Replication of Rous Sarcoma Virus in Synchronized Cells

By BARBARA HOBOM-SCHNEGG, HARRIET L. ROBINSON and W. S. ROBINSON

Department of Medicine, Stanford University, Palo Alto, California 94305

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SUMMARY

When synchronized chicken embryo cells were infected with Rous sarcoma virus in the the G-I period of the cell cycle, virus replication was delayed by several hours compared with cells infected in the S period, and virus release began only after mitosis. The delay in replication was not due to a change in the rate of virus adsorption and penetration. There was no delay in replication of Newcastle disease virus, indicating that the cells in G-I were able to support replication of other RNA viruses. The shortest latent period for RSV (~2 to 14 hr) occurred when cells were infected between S and mitosis. The latent period increased for cells infected after the maximum of mitosis. Cells which had been in culture for more than 15 hr rapidly lost their susceptibility to infection by RSV.

INTRODUCTION

Although Rous sarcoma virus (RSV) is known to be an RNA virus (Robinson, Pitkanen & Rubin, 1965), its replication is blocked by certain metabolic inhibitors which do not affect the replication of other RNA viruses. Actinomycin D, an inhibitor of DNA dependent RNA synthesis, inhibits the production of infectious RSV (Temin, 1963; Bader, 1965; Vigier & Goldé, 1964) and probably the synthesis of viral RNA (Robinson, 1967) at any time after infection of chick embryo cells in tissue culture. In contrast, the replication of most RNA viruses is not interrupted by actinomycin D (Reich et al. 1962; for review see Robinson & Duesberg, 1968) although a few RNA viruses such as influenza virus are blocked when the drug is added soon after infection but not at all when added three, four or more hours after infection when virus RNA synthesis has begun (Barry, 1964; Duesberg & Robinson, 1967). The sensitivity of RSV to actinomycin D at any time after infection has limited biochemical studies of RSV replication. Nucleoside analogues like cytosine arabinoside (ara C) and other agents known to inhibit DNA synthesis in chick embryo cells also have been reported to prevent the production of RSV (for reviews see Bader, 1967; Temin, 1967b). These observations suggest that RSV replication may depend upon host cell functions not required by other RNA viruses. It has been suggested that this function is DNA synthesis (Temin, 1964a, Bader, 1965). Temin (1964a) has further speculated that the replication of RSV RNA involves a DNA 'provirus' although there is no direct evidence to date for either hypothesis.

In order to understand the relation of host cell functions to RSV replication, we have studied the time course of RSV replication after infection of partially synchronized chick embryo cells in different parts of the cell division cycle. We have found that RSV replication is delayed when cells are infected in the pre-DNA synthesis period (G-I) compared with infection during the period of DNA synthesis (S). Newcastle disease virus (NDV), another RNA virus, replicates over the same time course when cells are infected in either G-I or S.
METHODS

**Chemicals.** Deoxycytidine was purchased from Calbiochem, Los Angeles, California; cytosine arabinoside from Sigma Chemical Company, St Louis, Missouri, thymidine [methyl-3H]- and [5-3H]uridine from New England Nuclear Corp., Boston, Massachusetts.

**Virus.** The Bryan high titre strain of Rous sarcoma virus, RSV (RAV)I, was grown in chick embryo fibroblast cultures in growth medium 1 to produce virus stocks with 10⁶ to 10⁷ focus forming units (f.f.u./ml.). The L-Kansas strain of Newcastle disease virus was grown in chick embryos as previously described (Bratt & Robinson, 1967).

**Cell culture.** RIF free 10-day-old chicken embryos from Kimber Farms Hatchery, Niles, California, were cultured as described by Rubin (1960a).

