Effect of Rous Sarcoma Virus Infection on Recovery of Nucleolar RNA, Ribonucleoprotein, and DNA Synthesis from Inhibition by Actinomycin D

R. Gerald Suskind, Thomas W. Pry, and Giancarlo F. Rabotti

Laboratory of Experimental Pathology, National Institute of Arthritis and Metabolic Disease, and Viral Biology Branch, National Cancer Institute, NIH, Bethesda, Maryland 20014

SUMMARY

The effect of Rous sarcoma virus (RSV) infection on the recovery of nucleolar RNA and protein synthesis from selective inhibition by actinomycin D was investigated using radioautographic technics. A transient increase in the grain count over nucleoli of actinomycin-treated cells, pulse-labeled with tritiated uridine, was observed in cells 10 to 18 hours after infection with Bryan strain RSV. The proportion of the total RNA synthesized in the nucleolus at that time is greater than in infected or uninfected cells not treated with actinomycin. In mock-infected cells, or in cells exposed to inactivated virus, inhibition of nucleolar RNA synthesis persists for 24 hours after removal of the inhibitor. During this period, actinomycin inhibits both the accumulation of protein label in the nucleolus as well as cellular DNA synthesis, and this inhibition is identical in infected and in uninfected cells. However, 48 to 72 hours after infection, a period coincident with the appearance of some transformed cells, DNA synthesis is significantly greater in infected cells not previously inhibited by actinomycin than in uninfected cells, whether actinomycin-treated or not. It is therefore concluded that RSV infection directly results in the transient synthesis of a nucleolar RNA that is not inhibited by actinomycin under these conditions, whereas the concomitant synthesis of nucleolar protein and cellular DNA is inhibited. The possible relationship of these findings to the formation of the mature virus particle and to the development of transformation is discussed.

INTRODUCTION

Despite intensive investigation, the cellular pathology underlying the "neoplastic" transformation of cells infected with Rous sarcoma virus (RSV) remains poorly understood, and relatively little is known about the site and manner of viral RNA synthesis (15, 16, 23–26, 32, 33, 38–40; S. W. C. Fiske and F. Gaguenau, unpublished data). Studies with metabolic inhibitors of RNA synthesis, such as actinomycin, and inhibitors of DNA and protein synthesis have shown a complex interdependence of infectivity and transformation with cellular DNA and protein synthesis (2–4, 14, 20, 38, 39, 42, 43); however, the mechanism of inhibition is still incompletely understood. Early administration of actinomycin results in inhibition of cellular transformation (4, 38, 42) and will prevent RNA labeling of the purified virus particle (23). It is unclear, however, whether this results from inhibition of intracellular viral RNA synthesis (39) or from host-dependent functions necessary in the assembly of the viral RNA (23). Resolution of this question by the use of physical separation technics is complicated by the fact that the amount of intracellular viral RNA is minuscule in comparison with that of host RNA and that viral RNA appears to be rapidly degraded (23–25). Quantitative radioautography, although cumbersome and lacking in the molecular specificity of physical separation technics, is a rather sensitive method for detecting small or transient differences in intracellular localization.

RNA synthesis in the nucleolus can be selectively inhibited by low doses of actinomycin, probably as a result of a special affinity of actinomycin for guanosine- and cytosine-rich template sites of precursor ribosomal RNA (21, 30, 34, 35). Previous investigations have shown that a concomitantly labeled protein, assembled or synthesized in the nucleolus, is also selectively inhibited by actinomycin (34, 35). These effects, as well as the delayed inhibition of DNA synthesis (6, 19, 25, 29), are reversible by removal of the inhibitor (6, 19, 29, 42). In this report, low doses of actinomycin were employed as a selective inhibitor of nucleolar RNA synthesis in the expectation that subsequent RSV infection might reveal discrete differences in the rates of nucleolar RNA and protein synthesis between infected and uninfected cells which in turn might elucidate some early intracellular events of infection. By studying the rate of recovery of nucleolar RNA synthesis from prior inhibition with actinomycin, a significant and consistent transient increase in the labeling of the nucleolus was found in infected cells at a time when synthesis of nucleolar RNA remained inhibited in mock-infected controls. This increase of...
nucleolar RNA synthesis in infected cells is associated with a depression of nucleolar protein and DNA synthesis, and it precedes the increase of DNA synthesis accompanying cellular transformation (36).

