Virus Production and Release, Cell Longevity, and Cloning Efficiency of Chicken Embryo Fibroblasts Infected with Rous Sarcoma Virus

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Continuous virus production is a characteristic of chicken embryo fibroblasts (CEF) infected and transformed by a nondefective Schmidt-Ruppin subgroup A Rous sarcoma virus. This virus production has been examined with particular attention to the amount of newly budded virus which remained cell-associated, and to the amount and degree of viral aggregation at the cell surface and in the fluid tissue culture medium. The total biologically active virus associated with a Schmidt-Ruppin subgroup A Rous sarcoma virus-infected CEF culture was divided almost equally between that portion of virus which was present in the fluid medium and that portion which was cell-associated. Various mechanical and enzymatic methods were used to remove cell-bound virus and to disperse aggregates of virus in the tissue culture medium to assess cell production of virus per hour accurately, which was determined as an average of 16.4 focus-forming units per cell per hour. With appropriate culture conditions, it was found that Schmidt-Ruppin subgroup A Rous sarcoma virus-infected and -transformed CEF replicated faster, could be passaged more times, and grew to higher cell densities than did normal CEF and CEF infected with a subgroup A Rous associated virus. Subgroup A Rous sarcoma virus-infected CEF cloned with much lower efficiency than did subgroup A Rous associated virus-infected CEF or normal CEF. Experiments employing a temperature-sensitive mutant of subgroup A Schmidt-Ruppin Rous sarcoma virus- and Rous associated virus-infected CEF indicated that the poor cloning efficiency of Schmidt-Ruppin subgroup A Rous sarcoma virus infected cells was not due to the constant production of virus but was probably related to some property associated with transformation of the cell by Rous sarcoma virus.

The question of the role of virus production and release in the growth of virus-caused tumors is an important aspect in our understanding of the characteristics of these malignancies. It has previously been shown that chicken embryo fibroblasts (CEF), subsequent to infection by Rous sarcoma virus (RSV), do not form continuous cell lines and, in fact, possess a shorter life span in vitro than the normal CEF, from which they were derived under certain cultural conditions in contrast to other malignant cells (17). Also, it has been shown that tumor production and proliferation in vivo is primarily dependent upon recruitment of normal host cells by infection of adjacent cells through the release of virus by the tumor cells (13, 16, 17). A possible reason for the limited life span of these tumor cells in vitro, and possibly in vivo, could be the continuous production of virus, which involves not only the added cellular burden of synthesis of viral components but also the constant replacement of cellular cytoplasmic and membrane constituents which are lost as the virus buds from the cell (10). These interesting characteristics of this malignant cell in vitro are the subject of this investigation.

The rate of virus production by CEF infected and transformed with a helper-independent strain of RSV was studied, taking into consideration the cohesion of virus to the cell surface to assess true virus production. Viral aggregates both at the cell surface and free-floating in the tissue culture fluid were dispersed to accurately measure the virus content.

Along with the establishment of unrestricted growth potential, oncogenic transformation by other tumor viruses of cells in vitro has usually been associated with an increased plating efficiency of the transformed cell as compared to the normal cell. It was therefore of interest to
assess the capacity of RSV-transformed CEF to form clones in vitro in comparison with their normal counterparts.

The avian leukemia-sarcoma complex provides an ideal system to study the virus-cell interactions as they relate to the growth capacity of infected cells, since cell transformation is rapid and virus titration methods are accurate. Also, the effects of virus production can be separated from cellular transformation when CEF are infected by a nontransforming leukemia virus (Rous associated virus), in contrast to infection of CEF by a sarcoma virus of the same subgroup that provides a cell which is producing virus and which is transformed. Finally, infection of CEF by the appropriate temperature-sensitive mutants of RSV allows one to selectively study virus production with or without cellular transformation, depending on the temperature of incubation.

MATERIALS AND METHODS

Viruses. The Bryan strain of RSV (subgroup A of Rous associated virus [RAV-1]) was derived from virus C-T 845 supplied by W. R. Bryan (National Cancer Institute, Bethesda, Md.). A subgroup A Schmidt-Ruppin strain of RSV (SR-RSV-A) was supplied by E. Reich (The Rockefeller University, New York, N.Y.). The temperature-sensitive mutant, ts-68, of a subgroup A Schmidt-Ruppin strain (RSV ts-68) and the wild-type virus from which the mutant was derived were the kind gift of H. Hanafusa (The Public Health Research Institute, New York, N.Y.). The nonfocus-forming RAV-1 virus was originally supplied by J. P. Bader (National Cancer Institute, Bethesda, Md.).