**Tissue culture media.** Tris + saline buffer contained pH 7.0 tris.HCl 9.3 g., Na₂HPO₄ 0.1 g., CaCl₂ 0.1 g., MgCl₂.6H₂O 0.1 g., and water to make 1 l. Growth medium 1 contained 199 medium 84 parts, tryptose phosphate broth 10 parts, calf serum 4 parts, chick serum 1 part and 2.8% NaHCO₃ 1 part. Growth medium 2 for synchronized cultures contained 199 medium 83 parts, tris + saline buffer 9.5 parts, tryptose phosphate broth 0.5 parts, calf serum 4 parts, chick serum 1 part and 2.8% NaHCO₃ 1 part. Primary medium contained 199 medium 95 parts, tryptose phosphate broth 2 parts, calf serum 1 part, chick serum 1 part, and 2.8% NaHCO₃ 1 part. Soft agar for primary cultures contained 199 medium 95 parts, tryptose phosphate broth 2 parts, calf serum 1 part, chick serum 1 part, 2.8% NaHCO₃ 1 part and Difco agar 0.36%, Scherer's hard agar for focus assay overlay contained Scherer's medium 83 parts, tryptose phosphate broth 10 parts, calf serum 4 parts, chick serum 1 part, 2.8% NaHCO₃ 2 parts and Difco agar 0.75%.

**Antiserum.** Chicken antiserum to RAV-I was prepared as described by Hanafusa, Hanafusa & Rubin (1964) and was inactivated for 30 min. at 56° before use. The k value of the antiserum was 0.8 x 10⁸ min⁻¹ at 40°.

**Virus assay.** RSV was assayed by focus formation in tissue culture as described by Rubin (1960a) except that Scherer's hard agar was used to cover the monolayers. The plaque assay of NDV was described by Bratt & Robinson (1967).

**Cell counting.** Cell monolayers in 60 mm. Petri dishes were washed once with tris + saline and incubated with 2 ml. of 0.125% trypsin in tris + saline at 37° for 7 min. Cells were then removed from the dish, mixed well, diluted and counted in a Coulter Electronics Model B cell counter.

**Mitotic index.** Cell monolayers in 60 mm. Petri dishes were washed once at appropriate times with tris + saline buffer, fixed with methanol and stained with Giemsa stain. Cells in metaphase and anaphase were scored among 1000 cells counted per culture.

**DNA and RNA synthesis.** DNA and RNA synthesis were determined by adding 5 μCi [methyl-3H]-thymidine (5Ci/m-mole) or 5 μCi [5-3H]-uridine (25 Ci/m-mole) respectively to the cell monolayers in 60 mm. Petri dishes containing 5 ml. of growth medium 2 and incubating the cultures for 1 hr at 40°. The monolayers were washed three times with tris + saline and the cells were removed with a solution of 1% sodium dodecyl sulphate in 0.1 M-NaCl, 0.01 M-tris (pH 8.2) and 0.001 M-EDTA. The aqueous solution was extracted once with buffer-saturated phenol and trichloracetic acid precipitable radioactivity was determined as previously described (Bratt & Robinson, 1967).
RESULTS

Synchronization of chick embryo cell cultures

Chick embryo cells were synchronized in tissue culture without using chemical inhibitors with metabolic effects which may not be completely understood or be completely reversible. Primary cultures from 10-day-old chick embryos were started with $6 \times 10^6$ cells per 100 mm. plastic tissue culture dish in 15 ml. of primary medium. The cultures were incubated at $37^\circ$ in an atmosphere of 2 % CO$_2$ for 3 days. On the fourth day the monolayers were confluent (approximately $1.5 \times 10^7$ cells per culture) and the medium was replaced with 15 ml. of soft agar. There was no increase in cell number during four more days at $37^\circ$ (Table 1). Secondary cultures started from 6 and 8 day old primary cultures appeared to be healthy 2 and 4 days respectively after overlaying with agar and grew with the same doubling time (17 to 20 hr) as the primary cells before they were covered with agar (Table 1). In contrast to primary cells cultured under agar, cultures which were changed to growth medium 1 on day 4 continued to increase in cell number and in a short time lost their capacity to grow (Table 1). Thus it appeared that in the cultures under agar cell growth was arrested and the cells retained full capacity for growth. The primary cells under agar were found to be arrested in G-1 or the pre-DNA-synthesis period of the cell cycle (Prescott, 1969) and after transfer to low cell density grew in a partially synchronous fashion (Fig. 1a).

