEF were grown in Dulbecco-modified Eagle medium (Flow Laboratories, Rockville, Md.) containing 10% tryptose phosphate broth (ET medium) supplemented with fetal calf serum and heat-inactivated chicken serum (GIBCO Laboratories, Grand Island, N.Y.) in plastic tissue culture dishes (Nunc Plastics, Copenhagen, Denmark).

All cells were prepared from primary cultures of a single embryo from a 10- to 11-day-old embryonated egg, using standard procedures. SPAFAS CEF (SPAFAS, Storrs, Conn.) were C/E, avian leukosis virus-free, chicken helper factor negative (10), and group-specific antigen negative (8). Reaseheath C-line CEF (Houghton Research Station, Houghton, Middlesex, England) were C/AE, avian leukosis virus-free, and chicken helper factor and group-specific antigen negative. Line 15_B CEF (U.S. Department of Agriculture Regional Poultry Research Laboratory, East Lansing, Mich.) were C/C, avian leukosis virus-free, and chicken helper factor and group-specific antigen negative. All cells were used between passages 5 and 10 after primary culture.

A single clone of the wild-type Prague strain of Rous sarcoma virus, subgroup C (PR-RSV-C), obtained from J. Wyke, was used throughout this study. Serum-free stocks of the virus were prepared in ET medium containing 0.1% bovine serum albumin. Virus prepared in this manner had a titer of 1×10^6 to 5×10^6 focus-forming units/ml on C/E cells and a titer of <10 focus-forming units/ml on C/C cells.

A single clone of a temperature-sensitive (*ts*) mutant of the Prague strain of RSV, subgroup A, defective for maintenance of transformation (*ts*LA24PR-A; 17) was prepared from stock virus obtained from J. Wyke. The titer of this virus was 0.5×10^7 to 1×10^7 focus-forming units/ml at 35°C on C/E cells and <10 focus-forming units/ml at 41°C on C/E cells or at 35°C on C/AE cells.

Stationary CEF. SPAFAS C/E CEF were prepared by depletion of serum factors from growth medium, using a modification of the method described previously (7, 14). Cells were plated at 1.2×10^6 cells per 50-mm dish containing 0.5% fetal calf serum and placed at 41°C in a humidified 5% CO₂ atmosphere. Four hours after plating, the medium was changed to 3 ml of ET medium containing 0.5% horse serum (Flow Laboratories), 0.5% fetal calf serum, and 0.02 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, pH 8.3. The cells were used between 48 and 72 h after plating. At this time, $<1\%$ of the cells showed nuclear labeling by autoradiography after 24 h in [³H]thymidine-containing medium, using conditions described previously (7). Cultures of stationary CEF infected with and producing *ts*LA24PR-A were prepared in a similar manner by plating CEF at the second passage after infection with the virus. Cells infected with *ts*LA24PR-A interfered specifically with superinfection by a wild-type Prague strain of RSV

(PR-RSV-A). The titer of PR-RSV-A was reduced 10^4 -fold compared with the titer on uninfected cells from the same embryo, whereas the titer of PR-RSV-C was reduced only 2- or 3-fold. These results indicate that the majority, if not all, of the cells were infected with *ts*LA24PR-A. Cultures of both uninfected and *ts*LA24PR-A-producing SPAFAS C/E CEF were used at passages 6 and 7 after primary culture to prepare stationary cells. Stationary cells were stimulated to divide by addition of ET medium containing 10% fetal calf serum. The number of cells per culture dish and the rate of DNA synthesis at different times after addition of serum-containing medium were determined by using a Coulter Counter and [³H]thymidine pulse-labeling as described previously (9). In some experiments, cells were exposed to ET medium containing both 10% fetal calf serum and 5×10^{-7} M colchicine.

