

Cell Cycle-Dependent Activation of Rous Sarcoma Virus-Infected Stationary Chicken Cells: Avian Leukosis Virus Group-Specific Antigens and Ribonucleic Acid

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Stationary chicken embryo fibroblasts exposed to Rous sarcoma virus (RSV) remained stably infected for at least 5 days, but they did not release infectious virus or become transformed until after cell division. These infected stationary cells did not contain avian leukosis virus group-specific antigens or ribonucleic acid (RNA) hybridizable to deoxyribonucleic acid (DNA) made by the RSV endogenous RNA-directed DNA polymerase activity.

Rous sarcoma virus (RSV) appears to replicate through a deoxyribonucleic acid (DNA) intermediate, the DNA provirus (14). Initiation of RSV production requires two types of early DNA synthesis, one viral and one cellular (12). In stationary chicken cells exposed to RSV, viral DNA synthesis occurs, whereas cellular DNA synthesis does not. Therefore, there is no cell division or virus production. The present studies were undertaken to determine whether either avian leukosis virus group-specific (gs) antigens or viral-specific ribonucleic acid (RNA) were present in stationary RSV-infected chicken cells.

MATERIALS AND METHODS

General experimental procedures were the same as those previously described for this laboratory (12, 13).

Cell culture. Primary cultures of fibroblasts were prepared from 12-day-old White Leghorn chicken embryos (Sunnyside Hatchery Co., Oregon, Wis.) and were grown in modified Eagle medium with 20% tryptose phosphate broth (ET medium) and 5% calf or fetal bovine serum. Secondary and later cultures of chicken cells were prepared at 5.0×10^5 cells per 60-mm plastic dish (Falcon Plastics, Division of B-D Laboratories, Inc., Los Angeles, Calif.) and at 2.0×10^6 cells per 100-mm dish. Cultures of stationary cells were prepared at 1.0×10^6 cells per 60-mm plastic dish or at 4.0×10^6 cells per 100-mm dish in ET medium and incubated for 3 days at 38 C in a humidified CO₂ incubator.

Viruses. Schmidt-Ruppin (SRV) and B77 virus were previously described (1). A heat-resistant strain of SRV (SRV-HR), at approximately 1×10^8 focus-forming units (FFU) per ml, was obtained from D. Boettiger (Ph.D. thesis, Univ. of Wisconsin, Madison,

1972). Virus was concentrated by centrifugation and purified by equilibrium density gradient centrifugation in 15 to 65% sucrose gradients in 0.01 M tris (hydroxymethyl)aminomethane-0.001 M ethylenediaminetetraacetic acid (pH 7.5) buffer.

Antisera. Two antisera were used. For immunofluorescence, rabbit immunoglobulin G (IgG) antiserum prepared against Tween-ether-disrupted avian myeloblastosis virus (AMV) was supplied by R. Nowinski. This serum had a fluorescence titer of 1:150 on uninfected chicken cells and a titer of 1:2000 on SRV-infected chicken cells. Since the rabbit anti-AMV antiserum contained antibodies that reacted with both normal cellular components and virus-specific antigens, *in vivo* absorption was carried out to eliminate nonviral fluorescence (8). Absorption was carried out in 2-week-old quail by intraperitoneal injection of 0.5 ml of antiserum followed by exsanguination 6 hr later. Sera were collected and stored at -70 C. This procedure reduced the titer of fluorescence on normal chicken cells to 1:8, whereas viral-specific fluorescence had a titer of 1:500. (Absorption in 1-week-old chickens resulted in a large reduction of fluorescence on both uninfected and infected chicken cells.) For testing cell preparations for avian leukosis virus gs antigens, cells were incubated with a 1:500 dilution of rabbit anti-AMV (purified IgG) or with a 1:128 dilution of rabbit anti-AMV (purified IgG) absorbed in quail.

The antiserum used in complement fixation was prepared against avian leukosis virus gs antigens in a rabbit by using ether-Nonidet P-40 (Shell Chemical Co., New York, N.Y.)-disrupted SRV. The antiserum was absorbed twice with sheep red blood cells and inactivated at 56 C for 30 min. The complement-fixing titer of the antiserum was 1:4 on 4 units of antigen prepared from uninfected quail, rat, or mouse cells, and 1:16 on 4 units of antigen from uninfected

chicken cells. The titer on 4 units of antigen prepared from chicken cells infected with SRV or from chicken cells found to release avian leukosis virus "spontaneously" was 1:128. For testing antigen preparations, the antiserum was used at 1:64 and 1:128 dilutions.