Cells. CEF were cultured from embryonated eggs obtained from R. E. Lugunbuhl (University of Connecticut, Storrs, Conn.) and were supplied by the special Virus Cancer Resource Program (National Cancer Institute, Bethesda, Md.) as previously described (11). All cell cultures were maintained in growth medium consisting of nutrient mixture F-12 and saline G (3) supplemented with 10% tryptose phosphate broth, 8% newborn calf serum, and 2% chicken serum. Usually, cells growing on culture dishes were infected directly by incubating the cells with a virus inoculum, including diethylaminoethyl-dextran, for 1 h at 39 C (5). Cultures to be used for the determination of virus production rates were maintained in culture for 10 days to insure uniform infection and transformation prior to use. On the other hand, cells to be used for cloning experiments were infected at 4 C while in suspension for 1 h and were cloned, immediately after infection and 4 days after infection, in culture.

Transforming virus and the percentage of cells within a population, which were previously infected and transformed, were also assayed by previously reported methods (24). To assay the presence of RAV-1, the nontransforming virus, susceptible first-passage CEF were infected with several dilutions of RAV-1 and, 7 days later, these cells were challenged with a standard inoculum of the Bryan strain RSV(RAV-1) (20).

Viability of cells to be plated for cloning experiments was determined by counting, with the use of the erythrosin-B exclusion technique previously reported (15).

It has been found that the viral budding process ceases when the temperature at which virus-producing cells are incubated is lowered just a few degrees below 37 C (personal communication, G. de Thé); this was confirmed by experimental tests. To insure the cessation of viral budding, at the beginning of an experimental protocol cultures to be analyzed for viral production rates were chilled to 7 C for 20 min.

Cloning normal and infected cells. Portions (0.2 ml) containing 100 viable normal CEF or 400 viable infected cells were dispensed in 60-mm plastic tissue culture dishes containing 5.0 ml of cloning medium. Cloning medium was comprised of equal volumes of fresh F-12 growth medium and the conditioned medium previously exposed to normal CEF (21), along with viral strain-specific antibody to a final dilution of 1:50 to prevent the spread of virus infection in the culture.

Staining and counting attached cells. A duplicate set of plates was prepared at the time of cloning to determine the number of cells in the cloning inoculum which attached to culture dishes within 24 h of plating. Attached infected and normal cells were fixed in situ for 10 min with the addition of 0.1 ml of formaldehyde solution to the growth medium. After the cells were rinsed in a stream of distilled water, they were stained with 3.0 ml of Delafield hematoxylin solution and counted with the aid of a microscope.

Soft agar culture. SR-RSV-A-infected cells were grown as colonies in soft agar according to methods previously reported (7).

Releasing virus from cells or aggregates. Aggregates of virus were dispersed and virus was removed from cell surfaces with 0.25% (wt/vol) trypsin, 2x crystallized, by a 10-min incubation at 37 C. The proteolytic activity of the enzyme was stopped by the addition of soybean trypsin inhibitor. Alternatively, aggregates of virus were removed from cells mechanically by sonic oscillation. Samples, chilled in an ice-water slush, were sonically treated at 20 kc (approximately 3.75 A) for 10 s, and then 1 min was allowed for the sample to cool before sonication was resumed. Each sample received three cycles of sonic treatment and cooling before virus assay.

RESULTS

Virus production rate. The calculation of virus production rates was based on the steady state equation of Rubin (19). This equation states that the constant rate of virus produced is directly proportional to the product of the biologically active virus present and the thermal inactivation constant of the virus, and indirectly proportional to the number of viable cells within the culture at the time the virus is
harvested. The total biologically active virus
associated with a culture was taken to be the
sum of the trypsin-treated virus in the growth
medium plus the cell-associated virus released
by sonication.

Although various enzymes were used to dis-
perse virus, trypsin or sonic treatment were
found to be the most efficient means of dispers-
ing virus contained within aggregates free-float-
ing in the growth medium (as measured by the
increase in infectious titer of the growth me-
dium subsequent to trypsin treatment), and
sonication was found to be a somewhat more
efficient means of releasing virus from infected
cells at 18 h. By employing these methods, it
was found that the total biologically active virus
associated with SR-RSV-A-infected CEF was
roughly equally divided between that portion of
virus which was contained in the growth me-
dium and that portion of virus which was
cell-associated (Table 1).

The rate of virus thermal inactivation was
determined with portions from a pool of SR-
RSV-A suspended in growth medium which had
been sonically treated prior to incubation and
then incubated at 39 °C. Samples were removed
at various intervals during 56 h of incubation.
The number of focus-forming units (FFU) of
biologically active virus remaining after each
interval of incubation was determined by focus
assay. The biological half-life of SR-RSV-A
under these conditions was calculated to be 2.5
h in three separate determinations, which is
similar to previous data (4).