Virus assays. Culture medium was harvested at different times and assayed for production of avian sarcoma virus (ASV), using a standard focus assay (16). To prepare such samples, duplicate culture dishes were washed twice with 0.05 M Tris-hydrochloride (pH 7.5) containing 0.14 M NaCl, and 2 ml of ET medium either containing (dividing cells) or not containing (stationary cells) 5% fetal calf serum was replaced. After 2 h of incubation at 41°C, the culture media from the duplicate dishes were pooled and stored at -70°C until assayed. For some samples, culture dishes were first washed twice with 0.05 M glycine-hydrochloride (pH 2.2) for 30 s before washing with 0.05 M Tris-hydrochloride (pH 7.5) containing 0.14 M NaCl (11). A drop of fetal calf serum was added to samples collected in ET medium before storage. Washing virus-producing or virus-infected cells with the glycine-hydrochloride (pH 2.2) buffer completely removes virus bound to the cell surface (11; Humphries, unpublished data). Virus harvests carried out in such a manner contain, therefore, only newly synthesized progeny.

A standard infectious-center assay was used to determine the number of infected cells at different times after exposure to PR-RSV-C (18). Cells were washed twice with 0.05 M Tris-hydrochloride (pH 7.5) containing 0.14 M NaCl, collected after trypsinization, and washed twice in ET medium. After dilution in ET medium containing 2% fetal calf serum, 2% calf serum, and 2 µg of Polybrene per ml, cells were plated on different feeder layers containing 0.3×10^6 cells per 35-mm plate and overlaid 2 h after plating. Assays were carried out in duplicate, with 10^1 to 10^5 cells plated per dish. For some assays, cells in culture were treated with mitomycin C (Sigma Chemical Co., St. Louis, Mo.) at 20 µg/ml for 1 h before preparation for plating on feeder layers. Treatment of CEF with mitomycin C under these conditions prevents division in $>99\%$ of the cells while allowing the majority of the cells to remain metabolically active for 5 or 6 days (18).

RESULTS

Characterization of stationary CEF producing *ts*LA24PR-A. SPAFAS C/E CEF were infected with mutant *ts*LA24PR-A and used to

prepare cultures of stationary cells at 41°C. After the depletion of serum growth factors from the medium, the number of cells per culture dish remained constant for 2 to 3 days. Stimulation of these cells with fresh growth medium containing 10% fetal calf serum resulted in a 50- to 100-fold increase in DNA synthesis approximately 12 h after addition of serum and in division of 90 to 95% of the cells by 20 h (Fig. 1). Addition of colchicine to the cells at the time of serum addition did not affect the first period of S-phase DNA synthesis, whereas cell division and the second period of DNA synthesis characteristic of the untreated cells were almost completely inhibited (Fig. 1). An apparent increase in the cells per culture dish is due in large part to cell blebbing, as determined by phase microscopy. The basic features of the *tsLA24PR-A*-infected CEF cell cycle are, therefore, similar to the cell cycles of uninfected or avian leukosis virus-infected CEF described previously (7).

We wanted to determine whether the cell division required for activation of an ASV pro-

virus would be eliminated if the host cell was already producing a closely related virus. Therefore, it was important both that the majority of the cells in the culture were infected with *tsLA24PR-A* and that these infected cells continued to release high titers of newly synthesized virus after they were arrested in G_0 by depletion of the serum factors from the growth medium, during the cell cycle after serum stimulation, and after they were exposed to concentrations of colchicine sufficient to block mitosis.

To demonstrate that most of the cells were infected, stationary *tsLA24PR-A*-infected CEF were prepared for an infectious-center assay. Serum-stimulated and control nonstimulated cells, either treated with mitomycin C or not treated, were assayed for their ability to act as infectious centers on C/E cells at 35°C. Figure 2 presents data demonstrating that both stationary cells and serum-stimulated cells throughout the stages of the cell cycle, either with or without mitomycin C treatment, score equally well as

FIG. 1. Cell cycle of CEF producing *tsLA24PR-A*. CEF were exposed to *tsLA24PR-A*, grown for 8 to 10 cell generations, and arrested in G_0 by depletion of serum factors from the growth medium at 41°C. Twenty-four to forty-eight hours after G_0 arrest, some cultures were changed to ET medium containing 10% fetal calf serum (●), some cultures were changed to ET medium containing 10% fetal calf serum and 5×10^{-7} M colchicine (◆), and some cultures remained unchanged (○). At different times after addition of serum, the number of cells per culture (---) and the amount of incorporation of [3 H]thymidine (—) were determined. The data are plotted on the abscissa (hours after serum) relative to the time serum was added to serum-stimulated cultures.