Complement fixation. Complement fixation was carried out by the microtechnique of Sarma (11). Fresh serum obtained from guinea pigs was absorbed twice with 0.3 ml of packed sheep red blood cells per 1 ml of complement and was frozen at -70°C in 0.2-ml fractions. Rabbit anti-sheep hemolysin (Miles Laboratories, Kanakee, Ill.) was inactivated at 56°C for 30 min. Antigens were prepared after two washes with Veronal buffer. Cells, 2×10^7 per ml, were frozen and thawed three times, extracted with an equal volume of ether, and centrifuged for 1 hr at $20,000 \times g$.

Indirect immunofluorescence. Indirect immunofluorescence was used to test cell preparations for viral antigens by the method of Hilgers et al. (8).

Immunofluorescence absorption test. Cells were also examined for the presence of viral antigens by their ability to absorb the positive fluorescence from a known standard system by the method of Hilgers et al. (8). Antigens were prepared from cells as described above for complement fixation. Antiserum was used at a fourfold dilution below the endpoint of viral-specific fluorescence on standard positive cells. Fifty μl iters of antisera were absorbed overnight at 4°C with various amounts of lyophilized antigen protein. Antiserum without antigen was included as a positive control. These antisera preparations were then used in indirect fluorescence tests on standard positive cells.

Polymerase reaction. The standard polymerase reaction of Temin and Mizutani (16) was used to produce large amounts of radioactive virus DNA product.

Reactions were carried out in a total volume of 2.50 to 6.50 ml of reaction mixture containing 160 μg of purified virus per ml and 20 μCi each of ^3H -thymidine-5'-triphosphate (13.4 Ci/mmol; Schwarz BioResearch, Inc.) and ^3H -deoxycytidine-5'-triphosphate (22.5 Ci/mmol; Schwarz BioResearch, Inc.) per ml. The reaction mixture was distributed in 0.5-ml samples and incubated at 40°C for 1 hr, after which the reaction mixtures were pooled for nucleic acid extraction. Approximately 6,000 counts/min of trichloroacetic acid-precipitable product corresponded to 1 ng of DNA.

Nucleic acids and hybridizations. The procedures used for extraction of nucleic acids from polymerase reactions, purified virus, and cells by using diethylpyrocarbonate (Baycovin; Bayer, Leverkusen, Germany) and sodium dodecyl sulfate and for RNA-DNA hybridizations have been described by Coffin and Temin (3, 4). Control hybridizations between uninfected chicken cell RNA and B77 virus product DNA contained a background of 3 to 5% of the counts banding at a density equal to or greater than 1.54 g/ml. Percent hybridization with virus-infected chicken cell RNA was defined as the fraction of counts banding at a density equal to or greater than 1.54 g/ml minus the background of parallel hybridizations with uninfected chicken cell RNA. The density

of 1.54 g/ml gave a slightly lower background compared with the density of 1.53 g/ml used earlier (3, 4). Approximately 45 to 50% of the total B77 virus product DNA used in these studies was shown to hybridize with a large excess of viral RNA. However, in the experiments reported here with cell RNA, lower levels of hybridization were achieved because there were lower concentrations of viral-specific RNA. Recovery of labeled DNA from gradients was always greater than 65 to 70%. Previous reports (3, 6, 7) have established that hybrids between RSV product DNA and viral-specific RNA band in the RNA region of Cs_2SO_4 gradients after equilibrium centrifugation.

DNA complementary to the RSV RNA, "minus" DNA, was prepared as previously described by Coffin and Temin (5), by hybridization of total virus product DNA with excess viral RNA and alkali hydrolysis of the fraction of DNA banding with a density greater than 1.65 g/ml after Cs_2SO_4 equilibrium centrifugation.

RESULTS

Absence of avian leukosis virus gs antigens in RSV-infected stationary chicken cells. RSV-infected stationary chicken cells contain the genome of RSV, but do not release infectious virus (12). These cells were tested by using three immunological techniques, indirect immunofluorescence, complement fixation, and immunofluorescence, to determine whether they contained avian leukosis virus gs antigens.