Virus production was measured with CEF
that had been maintained in culture for 10 days
after infection, which insured maximal infec-
tion and transformation of all cells within the
culture. Before viral harvest (18 h), the infected
cells were replated on 60-mm culture dishes at
a density of approximately 3.0 x 10⁶ cells per
culture dish. This plating density resulted in a
monolayer with no cellular overlap at the time
the virus was harvested, which was comparable
with RAV-infected or normal cells. The virus
measured in these experiments was the total
virus produced by the cells in the 18-h interval
between replating the cells and viral harvest
(Table 1).

The average constant production rate from
six different experiments was 16.4 FFU per cell
per h.

The nature of the association between
virus and transformed cell surfaces. Since
the amount of cell-associated virus contrib-
uted significantly to the total amount of virus
produced by SR-RSV-A-infected and trans-
formed CEF, the tenacity with which this
virus was bound to cell surfaces was investi-
gated. Cultures were chilled to prevent fur-
ther virus replication and release, and then
they were studied for virus content. First, a
determination of the amount of virus which
could be removed from cells by simply wash-
ing the infected cell sheet was made. This was
accomplished by adding 3.0 ml of tris (hydroxy-
methyl)aminomethane (Tris) A buffer (pH 7.4)
to each of two duplicate plates, which was as-
sayed for virus content with and without sonic
treatment. Cell sheets were washed with Tris
A three times.

To determine the amount of virus still
adhering to the same cells, they were ex-
posed to three cycles of trypsin treatment
(0.25% wt/vol), as this enzyme has been pre-
viously shown by Allen (1) to release virus ad-
hering to cells. Portions (3.0 ml) of the 0.25%
(wt/vol) trypsin was added to each of two cul-
tures, and suspended cells were placed in a
37 °C water bath for 10 min. The proteolytic
activity of the enzyme was stopped by the addi-
tion of soybean trypsin inhibitor. The cells were
removed from suspension by centrifugation, and
a portion of the supernatant was removed for
viral assay with and without sonic treatment.
The remaining supernatant was decanted and
fresh, and prewarmed trypsin was used to re-
suspend the cell pellet, initiating another cycle
of trypsin treatment.

Finally, any virus which remained cell-as-
associated after three washes with Tris A and
three cycles of trypsin treatment was re-
leased by sonically treating the remaining cells.
The composite results of two representative
experiments are presented in Table 1. The
data indicate that washing the cells with Tris
removed significant amounts of virus, but
that subsequent trypsin treatments released
more firmly bound virus; with both of these
treatments, significant amounts of virus re-
maind firmly bound to the cell. Furthermore,
the virus was released in aggregates since sonic
treatment resulted in a significant enhance-
ment of the titers. However, since only a maxi-
mum of 65% of the total cell-associated virus
could be recovered, significant viral inactiva-
tion must have occurred during the experimen-
tal manipulations.

Longevity studies. Since some insight had
been gained into the true rate of virus produc-
tion by CEF infected and transformed by SR-
RSV-A, the effect of continual virus production
on cells infected with a transforming virus and a
nontransforming virus (RAV-1) was assessed.
Approximately 3.0 x 10⁶ CEF cultured from
individual embryos and growing in 100-mm
<table>
<thead>
<tr>
<th>Expt</th>
<th>Titer in growth medium (FFU/ml)</th>
<th>Titer released by sonication (FFU/ml)</th>
<th>Tris A washes (FFU/ml)</th>
<th>Trypsin treatment (FFU/ml)</th>
<th>Titer released from remaining cells by sonication (FFU/ml)</th>
<th>Trypsin to Tris A ratio</th>
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<tr>
<td>I</td>
<td>$1.7 \times 10^7$</td>
<td>$1.2 \times 10^7$</td>
<td>$1.4 \times 10^4$</td>
<td>$10^4$</td>
<td>$8.0 \times 10^3$</td>
<td>$3.3 \times 10^4$</td>
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<td></td>
<td></td>
<td>$4.7 \times 10^4$</td>
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<td>$9.0 \times 10^4$</td>
<td>$5.7 \times 10^4$</td>
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<td>$4.0$</td>
<td>$11.2$</td>
<td>$1.7$</td>
<td>$3.4$</td>
<td>$8.4$</td>
</tr>
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<td>$1.1 \times 10^7$</td>
<td>$8.5 \times 10^6$</td>
<td>$10^6$</td>
<td>$4.0 \times 10^4$</td>
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<td>$8.8 \times 10^4$</td>
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<td>$1.7$</td>
<td>$2.0$</td>
<td>$1.3$</td>
<td>$3.1$</td>
<td>$5.9$</td>
</tr>
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</table>