Synchronized cultures of cells for our experiments were prepared from primary cultures 2 days after they were overlaid with nutrient agar by removing the cells with trypsin and transferring them to secondary cultures at a concentration of $9 \times 10^6$ cells/60 mm. plastic tissue culture dish in 5 ml. of growth medium 2 for incubation at 40°. Two hours after cell transfer the medium on all secondary cultures was changed to insure that the same number of cells would be attached to each dish for subsequent procedures. Immediately after cell transfer almost no DNA synthesis was detected by incorporation of $[^3H]$thymidine (or $[^32P]$O$_4$) into DNA, no mitoses and no increase in cell number were observed, but significant RNA synthesis was detected by $[^3H]$uridine incorporation into RNA (Fig. 1a). DNA synthesis (and the S period of the cell cycle) (Prescott, 1969) began between 6 and 8 hr after cell transfer and was greatest at 13 hr (Fig. 1a). The greatest increase in DNA synthesis in different experiments was 20 to 50 times the rate observed in G-1 and occurred between 11 and 13 hr after cell transfer. From 14 hr on, a sharp increase in mitotic index was observed.

### Table 1. Growth of primary chick embryo cells maintained under nutrient agar

<table>
<thead>
<tr>
<th>Medium of primary culture changed on day 4 to:</th>
<th>Age of primary culture (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth medium 1</td>
<td>4</td>
</tr>
<tr>
<td>No. of cells in primary culture</td>
<td>$1.7 \times 10^7$</td>
</tr>
<tr>
<td>doubling time of secondary cells*</td>
<td>19 hr</td>
</tr>
<tr>
<td>Nutrient Agar</td>
<td>6</td>
</tr>
<tr>
<td>No. of cells on primary plate</td>
<td>$2.5 \times 10^7$</td>
</tr>
<tr>
<td>doubling time of secondary cells*</td>
<td>72 hr</td>
</tr>
<tr>
<td></td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>$1.5 \times 10^7$</td>
</tr>
<tr>
<td></td>
<td>20 hr</td>
</tr>
</tbody>
</table>

* Doubling time of secondary cells was determined from the 3 day growth curve of cells seeded at $1 \times 10^5$ cells per 60 mm. dish in growth medium 1.
Fig. 1. Production of RSV and NDV by synchronously growing chick embryo cells. Primary cultures of cells from a single chick embryo were grown under agar and then transferred to make many synchronized secondary cultures. (a) At appropriate times after cell transfer identical secondary cultures were used to determine cell number (○—○; duplicate plates), mitotic index (△—△; single plates) and TCA precipitable tritium incorporated into cell monolayers after incubation with [methyl-3H]thymidine (●—●) or 5-[3H]uridine (○—○). (b) Identical secondary cultures were infected with RSV (RAV), (2 f.f.u./cell) or Newcastle disease virus (2 p.f.u./cell) at 2 hr (●—●) and at 12 hr (○—○) after cell transfer, as shown by arrows in Fig. 1a. At appropriate times samples of medium were removed from infected cultures, frozen and later assayed for RSV (——) and Newcastle disease virus (———).
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until a maximum was reached at 19 hr followed by a decline. After the mitotic index began increasing the cell number increased in a linear manner and doubled over an 8 hr period between 17 and 25 hr after cell transfer. The peak of mitoses which extended over a 6 to 8 hr period and the 8 hr period during which the cells doubled in number indicates that the cells were less synchronous at the time of mitosis than during the first 12 hr after cell transfer. The loss of synchrony probably reflects the heterogeneity of the cell population in primary chick embryo cultures where individual cells may have different division cycle times. The fact that the cell number did double over an 8 hr period indicates that at least most of the cells in the secondary cultures are proliferating. The synchronization of cells by this method was reproducible in many experiments.