Stationary chicken embryo fibroblast cells were exposed to SRV-HR, either ET medium or ET medium with serum was added, and various times after infection the percentage of cells containing gs antigens was determined by using indirect immunofluorescence (Fig. 1). Whereas more than 80% of the cells in the serum-containing cultures became positive for gs antigen within 48 hr, cultures of stationary cells were never more than 10% positive even after 130 hr. At 84 hr after infection, serum was added to cultures of stationary cells, and the cells were examined for gs antigens. These cells were 50% positive for gs antigen within 48 hr after addition of serum. The low level of positive cells in the cultures of stationary cells and the low level of virus produced in these cultures are most probably the result of division occurring in a small proportion of the population (15). This interpretation is supported by the observation that autoradiography of cultures of stationary cells labeled with ^3H -thymidine for 24 hr showed approximately 10% of the nuclei were labeled (data not shown).

Infected stationary cells were also tested for gs antigens by complement fixation (Fig. 2). Again no gs antigens were found.

To demonstrate the specificity of the immunological tests described above, immunofluorescence

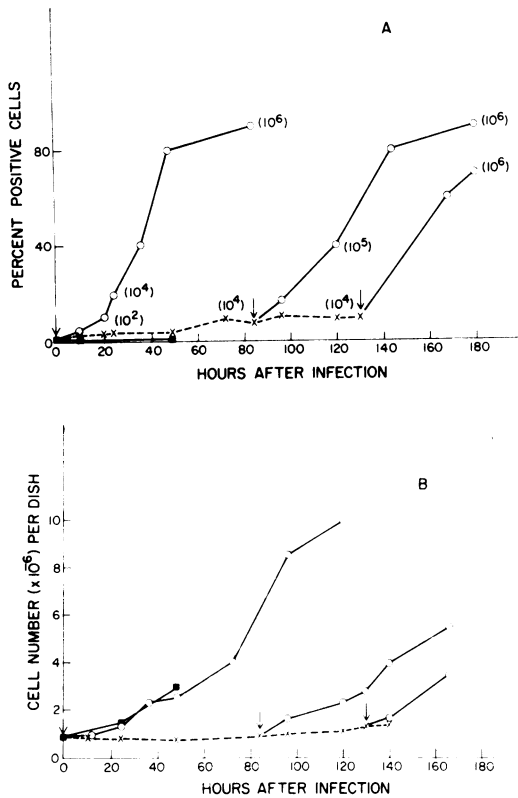


FIG. 1. Absence of avian leukosis virus group-specific antigens from RSV-infected stationary chicken cells. A, Stationary chicken embryo fibroblasts, 1.0×10^6 cells per 60-mm culture dish, were exposed to SRV-HR at a multiplicity of 1 FFU/cell, and the inoculum was replaced with ET medium (X) or ET medium with 4% fetal bovine serum (O). At various times after infection, cells were harvested and examined for gs antigens by using indirect fluorescence. Rabbit anti-AMV (purified IgG) was used at a 1:500 dilution. Each point represents 300 cells. At 84 and 130 hr after infection, serum-containing medium was added to cultures without serum (\downarrow). Culture fluids were not changed during the experiment. Virus titers (indicated in parentheses) represent FFU per ml of culture fluid at the time of cell harvest. Uninfected cells were incubated in medium with 4% fetal bovine serum for 48 hr and used as a negative control (\blacksquare). B, Cell numbers per culture dish were determined for the infected cultures with ET medium (X), for the infected cultures with ET medium with 4% fetal bovine serum (O), and for the uninfected cultures with ET medium with 4% fetal bovine serum (\blacksquare) described in A.

fluorescence was carried out with the antigen preparations from the experiment described in Fig. 2. The antigens were used exactly as prepared for complement fixation. The immunoabsorption was carried out by using overnight absorption of