* One set of cultures was used for determination of the virus present in the 18-h growth medium and for determination of the amount of virus which could be released from cell surfaces by three sequential Tris A washes, followed by three sequential 10-min trypsin treatments, followed by sonication of the remaining cells. An identical set of cultures was used for the determination of the amount of virus which could be released from cell surfaces by sonication alone after the growth medium was removed. In addition, portions of individual Tris washes and trypsins were sonicated and compared to portions before sonication. All virus titers expressed in FFU/ml are adjusted to FFU/ml present in a total volume of 6.0 ml. Therefore, all virus titers are directly comparable.

* The ratios of I-Sonicated/II and II-Sonicated/II indicate the factor by which the infectious titer of each Tris wash and trypsinize was increased by sonication.

* The ratio was calculated by dividing the sum of the virus titers obtained from individual Tris washes, trypsinizations, and sonication of the remaining cells by the virus titer obtained by the sonication of cells alone.
plastic culture dishes were infected with either 30 FFU of SR-RSV-A or 30 infectious units of RAV-1 per cell. A third plate was mock-infected with viral diluent and served as a normal control culture. Two days after infection, all cultures were refed with fresh growth medium. From this point until the termination of the experiment, normal cell cultures and RAV-1-infected cultures were refed every second day, which produced maximal growth, whereas SR-RSV-A-infected cultures were refed daily after 50% or more of the cells in the culture were morphologically transformed since it was found that RSV-infected cells grew much better on a daily refeding schedule. When cell confluency was reached, each set of cells was subcultured by replating 20 to 33% of the cells in the confluent monolayer to obtain comparable numbers of control and infected cells. Throughout the entire experimental period, the average number of normal and RAV-1-infected cells in the culture plates at the time of subculture was 10^7, whereas the average number of SR-RSV-A-infected cells at the time of subculture was 1.5 \times 10^7 (which was low since some transformed cells detached from the culture dishes and were removed as the medium was changed). At weekly intervals throughout the experimental period, portions of growth medium were removed from the RAV-1- and SR-RSV-A-infected cultures and analyzed for virus content.

The results of four experiments, with the same viruses but with cells from four separate individual embryos, are summarized in Table 2. The results from all four cultures are similar. The number of times that the normal cells and the RAV-1-infected cells could be passaged and continue to replicate was about the same, whereas the SR-RSV-A-infected cells could be subcultured three to four more times than the RAV-1-infected and normal cells. In addition, the normal cells required more days in culture to reach the last subculture than did SR-RSV-A-infected cells, with the exception of cells of embryo III. These data indicated that normal cells divided at a slower rate than did the SR-RSV-A-infected cells. The capacity of all cells to divide was limited since they stopped

<table>
<thead>
<tr>
<th>Embryo and infecting virus</th>
<th>No. of days in culture</th>
<th>No. of passages</th>
<th>No. of days in culture to last passage</th>
<th>Total expanded cell no.</th>
<th>No. of days in culture^c</th>
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<tr>
<td></td>
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<td></td>
<td></td>
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<td>10^3</td>
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<tr>
<td>I</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Control</td>
<td>175</td>
<td>14</td>
<td>80</td>
<td>5.9 \times 10^{15}</td>
<td>175</td>
</tr>
<tr>
<td>RAV-1</td>
<td>175</td>
<td>13</td>
<td>64</td>
<td>2.4 \times 10^{15}</td>
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<td>74</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
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<td>12</td>
<td>80</td>
<td>1.2 \times 10^{15}</td>
<td>175</td>
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<tr>
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<td>12</td>
<td>66</td>
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<td>60</td>
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<tr>
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<td>15</td>
<td>58</td>
<td>7.6 \times 10^{16}</td>
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<tr>
<td>III</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>161</td>
<td>12</td>
<td>66</td>
<td>7.1 \times 10^{13}</td>
<td>66</td>
</tr>
<tr>
<td>RAV-1</td>
<td>69^a</td>
<td>16</td>
<td>63</td>
<td>6.5 \times 10^{16}</td>
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<td>89</td>
<td>8.3 \times 10^{18}</td>
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<tr>
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<td>15</td>
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<tr>
<td>RAV-1</td>
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<td>18</td>
<td>57</td>
<td>5.8 \times 10^{18}</td>
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<tr>
<td>SR-RSV-A</td>
<td>161</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

* Normal cells and cells infected with SR-RSV-A or RAV-1 cultured from a single embryo in each experiment were maintained in culture for either 175 or 161 days. The growth medium from all the infected cultures was assayed for virus content at weekly intervals.