**RSV replication in cells infected in G-1 and S**

Secondary cultures of the synchronized cells (Fig. 1a) were infected with RSV (RAV)\(_1\) 2 hr after cell transfer (cells in G-1) and 12 hr after transfer (cells in S) in order to determine the sensitivity of the cells to RSV infection. After a virus adsorption period of 30 min. at 40° the monolayers were washed several times and RSV (RAV)\(_1\) neutralizing antibody in 5 ml. of growth medium was added. Twelve hours later the monolayers were overlayed with hard agar and foci were counted after 6 days. The same number of foci was formed on monolayers infected 2 hr and 12 hr after cell transfer, indicating that G-1 and S period cells were equally susceptible to RSV infection (Table 2).

**Table 2. Susceptibility of G-1 and S period cells to infection by RSV**

<table>
<thead>
<tr>
<th>Time of infection</th>
<th>Number of foci at 6 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hr after cell transfer</td>
<td>Period of cell cycle</td>
</tr>
<tr>
<td>2</td>
<td>G-1</td>
</tr>
<tr>
<td>12</td>
<td>S</td>
</tr>
</tbody>
</table>

Secondary cultures of the synchronized cells (Fig. 1) were infected with 0.2 ml. of a 1:1000 dilution of RSV (RAV)\(_1\) stock for 30 min. at 40° at the designated times after cell transfer. The cell monolayers were then washed three times, incubated with 5 ml. growth medium containing 0.015 ml. RAV-1 antiserum and 12 hr later the medium was replaced with Scherer's hard agar for focus counting 6 days later. Each number represents an average of two infected cultures with their experimental error.

Secondary cultures of the synchronized cells were also infected with RSV (RAV)\(_1\) at a multiplicity of 2 f.f.u./cell 2 hr and 12 hr after cell transfer in order to determine the time course of virus production by cells infected at each time. Samples of medium were removed from the cultures at appropriate times and frozen at −80°. Focus assay was done at the same time on all samples. RSV production began at the same time (24 to 26 hr after cell transfer) and followed the same time course with respect to cell transfer whether cells were infected in G-1 or 10 hr later in S (Fig. 1b). Cells infected at either time began producing virus only after almost all cells in the culture had divided. The indistinguishable time course of virus production by cells infected 2 hr and 12 hr after cell transfer was observed in several different experiments. Thus, although RSV appeared to adsorb to cells and become insensitive to antibody at the same rate in G-1 and S period cells (Table 2), virus production was delayed in cells infected in G-1.

**Newcastle disease virus replication in cells infected in G-1 and S**

To determine whether Newcastle disease virus, another RNA virus, replicates like RSV in synchronized cells, secondary cultures of the same cells used for RSV infection were
infected with Newcastle disease virus at a multiplicity of 2 p.f.u./cell at 2 hr and at 12 hr after cell transfer. As with the cells infected with RSV, samples of medium were taken from the cultures at appropriate times, stored frozen at -80° and assayed at the same time for plaque forming virus. Newcastle disease virus production began shortly after infection and increased rapidly in both G-1 and S period cells (Fig. 1 b). This indicated that Newcastle disease virus and RSV replication were different in that RSV was dependent on, and Newcastle disease virus independent of, the cell division cycle.

![Graph showing replication](image)

**Fig. 2.** Synchronous secondary cultures were prepared and mitotic index (○—○) determined as in the experiment in Fig. 1. Cultures were infected with RSV (RAV) at 12 hr (●—●), 15 hr (○—○), 19 hr (△—△) and 23 hr (□—□) after cell transfer as indicated by the arrows and virus production was measured as in the experiment in Fig. 1.