FIG. 2. Percentage of stationary *tsLA24PR-A*-producing cells plating as infectious centers. Stationary *tsLA24PR-A*-producing cells were prepared as in the legend to Fig. 1. At different times after addition of serum, serum-stimulated cells treated with mitomycin C (■) or not treated (●) and stationary cells treated with mitomycin C (□) or not treated (○) were assayed for infectious centers on C/E cells at 35°C (—) or 41°C (---). The data are plotted on the abscissa (hours after serum) relative to the time serum was added to serum-stimulated cultures.

infectious centers at 35°C. Controls plated at 41°C on C/E cells (Fig. 2) and at 35°C on C/AE cells (data not shown) show that the virus is both temperature sensitive for transformation and characterized by a subgroup A glycoprotein. The results demonstrate that, within experimental error, the majority, if not all, of the stationary cells were infected. These results are further supported by the 10^4 -fold reduction in the titer of PR-RSV-A on these cells compared with the titer on uninfected cells, as mentioned earlier (data not shown).

The expression of *tsLA24PR-A* was studied further by analysis of the release of infectious progeny by stationary cells, dividing cells, and colchicine-treated cells throughout the cell cycle described in Fig. 1. Virus samples to be assayed were prepared in two different ways. Before fresh medium was added, the cells were washed twice with either Tris-hydrochloride (pH 7.5) buffer or glycine-hydrochloride (pH 2.2) buffer. The latter procedure removed all membrane-bound virus at the start of the harvest. Figure 3 shows that all of the cultures released 10^5 to 10^6 focus-forming units of virus per ml throughout the cell cycle. Whereas the experiment described in Fig. 3 shows that two- to fivefold more virus was released from dividing and colchicine-treated cells than from stationary cells, the results from several experiments revealed no consistent pattern (data not shown). The amount of cell-associated virus was usually two- to fivefold greater than that detected after the glycine-hydrochloride (pH 2.2) wash.

From an analysis both of the capacity of stationary and dividing cells to act as infectious centers, with or without mitomycin C treatment, and of the amount of virus released from these cells during the cell cycle, it is evident that neither the phase of the cell cycle nor colchicine-induced metaphase arrest significantly alters the expression of an integrated ASV once the initial activation is completed. Other experimental evidence in support of these observations has shown that pulse-labeled RNA obtained from stationary, dividing, or colchicine-treated avian leukosis virus-producing cells contained similar amounts of newly synthesized viral RNA (7). The expression of the ASV provirus appears to be independent of both the cell cycle and the presence of the inhibitor colchicine. Therefore, CEF prepared for experimentation after the initial cell division and activation of virus production will be referred to as ASV-producing CEF.

PR-RSV-C superinfection of stationary *tsLA24PR-A*-producing CEF. To determine whether complete expression of one ASV provirus within an infected cell eliminates the re-

FIG. 3. Production of progeny virus from stationary, dividing, and serum-stimulated, colchicine-treated, *tsLA24PR-A*-producing CEF. Stationary *tsLA24PR-A*-producing CEF were changed to ET medium containing 10% fetal calf serum (●) or to ET medium containing 10% fetal calf serum and 5×10^{-7} M colchicine (◆) or left unchanged (○). At different times after addition of serum, cultures were washed with either a pH 7.5 (—) or a pH 2.2 (....) buffer, and fresh medium was readded for harvesting progeny virus as described in the text. Samples were assayed at 35°C on C/E CEF. The data are plotted on the abscissa (hours after serum) relative to the time serum was added to serum-stimulated cultures.