* Total number of cells which the cell line could have produced during its culture life if no cells had been discarded at the time of subculture. This figure was calculated on the proportion of cells discarded at each passage to the number of times each culture was passaged.

* Virus titers expressed in FFU per milliliter.

* The culture was lost to contamination on the day indicated.
dividing between 58 and 89 days in culture, depending on the single embryo used. The period during which cell multiplication took place represented approximately one-half of the total time the cells were maintained in culture before cells began to degenerate (161 to 175 days).

Under these culture conditions, SR-RSV-A-transformed cells had the potentiality of producing 60 to 100,000 times more cells than the normal cells from which they were derived (Table 2). The RAV-1-infected cells appeared to more closely resemble the growth characteristics of normal cells, as in cells from embryo I and II, or to hold an intermediate position between normal cells and SR-RSV-A-transformed cells of embryo III. Once the point had been reached (58 to 89 days in culture) at which rapid cell division was no longer apparent, the cells remained on the plate in stable numbers, with regular refeeding for approximately 100 days. During this portion of culture life, all cells increased in size and the normal cells and RAV-1-infected cells became highly vacuolated. Even though no net increase in the number of cells per culture was observed, the cells infected with RAV-1 and SR-RSV-A continued to produce virus. The SR-RSV-A-transformed cells had maintained a consistent, high level of virus production when the cells were rapidly dividing, but, with the cessation of cellular division, there was a concomitant reduction in the amount of virus produced. The observed decrease in virus production may have been a reflection of the reduction in cell number per culture, or it may have been a reflection of a reduction in the rate of virus production of the cells remaining in culture due to their nonreplicating state. However, it has been shown (23) that although initiation of virus infection is dependent on cell mitosis, later mitoses do not appear necessary for virus production.

Cloning studies. Preliminary observations in this laboratory have suggested that in the absence of feeder layers, RSV-infected and -transformed cells cloned with lower efficiency than did the normal cells from which they were derived. The role of continuous virus production alone, and continuous virus production concomitant with cellular transformation, was therefore studied in relationship to the reduced cloning efficiency of SR-RSV-A-infected cells in comparison to normal cells from a single embryo.

(i) Cloning SR-RSV-A-infected cells. First-passage CEF exposed to SR-RSV-A at high multiplicities of infection at 4 C were cloned immediately after infection and 4 days after infection, since cloning immediately after infection should allow time for clonal development before the infected cells became transformed, whereas cloning 4 days after infection would allow time for transformation to occur before the cells were tested for their capacity to form clones.

Normal CEF cloned with higher efficiency than did the SR-RSV-A-infected cells when cloned immediately after infection (Table 3). By 4 days after infection, the difference in cloning efficiencies was even more striking. Surprisingly, the cells within the infected clones had the same morphology as the cells within clones of normal cells, indicating that morphological transformation had not occurred in cells successfully growing as clones. It seemed possible, therefore, that the cells from the infected cultures which cloned were themselves not infected. However, several individual clones, all with normal cellular morphology, were isolated

<table>
<thead>
<tr>
<th>Exp#</th>
<th>Cloning efficiency (%)</th>
<th>Attachment (%)</th>
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<tr>
<td></td>
<td>Normal</td>
<td>Infected</td>
</tr>
<tr>
<td>Day of infection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>25</td>
<td>7.5</td>
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<tr>
<td>B</td>
<td>40</td>
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</tr>
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<td>C</td>
<td>33</td>
<td>32</td>
</tr>
<tr>
<td>D</td>
<td>34</td>
<td>33</td>
</tr>
<tr>
<td>Four days after infection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>30</td>
<td>1</td>
</tr>
<tr>
<td>B</td>
<td>35</td>
<td>1</td>
</tr>
<tr>
<td>C</td>
<td>44</td>
<td>35</td>
</tr>
<tr>
<td>D</td>
<td>48</td>
<td>36</td>
</tr>
</tbody>
</table>

*Experiments A and B were infected with RSV; C and D were infected with RAV-1.
*Normal cells (100) and infected cells (400) were plated on the day of infection and 4 days later in cloning medium supplemented with 2% chicken serum and 8% calf serum. Seven days after plating, the clones were counted on four plates each.
*Normal cells (100) and infected cells (400) were plated on the day of infection and, after 4 days in culture in cloning medium, were supplemented with 2% chicken serum and 8% calf serum. After plating (24 h), the cells were fixed, stained, and counted.
*ICA (infectious center assay) plates containing feeder layers of normal cells were plated with either 25 or 50 SR-RSV-A-infected cells on the day of infection or 4 days later and were overlaid with agar containing medium. Seven days later, the infectious centers were counted. The average for all plate counts within one experiment is presented.
and tested for the production of virus. All clones tested produced infectious virus which transformed susceptible CEF in virus assay. Infectious center assays were performed on infected cells both on the day of infection and 4 days after infection at the time of cloning, and at 4 days, all cells were releasing virus (Table 3). The decrease in cloning efficiency of infected cells was correlated with the increase in the number of cells which registered as infectious centers, confirming that they were infected. Two experiments in which cells infected with Bryan RSV (RAV-1) were used gave results similar to those obtained with SR-RSV-A.