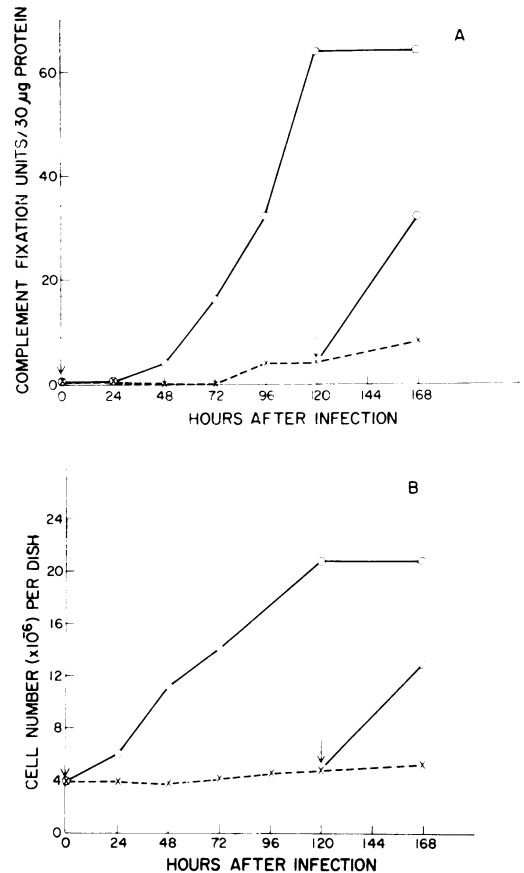


FIG. 2. Absence of avian leukosis virus group-specific antigens from RSV-infected stationary cells. A, Stationary cells, 4.0×10^6 cells/100-mm culture dish, were exposed to SRV-HR at a multiplicity of 1 FFU/cell, and the inoculum was replaced with ET medium (X) or ET medium containing 4% fetal bovine serum (O). At 24-hr intervals, cells were harvested and processed for complement fixation. Antigen preparations were normalized to 30 μ g of protein per 25 μ liters and assayed with complement fixation for gs antigens by using rabbit anti-SRV at a 1:64 dilution. Culture fluids were changed on all cultures 72 hr after infection. Serum was added to cultures with ET medium (\downarrow) 120 hr after infection. B, Cell numbers per culture dish were determined for the cultures with ET medium (X) and ET medium with 4% fetal bovine serum (O) described in A.

rabbit anti-AMV at 4 C with various amounts of lyophilized protein from different antigen preparations (Table 1). Only dividing cells at 72 and 120 hr after infection contained enough gs antigens to absorb specifically the capacity of the antiserum to react with the positive controls. The fact that equivalent amounts of fetal bovine serum and of

TABLE 1. *Detection of avian leukosis virus group-specific antigens by using immunoabsorption fluorescence*

Antigen preparation ^a	HAI ^b	Protein ^c (mg)	Presence of fluorescence ^d	Presence of gs antigen ^e
RSV-infected cells				
Stationary	24	500	+	-
Dividing	24	500	+	-
Stationary	72	500	+	-
Dividing	72	50	-	+
Stationary	120	500	+	-
Dividing	120	50	-	+
Fetal bovine serum		500	+	-
Uninfected chicken cells		500	+	-

^a Antigens prepared from cell extracts as described in Fig. 2 for complement fixation. RSV, Rous sarcoma virus.

^b Hours after infection.

^c Milligrams of protein used to absorb 50 μ liters of 1:500 rabbit anti-avian myeloblastosis virus (purified immunoglobulin G).

^d Observed presence of fluorescence on standard slides using antisera after absorption.

^e Presence of group \pm specific (gs) antigens in protein used for absorption (indicated by the absence of fluorescence).

uninfected chicken cell protein did not absorb the fluorescence illustrates the specificity of this test.

The results of these experiments demonstrate that gs antigens are present only in low amounts in cultures of RSV-infected stationary cells, whereas they are present in large amounts in dividing cells.

Absence of viral-specific RNA in stationary RSV-infected cells. To determine whether viral-specific RNA was present in RSV-infected stationary chicken cells, RNA-DNA hybridizations were carried out with RNA from infected stationary cells and DNA from the B77 virus endogenous RNA-directed DNA polymerase reaction. Sample gradients show the analysis of hybridizations between total B77 virus product DNA and RNA from stationary and dividing chicken cells 120 hr after infection with SRV-HR (Fig. 3). A control hybridization with uninfected chicken cell RNA is also included.

Figure 4 summarizes the results of hybridizations of RNA extracted 36 hr after infection from SRV-HR-infected stationary chicken embryo fibroblasts with whole B77 virus product DNA (Fig. 4A) and with the "minus" DNA isolated from whole B77 virus product DNA (Fig. 4B). In both sets of hybridizations, 150 μ g of RNA from infected stationary cells showed no

more than 0.5% hybridization above background values of 4.5 and 2.5% hybridization with uninfected chicken cell RNA. There was approximately a 100- to 150-fold difference between the amount of viral-specific RNA in RSV-infected dividing and RSV-infected stationary cells.

Figure 5 shows the results of hybridizations of B77 virus product DNA and various amounts of RNA extracted 72 and 120 hr after infection from stationary and dividing cells. RNA preparations from dividing cells 72 and 120 hr after infection gave approximately 21 to 22% hybridization at saturation. A 150- μ g amount of RNA from parallel stationary cells gave only 3 to 4% hybridization. Hybridization with uninfected chicken cell RNA gave a background value of 4%. There was at these times approximately 20-fold more RNA hybridizable with viral product DNA in RSV-infected dividing cells than in RSV-infected stationary cells.