quirement for a cellular division to activate a second ASV infection, we superinfected stationary *tsLA24PR-A*-producing cells with PR-RSV-C. Ten hours after infection, some cells were stimulated with fresh serum-containing medium. At different times after addition of serum, both serum-stimulated and control cells were either treated with mitomycin C or not treated and were assayed for infectious centers at 41°C on C/E cells. In this assay, an infected cell that is not treated with mitomycin C will be capable of division, production of progeny virus, and focus formation. However, since mitomycin C blocks cell division, an infected cell that is treated with mitomycin C will only be capable of focus formation if it is already producing virus at the time of treatment. Therefore, the percentage of untreated cells that register as infectious centers is a measure of the total number of cells that are infected, whereas the percentage of mitomycin C-treated cells registering as infectious centers is a measure of the number of

infected cells that are producing virus. To directly measure any effect on the production of PR-RSV-C due to the production of *tsLA24PR-A*, both *tsLA24PR-A*-producing and parallel uninfected CEF were exposed to PR-RSV-C. The results of this experiment are presented in Fig. 4.

The number of PR-RSV-C-infected cells increases gradually in serum-stimulated *tsLA24PR-A*-producing cultures as virus production begins and the infection spreads (Fig. 4A). Seventy hours after addition of serum, >95% of the cells plated as infectious centers if the mitomycin C treatment was omitted. In contrast, the percentage of stationary *tsLA24PR-A*-producing cells that register as infectious centers remains constant in time reflecting the initial level of PR-RSV-C infection. Samples of the PR-RSV-C-infected, *tsLA24PR-A*-producing stationary cells plated in parallel at 35°C revealed that 100% of the cells plated as infectious centers, confirming that all of the cells were producing *tsLA24PR-A* (data not shown). This result demonstrates that the production of *tsLA24PR-A* does not allow the initial PR-RSV-C infection to spread in cells in the absence of stimulation of the cells with serum-containing medium. Further, the rate of spread of the PR-RSV-C infection in *tsLA24PR-A*-producing cells

is indistinguishable from that in uninfected cells (Fig. 4A and B). These results indicate that the production of *tsLA24PR-A* does not affect the production of PR-RSV-C and suggests that cell division is required for activation of PR-RSV-C even in *tsLA24PR-A*-producing cells.

This conclusion is further supported by the results obtained from plating cells in infectious-center assays after mitomycin C treatment (Fig. 4). Although cells treated with mitomycin C remain metabolically active, they are rendered permanently incapable of cell division. Consequently, unless the newly infected cell has divided and activated the PR-RSV-C infection before mitomycin C treatment, expression of the virus is permanently blocked. Figure 4A shows that, initially, mitomycin C-treated stationary *tsLA24PR-A*-producing cells register 50- to 100-fold fewer PR-RSV-C infectious centers at 41°C than the corresponding untreated stationary cells. Therefore, although these cells are stably infected with PR-RSV-C, preventing cellular division with mitomycin C treatment blocks activation of the PR-RSV-C synthesis despite the continued production of *tsLA24PR-A* (see Fig. 2 and 3). Stimulation of these cells with serum gradually reduces the effectiveness of mitomycin C treatment in preventing cells from plating as infectious centers, suggesting that cell division is

FIG. 4. Appearance of PR-RSV-C infectious centers after infection of stationary *tsLA24PR-A*-producing or uninfected CEF. Stationary *tsLA24PR-A*-producing (A) or stationary uninfected (B) CEF were prepared as described in the legend to Fig. 1. The cells were exposed to PR-RSV-C, using a multiplicity of infection of approximately 0.05, and 10 h after infection, cultures were changed to ET medium with or without 10% fetal calf serum. At different times after addition of serum, dividing and stationary cells were either treated with mitomycin C or not treated and were assayed for infectious centers at 41°C on C/E CEF. Symbols: Dividing cells with (■) or without (●) mitomycin C treatment; stationary cells with (□) or without (○) mitomycin C treatment.