**RSV replication in cells infected about the time of mitosis**

Several experiments were done to determine the susceptibility of cells to infection by RSV and the time course of RSV replication when partially synchronized cells were infected at different times after the peak of DNA synthesis and about the time of maximum mitotic index (Fig. 2). Because in several experiments the peak of DNA synthesis was observed always between 11 and 13 hr after cell transfer, 12 hr was chosen as the first time after cell transfer for infection. Cells were also infected 15 and 19 hr after cell transfer, corresponding to times just before and after maximum mitotic index, and at 23 hr, after almost all cells had divided. The susceptibility of cells to infection by RSV and virus production by infected cells were measured as described for the experiment in Fig. 1.

Cells infected 12 hr after transfer (t = 12 hr) started releasing virus after the end of mitosis at about t = 26 hr or 14 hr after infection. Cells infected 3 hr later (t = 15 hr), just before the maximum mitotic index, started producing virus 3 hr later than cells infected at t = 12 hr and virus production followed a parallel course. The 14 hr latent period was the shortest observed for cells infected in any part of the cell cycle. The latent period for cells infected after the time of maximum mitotic index was greater than 14 hr, and the rate of virus
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production was significantly less than for cells infected before the peak of mitosis. The latent period for cells infected 19 and 23 hr after cell transfer was about 16 and 18 hr, respectively.

Table 3 shows an estimate for the rate of virus production by cells infected 12, 15, 19 and 23 hr after cell transfer and the susceptibility of cells at each time to infection by RSV measured by focus formation as described for the experiment in Table 2. The later the time of infection, the smaller the amount of virus produced during the first 29 hr after infection. Cells infected after the maximum of mitosis at 19 and 23 hr after cell transfer yielded 38% and 8% respectively of the focus forming virus produced by cells infected 12 hr after transfer. Similarly the number of foci resulting from the same inoculum of virus decreased with increasing time after cell transfer. Only 56, 49 and 20% as many foci developed on cells inoculated with RSV 15, 19 and 23 hr after cell transfer compared with cells 12 hr after transfer. Thus, the ability of the population of cells to develop foci and the capacity to produce virus decrease in parallel with increasing time when cells are infected between 12 and 23 hr after cell transfer.

Table 3. Susceptibility of cells to infection and production of virus by cells infected between 12 and 23 hr after cell transfer

<table>
<thead>
<tr>
<th>Time of infection</th>
<th>Foci at 6 days after virus infection</th>
<th>% of maximum foci</th>
<th>Yield (f.f.u.) in first 29 hr after infection</th>
<th>% of max. virus yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hours after cell transfer</td>
<td>Period of cell cycle</td>
<td>12</td>
<td>S</td>
<td>671 ± 13</td>
</tr>
<tr>
<td>15</td>
<td>Just before peak mitotic</td>
<td>377 ± 42</td>
<td>56</td>
<td>6 x 10^6</td>
</tr>
<tr>
<td>19</td>
<td>Just after peak mitotic index</td>
<td>261 ± 25</td>
<td>49</td>
<td>3 x 10^6</td>
</tr>
<tr>
<td>23</td>
<td>G-I of next cycle</td>
<td>133 ± 12</td>
<td>20</td>
<td>6 x 10^4</td>
</tr>
</tbody>
</table>

Secondary cultures of the synchronized cells shown in Fig. 2 were infected with 0.2 ml. of a 1:1000 dilution of RSV (RAV) stock for 30 min. at 40° at the designated times after cell transfer. Antiserum was added and 12 hr later agar for focus assay as described in Table 2.

Each number is the average for two infected cultures. Virus production was measured as described in Fig. 2.