The results of these experiments demonstrate that viral-specific RNA is present only in low amounts in cultures of RSV-infected stationary cells, whereas RSV-infected dividing cells contain 20- to 150-fold more viral-specific RNA.

DISCUSSION

Provirus formation occurs in stationary cells of RSV-infected chicken embryo fibroblasts, whereas cell cycle-dependent activation and release of infectious virus does not (12). The results presented above demonstrate that cultures of stationary cells contain only small amounts of avian leukosis virus gs antigens and viral-specific RNA. Although 90% of RSV-infected dividing cells contained gs antigens 48 hr after infection, only 10% of the cells in cultures without serum contained gs antigens 6 days after infection. Since a small proportion of cells in cultures without serum proceed through cell division, it is likely that the cells in cultures without serum that were positive for gs antigens had proceeded through mitosis. In parallel experiments, RSV-infected stationary cells contained only small amounts of viral-specific RNA compared to the amounts found in parallel dividing cells. At 36 hr after infection, dividing cells contained 100-fold more viral-specific RNA than found in stationary cells. Three and four days after infection, this ratio decreased to a 15 to 20-fold difference. It is likely that this decrease is the result of cell division in the cultures without serum. It appears that neither viral-specific RNA nor avian leukosis viral gs antigens are produced in stationary cells and that, until cell cycle-dependent activation, transcription of the provirus does not occur.

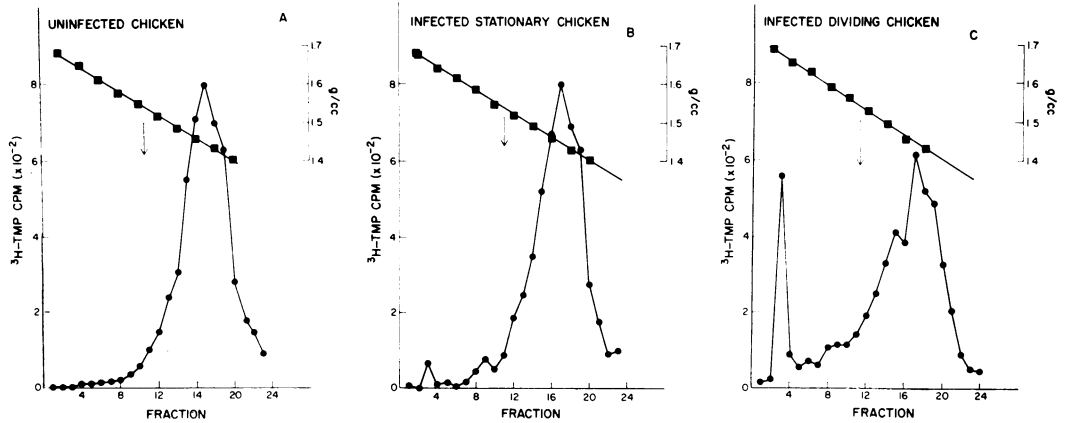


FIG. 3. Hybridization with B77 virus product DNA and RNA extracted 120 hr after infection from cultures of stationary and dividing SRV-HR-infected chicken cells. Stationary chicken embryo fibroblasts, 4.0×10^6 per 100-mm dish, were exposed to SRV-HR at a multiplicity of 100 FFU/cell, and the inoculum was replaced with ET medium or ET medium containing 4% fetal calf serum. At 120 hr after infection, cells were harvested, and the RNA was extracted. RNA was hybridized with 6,000 counts/min of B77 virus endogenous ^3H -labeled DNA product for 5 hr at 68 C, and the mixture was analyzed by Cs_2SO_4 equilibrium density gradient centrifugation. The hybridizations were carried out with RNA as follows: A, 150 μg of uninfected chicken cell RNA; B, 150 μg of infected stationary chicken cell RNA, 120 hr after infection; C, 75 μg of infected dividing chicken cell RNA, 120 hr after infection, plus 75 μg of uninfected chicken cell RNA. The arrows indicate the density of 1.54 g/ml.