MATERIALS AND METHODS

Tissue Culture

Secondary cultures of chicken fibroblasts were prepared using 9- to 12-day-old leukosis virus-free chick embryos (40) on 40 x 25 mm glass coverslips in 50-mm plastic Petri dishes (Falcon Plastics, Los Angeles, Calif.). Using Medium 109 (12) supplemented by 8% newborn gamma globulin-free calf serum and 10% tryptose phosphate 800,000 cells were plated; these were incubated at 39.5°C in an atmosphere of 5% carbon dioxide. After 12 to 18 hours, the attached cells were washed and infected.

Infection

The virus inoculum used was the Bryan high-titer Rous sarcoma virus strain CT-559 (7, 22) titering at 10^7.5 focus-forming units per 0.2 ml in chick fibroblast cultures (40). The cells were exposed for 3 hours to a 10^-2 virus dilution (1 ml per plate) in a diluent consisting of phosphate-buffered saline (Dulbecco) (10) and 2% horse serum. Estimating the plating efficiency to be 10% under these conditions (40), this dose was considered sufficient for the simultaneous infection of all cells. Growth medium was then replaced after a two-fold rinse. Uninfected control cells were treated identically except that these cells were exposed to either the diluent alone (mock-infected cells) or to the same virus inoculum inactivated in a water bath at 90°C.

Some cells were pretreated with actinomycin D (0.1 or 0.2 μg/ml) for a 3-hour period and were then washed with growth medium prior to infection. All control cells were given identical medium changes (41) and rinses to remove the inhibitor and/or virus suspension.

Radioisotopes

At varying times following actinomycin treatment and/or infection, some cells were pulse-labeled for 10 minutes with 10 μc/ml of uridine-5-3H (20 to 27 c/mmole, obtained from Schwarz BioResearch Inc., Orangeburg, N. Y.) prepared in Eagle's minimal essential medium (11). The cells to be labeled were previously rinsed with this medium in order to remove the nucleoside from the growth medium and were “chased” with growth medium containing an excess of 0.1 mg/ml of cold uridine. Other cells were pulse-labeled with 10 μc/ml thymidine methyl-3H (11.2 c/mmole) for 2 hours and chased briefly with growth medium containing an excess of 1 mg/ml of cold thymidine, or with 10 μc/ml lysine-4,5-3H (2.9 c/mmole), obtained from New England Nuclear Corporation, Boston, Mass.) prepared in Medium 109 without lysine (supplied by NIH Media Section) and chased with medium containing a 0.1 mg/ml excess of cold lysine. After labeling, all cells were washed five times with phosphate-buffered saline (Dulbecco) (10), fixed for 30 minutes in cold 5% glutaraldehyde (Polyscience, Inc., Rydal, Pa.), and buffered in 0.136 M sodium phosphate (18). After fixation the cells were again rinsed five times in cold distilled water, and, in the case of uridine-3H and thymidine-3H label, the acid-soluble nucleotides were removed by extraction with 0.5 N perchloric acid at 0–4°C for 30 minutes.

Radioautography

Radioautographs were prepared as previously described (34, 35) by coating the coverslip preparations with a fine-grain radiographic liquid emulsion (Ilford L-4, diluted 1:1.6) and exposing it at −20°C for 10–21 days. The radioautographs were developed in Ficq's dianaminophenol developer (13) and were stained with Harris' hematoxylin. Examination with phase-contrast optics, using a Zeiss Neofluar 100/1.30 objective, allowed recognition of nucleolar outlines in most cells and gave adequate resolution and heightened contrast to the small silver grains. The relative areas of nucleoli and nucleus were approximately determined for each cell by multiplying their respective largest and smallest diameters, as measured in an ocular micrometer grid. The grain counts over nucleoli, total nucleus, and cytoplasm per cell were then determined by averaging 5 or more counts per cell within an approximate range of 10%. The mean grain counts for samples of 25 randomly selected cells were thus established. Because of the greater consistency of the means of the ratio of nucleolar to nuclear grain counts (35) than that of the means of the separate grain counts, this ratio and the ratio per unit area were determined for each cell. Statistical comparison of the means was performed by variance analysis and standard “t" test, with the aid of a digital computer. The assumption of normality of the sample distribution underlying a “t" test comparison of the means appears justified by the fact that the differences between the mean and the median values (Table 1) are well within the limits of standard error (<2o'/π).