It was possible that cells from infected cultures did not clone as well as cells from normal cultures, because the infected cells did not attach as efficiently to culture dishes as did the normal cells. To investigate this possibility, duplicate plates were planted with the same number of cells, 100 normal and 400 infected, under the same conditions as the plates used for cloning. These cultures were incubated for 24 h, and then the attached cells were fixed to the culture vessels and stained with Delafield hematoxylin for counting. Two categories of cells which attached to plates were recorded. Individual cells with no other cells in close proximity were scored as single cells. Two cells contiguous with one another and possessing the same morphology were also scored as single cells, since the occurrence of two adjacent cells of identical morphology was interpreted as due to division during the 24-h period of culture. It was of interest to learn if a correlation could be made between the number of attached cells which divided in the first 24 h and the number of clones which developed by 7 days after plating, but no such correlation could be found. The percentage of cells plated that attached to culture dishes within the first 24 h of culture is shown in Table 3. In all cases, the infected cells attached to culture dishes as efficiently as normal cells, independent of whether the experiment was performed on the day of infection or 4 days after infection. Therefore, the poor cloning efficiency of infected cells was not due to an inability of the infected cell to attach to culture plates.

(ii) Cloning RAV-1-infected cells. It was important to know whether or not the failure of infected cells to clone was due to the continuous production of virus; therefore, the same cloning procedure was employed with cells infected with RAV-1 (Table 3). The cloning efficiencies of RAV-1-infected cells and normal control cells on the day of infection were practically identical. Four days after infection, when both sets of cells were again cloned, the cloning efficiency of the RAV-1-infected cells was slightly less than that of the control cells. However, the difference in cloning efficiencies was insignificant when compared with the differences in cloning efficiency observed between normal cells and SR-RSV-A-infected cells 4 days after infection. When several RAV-1-infected clones were monitored, all were found to produce significant amounts of virus. These experiments suggested that virus production per se was not responsible for the failure of RSV-infected cells to clone efficiently.

(iii) Cloning ts-68-infected cells. The role of cellular transformation in the failure of RSV-infected cells to clone as efficiently as normal uninfected cells could be examined using a temperature-sensitive mutant (ts-68) of SR-RSV-A. At 37°C, ts-68-infected cells behave as typically transformed cells, whereas at 41°C, ts-68-infected cells have a normal morphology and growth pattern; however, the ts-68-infected cells produce virus at both 37 and 41°C. Furthermore, the virus produced at either temperature was capable of transforming susceptible cells at 37°C. Therefore, transforming virus production was constant in ts-68-infected cells, and transformation would be regulated by changing the temperature at which the infected cells were incubated. At the time of cloning, either immediately after infection or 4 days after infection, duplicate plates of infected and normal cells were incubated at 37 and 41°C. In the 4-day interval, the cells for the test were incubated respectively at 37 and 41°C also.

The results of two such experiments are presented in Table 4. On the day of infection, ts-68-infected cells incubated at 41°C cloned with essentially the same efficiency as normal cells also incubated at 41°C, whereas ts-68-infected cells cloned with somewhat lower efficiency than did normal cells at 37°C. When cells were cloned at 41°C 4 days after infection, the ts-68-infected cells cloned almost as efficiently as normal cells. However, the cloning efficiency of ts-68-infected cells cultured at 37°C dropped dramatically when compared to normal cells grown at 37°C and ts-68-infected cells grown at 41°C. As in the cloning experiments with SR-RSV-A, the decrease in cloning efficiency of ts-68-infected cells incubated at 37°C was correlated with an increase in experiment II in the number of cells registering as infectious centers after infection, but not in experiment I where the initial rate was already 97%. The percentage of cells, infected and normal, which attached to plates within the first 24 h of culture on the day of infection and 4 days after infection was
Table 4. Normal and RSV ts-68-infected cells on day of infection and 4 days after

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Cloning efficiency (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Attachment (%)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>ICA (37 C)&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Infected</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>37 C</td>
<td>41 C</td>
<td>37 C</td>
</tr>
<tr>
<td>Day of infection</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>25</td>
<td>20</td>
<td>15</td>
</tr>
<tr>
<td>II</td>
<td>32</td>
<td>21</td>
<td>20</td>
</tr>
<tr>
<td>Four days after infection</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>28</td>
<td>18</td>
<td>3</td>
</tr>
<tr>
<td>II</td>
<td>31</td>
<td>33</td>
<td>7.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> Normal cells (100) and RSV ts-68-infected CEF (400) were plated on the day of infection and 4 days later. Four plates of normal CEF and four plates of RSV ts-68-infected CEF were incubated at 37 or 41 C, respectively. Seven days after plating, the clones were counted on four plates each.

