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Rate of Virus-Specific RNA Synthesis in Synchronized Chicken Embryo Fibroblasts Infected with Avian Leukosis Virus

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The rate of avian leukosis virus (ALV)-specific RNA synthesis has been examined in both uninfected and ALV-infected synchronized chicken embryo fibroblasts. RNA from cells labeled for 2 h with [^{3}H]uridine was hybridized with avian myeloblastosis virus poly(dC)-DNA, and the hybridized RNA was analyzed with poly(I)-Sephadex chromatography. Approximately 0.5% of the RNA synthesized in ALV-infected cells was detected as virus specific, and no more than a twofold variation in the rate of synthesis was detected at different times in the cell cycle. In synchronized uninfected chicken embryo fibroblasts, approximately 0.03% of the RNA synthesized was detected as virus specific, and no significant variation in the rate of synthesis was observed during the cell cycle. Treatment of ALV-infected chicken embryo fibroblasts with cytosine arabinoside or colchicine was used to block cells at different stages in the cell cycle. The rates of virus-specific RNA synthesis in cells so treated did not differ significantly from the rates in either stationary or unsynchronized virus-infected chicken embryo fibroblasts. These findings support the conclusion that after the initial division of an ALV-infected chicken embryo fibroblast and the initiation of virus RNA synthesis, the rate of virus-specific RNA synthesis is independent of the cell cycle.

Virions of the avian leukosis-sarcoma viruses (ALSV) contain an RNA genome. Infection of a chicken embryo fibroblast by ALSV is followed by the synthesis of a DNA copy of the genome, the provirus (12, 13), and synthesis of progeny genome RNA requires transcription of the DNA provirus (9). It has previously been demonstrated that both production of progeny virus (5, 10) and synthesis of progeny virus RNA (6) do not occur until the infected cell has undergone one cell division. The requirement for division of the infected cell for production of ALSV progeny has been called activation (12). It has further been demonstrated that progeny virus can be labeled by precursors of RNA synthesis in cells made stationary after the activation event (6). It appears, therefore, that whereas activation of virus RNA synthesis requires division of the infected cell, continued synthesis of virus RNA does not require continued division of the infected cell. Thus, it appears that only the onset of ALSV RNA synthesis is affected by cell division.

It has, however, been suggested by Leong et al. (7), who used avian sarcoma virus (ASV)-infected chicken embryo fibroblasts synchronized after the initiation of virus production, that both the amount of intracellular ASV RNA and the release of progeny ASV vary during the cell cycle. Their results suggested that release of progeny virus was 5- to 10-fold greater in G_1 and that the amount of intracellular virus RNA was 5- to 10-fold greater in S than at the other times examined. The present studies were undertaken to examine more closely the effect the cell cycle had on the rate of ALSV RNA synthesis.

It has not generally been possible to synchronize cells transformed by ASV with growth medium free of serum growth factors (but see reference 7). Therefore, to examine the effect of the cell cycle on the synthesis of ALSV RNA, nontransformed chicken embryo fibroblasts producing avian leukosis virus (ALV) were synchronized as previously described (6). The rate of virus RNA synthesis was determined with the hybridization technique described by Coffin et al. (2) in which labeled RNA hybrids were detected by using poly(I)-Sephadex chromatography.

MATERIALS AND METHODS

Cells and viruses. Primary and later cultures of chicken embryo fibroblasts were prepared from single

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white Leghorn chicken embryos and grown in Dulbecco modified Eagle medium (Flow Laboratories, Inc., Rockville, Md.) with 10% tryptose phosphate broth (ET medium) and 5% calf serum (Flow Laboratories, Inc.) as previously described (8). The cells were grown at 38 C in a 5% CO_2 atmosphere. The cells used were C/E and were free of ALV.

The BAI strain A of avian myeloblastosis virus (AMV) was grown and purified as previously described (8). Rous-associated virus-49 (RAV-49) has been previously described (6).