In cells labeled with tritiated thymidine, the mean incidence per mille of labeled nuclei was determined from 5 rank-listed (X_1-X_5) counts of 1000 cells per slide. A linear estimate of the standard deviation of the mean (S_1) was calculated according to the formula S_1 = 0.3724 (X_5-X_1) + 0.1352 (X_4-X_3). The estimated significance of differences between the mean incidence (X_1, X_2) was calculated from the range (w) according to the formula:

\[ t_{d} = \frac{X_1 - X_2}{\frac{1}{2}(w_1 + w_2)} \]

RESULTS

Secondary cultures of leukosis virus-free chicken fibroblasts were treated for 3 hours with 0.1 or 0.2 μg/ml of actinomycin...
Effect of pretreatment with actinomycin D (0.1 μg/ml) on nucleolar RNA synthesis in Rous sarcoma virus (RSV)-infected and in mock-infected chicken fibroblast cells. One out of three representative experiments showing the rate of recovery of nucleolar RNA synthesis after inhibition by actinomycin D (0.1 μg/ml, 3 hr) in chicken fibroblast cells subsequently infected with Rous sarcoma virus or mock-infected with a 10-min pulse of uridine-3H.

Mean [Median] (n = 25) of the ratio per cell of the grain count over the nucleolus/grain count over the total nucleus ± standard deviation (σ)

\[
t_d = \frac{\text{Mean}_1 - \text{Mean}_2}{\sqrt{\frac{\bar{x}_1^2/n_1} + \frac{\bar{x}_2^2/n_2}}}
\]

\[t_{dc} = t_d \text{ relative to the control (column 2)}\]

\[p, (p_c) = \text{significance of difference of the means expressed as probability of random error}\]

Duplicate values represent separate samples from either the same or duplicate culture dishes. The \(t_d\) values of adjacent means are between the dividing vertical lines. The \(t_d\) values of nonadjacent means are on the outside of the respective indicator bracket. This is the same experiment as Charts 1 and 2.

and were washed and infected with Bryan strain RSV as described above. Control cells were either mock-infected in the suspending medium or exposed to a heat-inactivated virus suspension (Chart 3). At various times following infection, the cells were pulse-labeled with tritiated uridine for 10 minutes so that the label would appear only in the nucleus. Mean grain counts of the nucleolus and of the nonnucleolar areas of the nucleus were determined through radioautographs using groups of 25 randomly selected cells; statistical comparison was made by standard "t" test analysis (Table 1). Although the standard deviation for the absolute nucleolar grain counts is on the average of 52.4% of the mean, it is reduced to 26.1% (σ/√n = 5.2%) when the nucleolar grain count is expressed as the ratio (per cell) of the nucleolar to total nuclear grain count (Table 1 column 2; also see Ref. 36).

The effect of pretreatment with actinomycin on nucleolar RNA synthesis in infected and uninfected cells was compared in three experiments (Table 1; Charts 1–3, 5). In mock-infected cells there is a significant selective inhibition of nucleolar RNA synthesis in cells pretreated with actinomycin, but this inhibition is gradually reversed within 24 hours after removal of the inhibitor. However, in cells infected with RSV after actinomycin treatment, the inhibition is already reversed within 10 hours (Table 1, Chart 1). At that time nucleolar RNA synthesis in actinomycin-treated, RSV-infected cells shows a highly significant increase, above that of similarly treated but
Effect of Rous Sarcoma Virus Infection

Chart 1. (Same experiment as Table 1 and Chart 2.) Effect of actinomycin D (0.1 μg/ml for 3 hours) on nucleolar RNA synthesis per unit area in Rous sarcoma virus (RSV)-infected cells and in uninfected chicken fibroblast cells (uridine-3H, 10 min pulse label). Changes with time after infection of the mean grain count per unit area of nucleolus/nucleus expressed as percentage of the respective controls. GM, growth medium. IA, A--A, cells treated with actinomycin D (AD) and subsequently infected with RSV are compared with cells pretreated with AD and subsequently mock-infected. •--•, cells treated with AD and subsequently infected are compared with control cells, i.e., changes in growth medium instead of AD and mock-infected. •—•, cells treated with AD and subsequently mock-infected are compared with control cells. IB, •••••••• cells treated with AD and subsequently infected are compared with infected cells not treated with AD. v—v, infected cells not treated with AD are compared with control cells.