required for activation of the PR-RSV-C production. Seventy hours after serum stimulation, the number of cells plating as infectious centers increased from a background level of 0.01% to nearly 5% (Fig. 4A). Whereas part of this increase appears to result from division of the infected cell, secondary spread of the PR-RSV-C infection would also increase the number of infected cells. It should be emphasized, however, that the spreading infection should be as sensitive to mitomycin C treatment as was the initial infection. The rate at which PR-RSV-C infectious centers become resistant to mitomycin C treatment is nearly identical after PR-RSV-C infection of either uninfected or *tsLA24PR-A*-producing CEF (Fig. 4A and B).

This experiment demonstrates that both the initial activation of the PR-RSV-C infection in ASV-producing cells and the subsequent spread of that infection in these cells are dependent upon serum stimulation of the infected cells. The data presented in Fig. 4 indicate that whether the cells are infected or uninfected has no effect upon the percentage of cells plating as infectious centers (with or without mitomycin C treatment) during the course of the experiment. Furthermore, the PR-RSV-C infection is stable for 70 h in the stationary cells. Since viral DNA synthesis is not completed in these cells, it is not surprising that the infection does not spread in the absence of serum. However, after addition of serum, the provirus can be synthesized and integrated in approximately 9 to 12 h (Humphries et al., in press). Since the cells in this experiment divided every 15 to 20 h (data not shown), if the production of a second virus eliminates the requirement for cell division, the infection should spread considerably faster in the cells producing ASV than in the uninfected cells. With or without mitomycin C treatment, however, the PR-RSV-C infection spread equally in uninfected or *tsLA24PR-A*-producing cells, demonstrating that the presence of virus production did not affect the rate at which the PR-RSV-C infection spread and suggesting that the superinfecting virus required activation through cell division.

Production of PR-RSV-C after superinfection of *tsLA24PR-A*-producing cells requires cellular division. The experiment described in Fig. 4 suggests that cell division is required for production of a superinfecting ASV. Therefore, the experiment was repeated with the inhibitor colchicine. The results presented in Fig. 1 indicate that, in our experiments, colchicine specifically inhibits cellular division and has little or no effect on cellular S-phase DNA synthesis. Therefore, we exposed stationary cells

producing *tsLA24PR-A* to PR-RSV-C. Ten hours after infection, some cells were changed to serum-containing medium, some were changed to serum-containing medium with colchicine, and some were left in serum-depleted medium. At different times, fresh medium was added to washed cultures and harvested 2 h later. The samples were assayed at 41°C to determine when progeny PR-RSV-C could first be detected. The results presented in Fig. 5 show that progeny was first detected in serum-treated cultures approximately 24 h after addition of serum. Control stationary cells produced no detectable PR-RSV-C 60 h after infection. Production of PR-RSV-C from colchicine-treated cells was delayed for 15 h when compared with that produced from serum-stimulated cells. After the initial delay, virus production gradually increased in parallel with production from dividing

FIG. 5. Effect of colchicine on the appearance of progeny PR-RSV-C after infection of stationary *tsLA24PR-A*-producing CEF. Stationary *tsLA24PR-A*-producing CEF were prepared as described in the legend to Fig. 1. Cultures were exposed to PR-RSV-C, using a multiplicity of infection of approximately 0.05, and 10 h after infection, cultures were changed to ET medium containing 10% fetal calf serum (●) or ET medium containing 10% fetal calf serum and 5×10^{-7} M colchicine (◆) or left unchanged (○). At different times after addition of serum, cultures were washed with a pH 7.5 buffer, and fresh medium was added for harvesting virus progeny as described previously. Samples were assayed at 41°C on either C/E (—) or C/C (---) CEF.

cells. To demonstrate that the PR-RSV-C progeny initially produced in these cultures was synthesized in *tsLA24PR-A*-infected cells, the virus was assayed at 41°C on C/C CEF to detect PR-RSV-C released as a pseudotype carrying the subgroup A glycoprotein. The PR-RSV-C progeny from either dividing cells or colchicine-treated cells infected both C/E and C/C CEF with equal efficiency, indicating that the newly released virus existed as a pseudotype (Fig. 5). Assays of the same samples at 35°C showed that cells in all of the cultures were producing between 0.5×10^6 and 1×10^6 focus-forming units of *tsLA24PR-A* per ml (data not shown).