DISCUSSION

The results of our experiments with RSV show that RSV production starts at the same point in the cell cycle (after mitosis) and at the same rate whether cells are infected in G-I or 10 hr later in S (Fig. 1). This finding is similar to the result of Temin (1967a), who used cultures partially synchronized with depleted culture medium and high concentrations of thymidine and observed an increasing delay in RSV replication, or an increasing virus latent period, the greater the time before mitosis that cells were infected. In addition, we have shown that replication of NDV is independent of the time in the cell cycle when cells are infected and that our cells in G-I immediately after transfer are capable of supporting the replication of another virus (Fig. 1). Thus, the apparent dependence of RSV replication on the time in the cell cycle when cells are infected is not general for all RNA viruses. Further, we have shown that the same number of G-I and S period cells are infected during a 30 min. incubation period with RSV (RAV) followed by neutralizing antibody (Table 2). These results suggest that some step in virus replication, after virus penetration, does not proceed in G-I but is delayed until some time between 12 and 24 hr after cell transfer, i.e. between the peak of DNA synthesis when cells were infected in the S period and immediately after
mitosis when virus production began. A cell function (or functions) present between the peak of DNA synthesis in the S period and the end of mitosis when virus production begins, but not present in cells in G-1, is apparently essential for RSV replication but not for NDV replication.

On the basis of experiments with a number of inhibitors of DNA synthesis, Temin (1964a, b) and Bader (1965, 1966a, b) concluded that DNA synthesis is directly involved in RSV replication. Although this may be the correct interpretation of the experiments with inhibitors of DNA synthesis, there is no direct evidence for the involvement of DNA synthesis per se in RSV replication and other possible interpretations of the inhibitor effects must be considered. Inhibition of DNA synthesis with compounds such as thymidine has been shown to block progression of the cell cycle in the S period (Xeros, 1962; Bootsma, Budke & Vos, 1964). Thus, not only is DNA synthesis inhibited but cell functions associated exclusively with other parts of the cell cycle such as G-2 or mitosis may not be present in cells blocked by inhibitors of DNA synthesis.

Cytosine arabinoside (ara C), which has probably been used more than any other inhibitor of DNA synthesis in experiments with RSV, not only blocks progression of the cell cycle but its effects are irreversible and rapidly lethal to S period chick embryo cells (unpublished observations). Similar observations about the lethal effects of ara C on cells in the S period have been made with mammalian cells (Young & Fischer, 1968) and it has been shown to block irreversibly the DNA polymerase reaction in vitro (Momparler, 1969). The apparent reversibility reported for the effects of ara C on DNA synthesis in chick embryo cells after short periods of incubation with cells (Bader, 1965, 1967) could be explained by the lack of toxic effects of ara C on cells not actively synthesizing DNA. Removal of the drug before the onset of DNA synthesis permits chick cells to replicate in a normal fashion (unpublished observations). Thus, cells not synthesizing DNA during exposure to ara C (e.g. cells in G-1) could subsequently pass through the S period after removal of ara C and the apparent effect of ara C would be reversible inhibition of DNA synthesis.

Because inhibitors of DNA synthesis block progression of the cell cycle, more direct evidence is needed to determine whether DNA synthesis per se is essential for RSV replication or whether other cell functions during the S period or even later in the cell cycle during mitosis are involved.

There is some evidence that RSV replication may depend upon events during cell mitosis. Our data show that even after infection of cells many hours before mitosis RSV production does not begin before the end of mitosis. Temin (1967a) observed that colchicine in concentrations which arrest cells in mitosis blocks or delays RSV production. Whether colchicine acts directly on the virus or whether its primary effects are on a cellular function essential for RSV replication is not clear. Also it was not shown whether the colchicine effect is unique for RSV or whether it occurs with other viruses.

In our experiment in which cells were infected 15 hr or more after transfer, cell growth was not sufficiently synchronous to permit conclusions about the relation of RSV replication to later parts of the cell division cycle. Clearly, however, more than 15 hr after cell transfer cells rapidly lose in parallel their ability to form foci and to produce virus (Table 3), suggesting that they lose susceptibility to infection by RSV. Such a change in ability to form foci with increasing time of cells in culture was described by Rubin (1960b). The mechanism of this change is not known.
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REFERENCES


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