uninfected cells (Table 1). This is shown both as an increase in the absolute grain count over nucleoli and as an increase in the proportion of the total grain count synthesized in the nucleolus (Table 1). In contrast, the nonnuclear portions of the nucleus (nucleoplasm) show no significant differences in grain count (Chart 2). There was no difference between the effects of 0.1 and 0.2 μg/ml of actinomycin and unit area, and total measurements were also similar (Charts 3, 5). The kinetics of the relative changes of nucleolar RNA synthesis occurring within the first 24 hours of the infectious cycle in cells pretreated with actinomycin are more apparent when results are charted (Chart 1) as percentage of their respective controls. In actinomycin-treated cells the nucleolar proportion of the RNA label at 10 hours after infection is about twice that of uninfected actinomycin-treated cells (Charts 1, 3, 5). When corrected for changes of nucleolar volume during actinomycin treatment, there is also a significant transient increase of the nucleolar proportion of RNA label in infected actinomycin-treated cells compared with uninfected control cells not treated with actinomycin (t = 4.9, 2.73, and Charts 1, 3). Moreover, prior inhibition of nucleolar RNA synthesis in infected cells, at 10 hours, results in a small, transient, but consistent increase of nucleolar label above that of infected cells not previously inhibited (t = 3.3, 2.13 and Charts 1, 3); i.e., the rise

3 Additional tables are available upon request from the senior author.

Chart 2. Changes of the absolute mean grain count of the nucleolus and of the nonnuclear portions of the nucleus ("nucleoplasm") in relation to actinomycin treatment in infected and uninfected cells. A—A, Mean grain count over nucleolus in cells treated with actinomycin D (AD) and subsequently infected, expressed as percentage of count in cells treated with AD and mock-infected. •—•, Mean grain count over "nucleoplasm," in cells treated with AD and subsequently infected, expressed as percentage of that in cells treated with AD and mock-infected.
dissociated briefly by actinomycin from ribosomal ribonucleoprotein in the nucleus. As shown in Chart 5, there is an identical 50% reduction of DNA-labeled nuclei in infected and uninfected cells 10–18 hours after removal of actinomycin as well as a decrease in the relative density of the label per cell at 18 hours. However, during this period, nucleolar RNA synthesis of actinomycin-treated infected cells is increased twofold (Chart 5). This inhibitory effect of actinomycin on DNA synthesis in both infected and uninfected cells is reversed within 24 hours. RSV-infected cells which were not exposed to actinomycin showed a 2.5-fold increase in DNA synthesis at 72 hours (Chart 6), coincident with the appearance of some morphologically transformed cells (see also 28, 32). This increase of DNA label is significantly greater in RSV-infected cells at 48 hours (P < 0.0001) and 72 hours (P < 0.0005) than that seen in infected or uninfected cells previously exposed to low doses of actinomycin. These experiments show that pretreatment with low doses of actinomycin results in inhibition of nucleolar protein and cellular DNA synthesis at 10 to 18 hours postinfection in both infected and uninfected cells, but that nucleolar RNA synthesis is inhibited only in uninfected cells during this period which is apparently critical for the “fixation” of the transformed state (20).

in the nucleolar RNA label in actinomycin-treated RSV-infected cells precedes that of RSV-infected control cells by several hours (Chart 1). Using cells infected with heat-inactivated virus as the control, similar differences were obtained (Chart 3). For this reason, the possibility that these observations could result from the presence of a nonspecific growth factor in the virus suspension appears unlikely. It might be possible to account for the increment of RNA label in nucleoli of infected cells pretreated with actinomycin above that of control cells, in which nucleolar ribosome synthesis remains inhibited, by postulating that in infected cells RNA synthesis with a different sensitivity to actinomycin occurs in the nucleolus. Alternatively, it may be that these observations are the result of stimulation of cellular ribosomal RNA synthesis by the infectious process. However, this latter interpretation is difficult to reconcile with a differential inhibition by actinomycin.

In order to establish whether (ribosome-associated) protein in the nucleolus shows a similar increase after RSV infection, nucleolar grain counts were determined after a 15-minute pulse of tritiated lysine. This protein had been shown earlier to be inhibited concomitantly with rapidly labeled nucleolar RNA by low doses of actinomycin (34, 35). Chart 4 shows that the protein label of the nucleolus is, unlike nucleolar RNA, identically depressed by actinomycin in infected and uninfected cells at 10 hours and shows an equivalent rate of recovery within 24 hours. These observations suggest that some rapidly labeled nucleolar RNA in infected cells may be

<table>
<thead>
<tr>
<th>FFU</th>
<th>Postinfection 18 hours</th>
<th>24 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>510</td>
<td>105.3 ± 2.48</td>
<td>84.2 ± 8.15</td>
</tr>
<tr>
<td>610</td>
<td>114.6 ± 5.03</td>
<td>96.9 ± 12.21</td>
</tr>
<tr>
<td>710</td>
<td>123.5 ± 6.89</td>
<td>102.5 ± 10.52</td>
</tr>
</tbody>
</table>