The experiment described in Fig. 5 was repeated, and cells from the different cultures were plated for production of infectious centers. *tsLA24PR-A*-infected cells were exposed to PR-RSV-C. Ten hours after infection, some cells were treated with medium containing serum, some were treated with medium containing se-

FIG. 6. Effect of colchicine on the appearance of PR-RSV-C infectious centers after infection of stationary *tsLA24PR-A*-producing CEF. Stationary *tsLA24PR-A*-producing CEF were prepared as described in the legend to Fig. 1. Cultures were exposed to PR-RSV-C, using a multiplicity of approximately 0.05, and 10 h after infection, cultures were changed to ET medium containing 10% fetal calf serum (●) or ET medium containing 10% fetal calf serum and 5×10^{-7} M colchicine (◆) or left unchanged (○). At different times after addition of serum, cells were plated for infectious centers at 41°C on C/E CEF.

rum and colchicine, and some were left as controls. At different times after addition of serum, cells were plated for infectious centers at 41°C. The results presented in Fig. 6 show that colchicine inhibits the increase in infectious centers seen in serum-stimulated cultures. Approximately 1% of the cells are infected in cultures of either stationary cells or metaphase-arrested cells 45 h after infection, whereas 15% of cells are infected at the same time in cultures of serum-stimulated cells. Therefore, the PR-RSV-C infection spreads no more efficiently in serum-stimulated, colchicine-arrested, *tsLA24PR-A*-producing cells than in the stationary *tsLA24PR-A*-producing cells. Parallel samples plated at 35°C showed that all cells from the three cultures were producing *tsLA24PR-A* (data not shown).

These results further demonstrate that colchicine does not prevent the ultimate expression of the PR-RSV-C infection, but rather delays the appearance of progeny virus and the spread of infection. Thus, although progeny normally first appear at the time of cellular division, establishing a second round of infection, colchicine inhibits virus production for a period of 15 to 20 h so that only the cells originally infected register as infectious centers. The inability to completely block progeny synthesis is also seen in ASV infection of uninfected stationary cells (9) and appears to be related to the formation of "mininuclei" as discussed below.

We conclude from the results presented in Fig. 5 and 6, therefore, that under experimental conditions that allow synthesis and integration of the provirus, production of *tsLA24PR-A* does not eliminate the requirement for activation by cell division for a superinfecting PR-RSV-C. These results support a model characterizing the event(s) necessary for virus expression and requiring cell division as an event(s) controlling the provirus in a *cis*-acting fashion.

DISCUSSION

Experiments published previously have demonstrated that ASV infection of a sensitive cell requires cell division for the production of progeny virus (13). Experiments designed to characterize this requirement for division have demonstrated that transcription of the newly synthesized provirus does not occur until after cell division (8, 9). Based on these studies, it was postulated that initiation of transcription of the ASV provirus is controlled by a mechanism that requires at least one cellular division. Further work showed that, if infected cells were arrested in G_0 after initiation of viral RNA synthesis and

the release of progeny virus, the stationary cells continued to synthesize viral RNA at a rate very similar to that observed in dividing cells (7). In fact, the rate of viral RNA synthesis was not significantly altered by treatment of the cells with either cytosine arabinoside or colchicine. Hybridization analysis of pulse-labeled RNA estimated that rate to be between 0.5 and 1% of total cellular RNA synthesis. It appears, therefore, that the requirement for cellular division for expression of ASV is only a requirement for the initiation of RNA synthesis and not a requirement for continued viral RNA synthesis.