<sup>b</sup> Normal (100) and ts-68-infected CEF (400) were plated on the day of infection and 4 days later. After plating (24 h), the cells were fixed, stained, and counted.

<sup>c</sup> Infectious center assay (ICA) plates containing feeder layers of normal CEF were plated with either 25 or 50 RSV ts-68-infected CEF on the day of infection and 4 days later and were overlaid with agar containing medium. After 7 days of incubation at 37 C, the infectious centers were counted.

essentially the same regardless of the temperature of incubation.

The soft agar cloning technique was employed as a means of determining the effectiveness of incubation at 41 C in inhibiting the transformation of ts-68-infected cells. Although the technique was not quantitative under the conditions employed, it did provide a means of distinguishing transformed cells from normal cells. SR-RSV-A and ts-68-infected cells incubated at 37 C always formed a variable number of colonies in soft agar, whereas normal cells never formed a colony in soft agar. On the other hand, ts-68-infected cells incubated at 41 C either formed no colonies in soft agar or formed pinpoint colonies which were much smaller than the colonies formed by the same cells when incubated at 37 C. The fact that small colonies did form when ts-68-infected cells were incubated at 41 C suggested leakiness of the mutant. These results are similar to the experiences of Kawai and Hanafusa (6) working with the same temperature-sensitive mutant. This leakiness of the mutant probably also explains the differences observed in experiments I and II in the variations of the capacity of ts-68 virus-infected cells to clone at 41 C in different experiments.

On the other hand, the poor agreement of cloning efficiencies of normal and ts-68-infected cells incubated at 41 C might have been due to reversion of ts-68 to a wild-type virus during replication in new host cells. Virus was harvested from several individual clones of ts-68-infected cells incubated at 41 C, and it was tested for its capacity to form foci at 37 and 41 C. Even at high viral concentrations no foci developed at 41 C. Therefore, reversion to wild-type virus was ruled out as a probable cause for the somewhat reduced cloning capacity of ts-68-infected cells incubated at 41 C.

As was the case with SR-RSV-A-infected cells, the few clones that did develop from ts-68-infected cells at 37 C were all of normal cellular morphology. Furthermore, all clones tested produced infectious transforming virus.

**DISCUSSION**

Since it had been previously shown that large aggregates of virus become associated with the surfaces of CEF subsequent to infection and transformation by members of the avian leukosis-sarcoma complex (12), it was of interest to investigate the contribution of cell-associated virus to the total amount of biologically active virus produced by the cell, and to investigate the tenacity with which the aggregated virus was bound to the cell surface. Although three cycles of freezing and thawing of infected cells had been used previously to release cell-associated virus (25, 28), sonication seems to be more efficient than freezing and thawing in releasing and dispersing cell-associated viral aggregates. The amount of virus associated with cells infected with SR-RSV-A does not seem to be unique, since electron microscopy observation has demonstrated similar degrees of cell surface viral aggregation on CEF infected with Bryan (RAV-1) RSV, Fujinami RSV, and RAV-1 (12). Furthermore, since a significant amount of virus was released from cells by sonic
treatment after they had been subjected to three cycles of trypsinization, it suggested that the cell-associated virus is rather firmly bound to infected and transformed cells.

Even after Tris A washes, trypsinization, and finally sonic treatment, the total virus recovered from such treated cells represented only 65% of the virus that was liberated from duplicate cultures sonically treated directly after the growth medium was removed. This poor recovery was perhaps due to several factors. The sequential exposure of virus and cells to trypsin probably inactivated some of the virus (1), as did thermal inactivation during the procedure. Even though the cells which were treated with trypsin remained in one centrifuge tube during the entire procedure, cells were lost as indicated by viable cell counts at the end of the treatment period, probably due to cellular adherence to pipettes used after each centrifugation to resuspend the cells. However, the viability of cells after the trypsin treatment was between 80 and 90%, so they were unharmed.