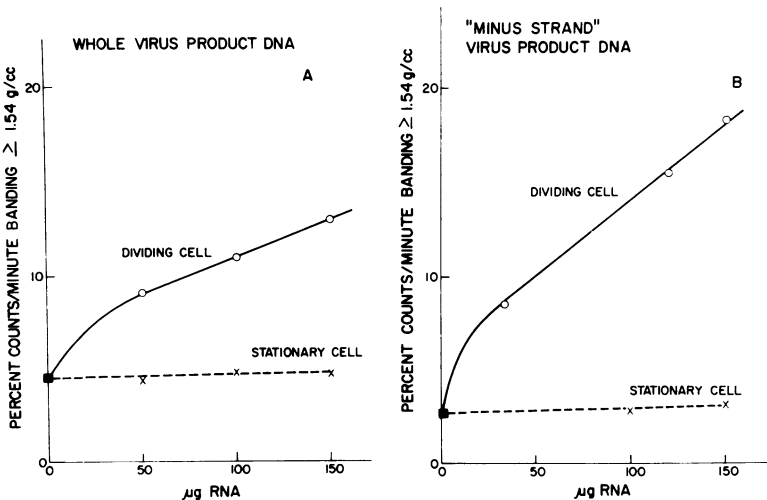


FIG. 4. Hybridization between B77 virus product DNA and RNA extracted 36 hr after infection from cultures of stationary and dividing SRV-HR-infected chicken cells. Stationary cells were infected as described in Fig. 3 and were harvested 36 hr after infection. A, Varying amounts of RNA from infected stationary cells (×) and infected dividing cells (○) were hybridized with 3,000 counts/min of endogenous ^3H -labeled B77 virus DNA product for 5 hr at 68 C and analyzed by Cs_2SO_4 equilibrium density gradient centrifugation. Varying amounts of uninfected chicken cell RNA were added so that all hybridizations were carried out in the presence of 150 μg of chicken cell RNA. A 150- μg amount of uninfected chicken cell RNA was also hybridized without added infected cell RNA (■). B, Duplicate hybridizations were carried out as in A except that 3,000 counts/min of "minus" strand B77 virus product DNA was used in each hybridization.

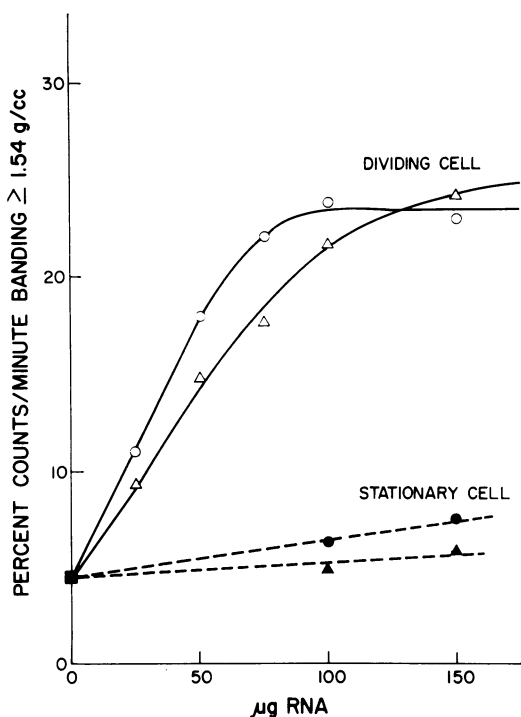


FIG. 5. Hybridization between B77 virus product DNA and RNA extracted 72 and 120 hr after infection from SRV-HR-infected chicken cells, dividing and stationary. Cells were infected, and RNA was extracted and hybridized with whole B77 virus product DNA as described in Fig. 3. Under these conditions, 1.5 µg of B77 virus RNA gave approximately 25 to 27% hybridization. The RNA species in the hybridization mixtures were: uninfected chicken cell RNA (■); infected stationary (▲) and dividing (△) chicken cell RNA at 72 hr after infection; infected stationary (●) and dividing (○) chicken cell RNA at 120 hr after infection.

The RNA-DNA hybridizations were carried out with an endogenous B77 viral DNA polymerase product capable of detecting approximately 20% of the viral RNA (5). Although the large amounts of viral-specific RNA found in dividing cells were not present in stationary cells, it is possible that some viral-specific RNA was in stationary cells which was not detected by the DNA used in this hybridization procedure.

Leong et al. (9) have reported that the rate of production of RSV-RNA varies with the stage of the cell cycle of the infected cells. This would be a second control of viral transcription once the transcription was activated by the initial cell division.

Cell cycle-dependent activation of differentiation has been reported in several other systems (2,

10, 17). It is possible that activation of transcription is involved in all these systems.

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