Boettiger and Temin showed in an analysis of the state of the ASV provirus in stationary cells that the newly synthesized provirus could be labeled with 5-bromodeoxyuridine (2). Later studies showed that this viral DNA did not represent a complete copy of the viral genome (6, 15). The complete DNA provirus was first detected in cells after stimulation with serum-containing medium (15). Whereas these studies demonstrated that newly synthesized proviral DNA was integrated into newly replicated cellular DNA, it was not possible to determine whether the provirus was integrated before or after the first cell division following infection. The authors were restricted in their conclusion, therefore, and could conclude only that serum-stimulated cellular functions were required for the synthesis and integration of the ASV provirus (15). In a more definitive analysis, experiments have documented that the newly synthesized provirus is integrated during S-phase cellular DNA synthesis before the first cell division (Humphries et al., in press). These studies show further that the presence of colchicine does not affect the integration process. The colchicine-sensitive event required for complete expression of the ASV provirus, therefore, appears to act upon an integrated but inactive provirus. As colchicine treatment inhibits the appearance of viral-specific RNA (9), the required event appears to control the initiation of transcription.

The experiments presented in this paper were designed to distinguish between two possible models concerning the nature of the requirement for a cell division for initiation of viral transcription. In one model, division is required for alteration of the cell in order to permit transcription. The activated cell might be permanently altered so that subsequent ASV infections might not require a division for initiation of transcription. Alternatively, the requirement might be provirus specific. Such a model would require that each infection be followed by a division in order to accomplish activation.

To distinguish between these two models, we

prepared CEF that were producing a mutant of the Prague strain of RSV that is temperature sensitive for transformation. These cells were arrested in G_0 by depletion of specific serum factors from growth medium. Experiments described here (Fig. 2 and 3) and elsewhere (7, 9) demonstrate that (i) stationary ASV-producing cells remain fully infected and release high titers of virus after G_0 arrest, (ii) stationary ASV-producing cells continue to synthesize virus-specific RNA, and (iii) serum stimulation or colchicine-induced metaphase arrest of stationary ASV-producing cells does not appreciably alter the rate of either viral RNA synthesis or virus production. It appears, therefore, that the phase of the cell cycle has no effect on the transcription of viral RNA or the release of infectious progeny once activation has occurred. The ASV-producing CEF ought, therefore, to provide an optimal environment in which to distinguish between the two models described above. The experimental system is complicated, however, by the fact that the provirus is not completely synthesized in stationary cells. To properly test the two hypotheses presented above, the cells must enter S-phase DNA synthesis to permit formation and integration of the provirus but must be prevented from completing cell division. The experiment described in Fig. 4 does not distinguish between a requirement for provirus integration or for provirus integration and cell division. The results, however, do suggest that the PR-RSV-C provirus requires activation through cell division since the infection spreads at the same rate in both uninfected and *tsLA24PR-A*-producing cells. This result is confirmed by the experiments presented in Fig. 5 and 6 showing that production of progeny PR-RSV-C after superinfection of stationary *tsLA24PR-A*-producing cells is completely dependent upon cellular division. The use of the inhibitor colchicine distinguishes between serum-stimulated events before mitosis and mitosis itself. In the experiments reported here, approximately 1% of the cells were infected with PR-RSV-C. To eliminate the possibility that our findings were the result of infecting a very minor population of *tsLA24PR-A*-producing cells, we repeated the experiments under conditions in which 10% or more of the cells were infected. The results showed that (i) the PR-RSV-C infection spreads only in serum-stimulated cultures, (ii) the appearance of progeny PR-RSV-C is inhibited by treatment of the cultures with colchicine, and (iii) the PR-RSV-C is released as a pseudotype carrying the subgroup A glycoprotein (data not shown). It appears, therefore, that cellular division is required for an event that controls the initiation of tran-

scription in a *cis*-acting fashion.