By using the techniques for release of virus from cells and disaggregation of virus clumps, a virus production rate of 16.4 FFU per cell per h for the SR-RSV-A strain employed here was obtained which was 10 to 100 times more than that reported from previous investigations. The study of Langlois et al. (8) is most pertinent since the conditions they employed most closely resembled those used here. They calculated a constant rate of virus production of 1 FFU per cell per h with the Bryan strain of RSV and of 0.15 FFU per cell per h for MC-29 virus. They, however, made no attempt to account for cell-associated virus, aggregated virus, or the rate of thermal inactivation. Also, different avian sarcoma-leukosis viruses were employed so that data are not directly comparable, because the SR-RSV-A strain was selected for high virus yield from infected cells.

The results of the long-term in vitro growth of normal CEF and CEF infected and transformed by SR-RSV-A differ somewhat from previous reports (9, 17) using other conditions for culture of cells where infected cells had a more limited life span than normal cells. By using the optimal culture conditions for normal and infected cell lines employed, we were able to maintain SR-RSV-A-transformed and virus-producing CEF in a state of active cell division for up to 89 days in culture. Normal cells were found to grow best with feeding every second day, probably due to the conditioning effect on the medium of cell growth (21), whereas daily refeeding of infected cultures may have limited the accumulation of the possible growth inhibitory substances produced by RSV-transformed cells previously described (22). Also, since RSV-transformed cells grow more rapidly than normal cells and use more glucose than normal cells (2, 14), the availability of glucose may be a growth limiting factor in cultures transformed by RSV. Therefore, daily feeding of SR-RSV-A-transformed CEF may provide sufficient glucose for continued cell growth and/or protection against the rapid drop in pH to levels which are not optimal for cell growth caused by the accumulation of lactic acid in RSV-infected cultures. The fact that transformed cells require daily feeding for optimal growth does indicate, however, that transformed cells are either more fastidious or that this is a consequence of the more rapid growth of the RSV-infected cells, apparently related to conversion of cells to the transformed state and not a result of continuous viral production since the RAV-1-infected cells behaved in a similar fashion to the normal cells.

The results of the cloning experiments clearly indicate that, in the absence of a cell feeder layer, SR-RSV-A-infected and -transformed cells do not clone with the same efficiency as the normal cells. The poor cloning efficiency of SR-RSV-A-transformed cells is in direct contrast to the enhanced cloning ability of mamalian cells infected and transformed by the deoxyribonucleic acid tumor simian virus-40 (29). Again, lack of cloning ability was associated with cellular transformation and not continual virus production, since RAV-1-infected cells and ts-68-infected cells, incubated at 41 C, cloned with practically the same efficiency as the normal cells from which they were derived. Although it was clearly shown that the poor cloning efficiency was not due to an inability of the infected cells to attach to plates within 24 h of plating or a failure of a significant number of the attached cells to initiate division during the first 24 h, we do not know at what point the RSV-transformed cells ceased to divide or die. Though transformed cells attached and began to multiply, it is possible that they might subsequently detach from culture plates due to their fibrinolytic activity, which is produced by a cell factor released by transformed cells interacting with a serum factor (27).

A striking feature of the clones which did develop from CEF infected with SR-RSV-A and ts-68, incubated at 37 C, was their normal cellular morphology, which suggests that reduced efficiency in cloning after RSV-infection is related to properties acquired during transformation by the virus. The fact that this is seen 4 days after infection, when all cells are producing virus as revealed by infectious center assay as well as the demonstration of virus release, eliminates the possibility that these clones are
derived from uninfected cells present in the culture.

Trager and Rubin (26) reported the appearance of clones of normal morphology when using cells infected with Bryan-RSV for 3 h, which were composed of 90% normal uninfected cells and 10% infected cells. These normal-appearing clones arose when the multiplicity of infection was low, and cell division occurred before virus multiplication had begun. With a feeder layer of irradiated CEF, the cloning efficiencies varied from 5 to 30% with no difference observed between infected and control cells. In contrast, Prince (18), with feeder layers of mouse L fibroblasts with CEF infected with Bryan-RSV for 72 h, found that infected cells cloned with a lower efficiency (1.4%) than normal cells (14%) in line with the experiments reported here. The differences in period allowed for infection of cells probably explains the variation in the results obtained in these investigations, since longer exposure to virus insures that almost all cells are infected. This is associated with the decreased cloning efficiency observed by Prince (18) and reported here.

The data show that whereas the SR-RSV-A-infected and -transformed cells have a comparable culture cell life to normal CEF, their cloning efficiency is reduced. This reduced cloning efficiency is related to the process of transformation of the cell by RSV and is not associated with virus production by the cell.

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LITERATURE CITED