Chart 4. Intranuclear distribution of lysine-3H (15 min pulse) in actinomycin-treated (0.2 μg/ml, 3 hr) chicken fibroblast cells infected with Rous sarcoma virus (RSV). Histogram of nucleolar grain count as percentage of nuclear grain count at 10, 18, and 24 hours postinfection. The grain count over nucleolus relative to that of the nucleolus and adjusted to unit area is shown in the lower histogram. Absolute grain counts ± standard error are shown in table above the histogram.

Grain Count Nucleolus ± σ

<table>
<thead>
<tr>
<th>FFU</th>
<th>Postinfection 18 hours</th>
<th>24 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>510</td>
<td>105.3 ± 2.48</td>
<td>84.2 ± 8.15</td>
</tr>
<tr>
<td>610</td>
<td>114.6 ± 5.03</td>
<td>96.9 ± 12.21</td>
</tr>
<tr>
<td>710</td>
<td>123.5 ± 6.89</td>
<td>102.5 ± 10.52</td>
</tr>
</tbody>
</table>

Chart 3. Intranuclear distribution of uridine-3H (10 min pulse) in actinomycin D-treated (0.2 μg/ml, 3 hr) chicken fibroblast cells infected with active Rous sarcoma virus (RSV) or heat inactivated RSV for 10 hours. Histogram of nucleolar grain count as percentage of nuclear grain count. Absolute grain counts ± standard error are shown in table above the histogram. FFU, focus-forming units.
DISCUSSION

The radioautographic experiments reported here were designed to investigate the functions of the nucleolus with respect to synthesis or assembly of RNA and protein in early stages of RSV infection (8, 15, 16; S. W. C, Fiske and F. Haguenau, unpublished data). When the rate of recovery of nucleolar RNA synthesis from selective inhibition by low doses of actinomycin (34, 35) is compared in infected and mock-infected cells, significant differences in RNA labeling of the nucleolus were observed. It has been shown that the rate of synthesis of rapidly labeled RNA in the nucleolus of cells infected with RSV after actinomycin inhibition is transiently increased, both absolutely and per unit area, above the level observed in actinomycin-treated cells mock-infected or exposed to inactivated virus. Also the proportion of total RNA synthesized in the nucleolus of these infected cells is greater per unit area than in uninfected control cells not previously inhibited. This increase of RNA synthesis in the nucleolus of actinomycin-treated, infected cells was observed 10 to 18 hours following infection and preceded a comparable relative increase in RSV-infected control cells by several hours.

It may be concluded from these findings that: (a) the observed increase of nucleolar RNA label is a result of infection, (b) the nucleolus in infected cells is transiently a site of assembly of rapidly labeled RNA with a different rate of synthesis than in control cells, and (c) this increase of nucleolar RNA synthesis is not inhibited by actinomycin in infected cells under these conditions. It is possible that this increment of nucleolar RNA label, not inhibited by low doses of actinomycin in infected cells, represents a viral RNA. An alternate possibility is that cellular ribosomal RNA synthesis is stimulated as a consequence of infection. Such a transient increase of nucleolar RNA synthesis could result from competition between a viral RNA and actinomycin for guanosine- and cytosine-rich template sites of ribosomal precursor RNA in the nucleolus (21, 30). The ultrastructural localization of this RNA and actinomycin (31) in the nucleolus of infected cells may distinguish between these interpretations.

The increase of nucleolar RNA synthesis in infected cells previously inhibited by actinomycin is not accompanied by a corresponding increase in nucleolar protein, but, in both infected and uninfected cells, nucleolar protein label is inhibited identically by actinomycin. In other cell systems, such protein

Effect of Rous Sarcoma Virus Infection
REFERENCES

AKNOWLEDGMENTS

ACKNOWLEDGMENTS

REFERENCES


27. Rogers, M. E. Ribonucleoprotein Particles in the Amphibian Oocyte Nucleus. Possible Intermediates in Ribosome Synthesis. J.
Effect of Rous Sarcoma Virus Infection


Effect of Rous Sarcoma Virus Infection on Recovery of Nucleolar RNA, Ribonucleoprotein, and DNA Synthesis from Inhibition by Actinomycin D

R. Gerald Suskind, Thomas W. Pry and Giancarlo F. Rabotti