It is important to point out that colchicine is only a temporary block on the synthesis of viral RNA and the release of progeny. It is clear, however, that cellular division is inhibited. Colchicine-induced metaphase arrest has been reported to prevent division but to permit the formation of mininuclei (3-5). During this process, the nuclear membrane begins to reform around chromosomes in an irregular manner, producing mininuclei containing a random assortment of chromosomes. Decondensation of these chromosomes follows producing a typical interphase chromosomal state. The time period required for formation of these mininuclei ranges from 15 to 20 h after metaphase arrest. Experiments published here (Fig. 5) and elsewhere (9) show that the appearance of progeny ASV is completely prevented for a period of only 15 to 20 hours. It may be that nuclear membrane reformation and chromosomal decondensation are required for a proviral specific event leading to the initiation of transcription of the integrated provirus through the formation of a stable transcriptional complex.

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LITERATURE CITED

1. Bishop, J. M. 1978. Retroviruses. *Annu. Rev. Biochem.* **47**:35-88.
2. Boettiger, D., and H. M. Temin. 1970. Light inactivation of focus formation by chicken embryo fibroblasts infected with avian sarcoma virus in the presence of 5-bromodeoxyuridine. *Nature (London)* **228**:622-624.
3. Borisy, G. G., and E. W. Taylor. 1967. The mechanism of action of colchicine. *J. Cell. Biol.* **34**:535-548.
4. Brachet, J., and A. E. Mirsky. 1961. The cell, p. 311, vol. 3. Academic Press, Inc., New York.
5. Ege, T., and N. R. Ringertz. 1974. Preparation of microcells by enucleation of micronucleate cells. *Exp. Cell Res.* **87**:378-382.
6. Fritsch, E., and H. M. Temin. 1977. Inhibition of viral DNA synthesis in stationary chicken embryo fibroblasts infected with avian retroviruses. *J. Virol.* **24**:461-469.
7. Humphries, E. H., and J. M. Coffin. 1976. Rate of virus-specific RNA synthesis in synchronized chicken embryo fibroblasts infected with avian leukosis virus. *J. Virol.* **17**:393-401.
8. Humphries, E. H., and H. M. Temin. 1972. Cell cycle-dependent activation of Rous sarcoma virus-infected stationary cells: avian leukosis virus group-specific antigens and ribonucleic acid. *J. Virol.* **10**:82-87.
9. Humphries, E. H., and H. M. Temin. 1974. Requirement for cell division for initiation of transcription of Rous sarcoma virus RNA. *J. Virol.* **14**:531-546.
10. Murphy, H. H. 1977. A new replication defective variant of the Bryan high titer strain of Rous sarcoma virus. *Virology* **77**:705-721.
11. Steck, F. T., and H. Rubin. 1966. The mechanism of interference between avian leukosis virus and Rous sarcoma virus. *Virology* **29**:628-641.
12. Temin, H. M. 1964. The nature of the provirus of Rous sarcoma virus. *Natl. Cancer Inst. Monogr.* **17**:557-570.
13. Temin, H. M. 1967. Studies on carcinogenesis by avian sarcoma viruses. V. Requirement for new DNA synthesis and for cell division. *J. Cell Physiol.* **69**:53-63.
14. Temin, H. M. 1971. Stimulation by serum of multiplication of stationary chicken cells. *J. Cell Physiol.* **78**:161-170.
15. Varmus, H. E., T. Padgett, S. Heasley, G. Simon, and J. M. Bishop. 1977. Cellular functions are required for the synthesis and integration of avian sarcoma virus-specific DNA. *Cell* **11**:307-319.
16. Vogt, P. K. 1969. Focus assay of Rous sarcoma virus, p. 198-211. In K. Habel and N. P. Salzman (ed.), *Fundamental techniques of virology*. Academic Press, Inc., New York.
17. Vogt, P. K., R. A. Weiss, and H. Hanafusa. 1974. Proposal for numbering mutants of avian leukosis and sarcoma viruses. *J. Virol.* **13**:551-554.
18. Weiss, R. A., W. S. Mason, and P. K. Vogt. 1973. Genetic recombinants and heterozygotes derived from endogenous and exogenous avian RNA tumor viruses. *Virology* **52**:535-552.