Cultures of RAV-49-infected chicken embryo fibroblasts were prepared as follows: 90-mm culture dishes containing 2×10^6 cells/dish were exposed to 0.5 ml of approximately 10^6 to 10^7 interference-inducing units/ml for 1 h at 38 C, and growth medium was added. The cells were grown and transferred at 3- to 4-day intervals and were used at the third transfer to prepare cultures of stationary cells. RAV-49-infected cells prepared in this manner were completely resistant to challenge with Prague strain of Rous sarcoma virus, subgroup C, at a multiplicity of 2 focus-forming units/cell, whereas Prague strain of Rous sarcoma virus, subgroup A, had the same titer on both the RAV-49-infected cells and on control uninfected cells.

Stationary cells and the cell cycle. Stationary chicken embryo fibroblasts infected with RAV-49 were prepared by depletion of serum growth factors (11) from growth medium containing a low serum concentration as previously described (6). Cells were plated at approximately 5×10^5 to 6×10^5 cells/50-mm dish, and 4 h after plating the medium was changed to 5 ml of ET medium containing 1.6% newborn calf serum (Flow Laboratories). Four to 5 days later, 2.5 ml of Dulbecco modified Eagle medium was added per dish, and the cells were used 8 to 9 days after plating. The cultures used were passage five and six after primary culture. Stimulation of the stationary cells was carried out by changing the depleted medium to ET medium containing 10% newborn calf serum.

The number of cells per culture dish was determined at different times after addition of serum with a hemocytometer. The cells were removed by trypsinization and counted. All determinations were done in duplicate.

The rate of DNA synthesis was determined at different times after addition of serum by changing the medium to 2 ml of Dulbecco modified Eagle medium containing 0.2 μCi of [*methyl*-^{3}H]thymidine per ml (15.5 Ci/mmol; Amersham, Buckinghamshire, England) for 30 min at 38 C. The dishes were then treated twice with 10% trichloroacetic acid at 4 C and washed with ether-ethanol (1:3), and the residue was taken up in 1 ml of 0.2 N NaOH. The radioactivity was determined by scintillation counting.

The number of nuclei synthesizing DNA at different times after addition of serum was determined by autoradiography. Cells were labeled with 2 μCi of [*methyl*-^{3}H]thymidine for 1 h at 38 C, washed, fixed, and overlaid with Ilford emulsion K5 (Ilford Ltd., Ilford, Essex, England). Approximately 4,000 cells/culture dish were examined.

Cells were blocked at different stages in the cell cycle by changing the medium to fresh ET medium containing 10% newborn calf serum and either 5×10^{-5} M cytosine arabinoside (Sigma Chemical Co., Surrey, England) or 5×10^{-7} M colchicine (Sigma Chemical Co.). Cells blocked in the cell cycle by treatment with cytosine arabinoside were released from the block by changing the medium to fresh ET medium containing 10% newborn calf serum and 10^{-4} M deoxycytidine (Sigma Chemical Co.).

Preparation of labeled RNA. Cultures of RAV-49-infected cells were labeled with [^{32}P]phosphate to prepare RAV-49 70*S* [^{32}P]RNA according to conditions previously described (8).

Cells infected with RAV-49 were labeled with [5-^{3}H]uridine (25 to 30 Ci/mmol; Amersham) to prepare RAV-49-infected cellular [^{3}H]RNA. The labeling was carried out with 500 μCi of uridine/ml, 0.25 ml per plate, for 2 h at 38 C in Dulbecco modified Eagle medium for stationary cells or Dulbecco modified Eagle medium and 5% dialyzed calf serum for dividing cells. Labeling was terminated by washing with cold 0.15 M NaCl-0.01 M sodium phosphate (pH 7.0). Cells were then trypsinized, washed, and frozen at −20 C until used.

Virus 70*S* RNA was prepared from AMV and ^{32}P-labeled RAV-49 according to conditions previously described (8). The specific activity of RAV-49 70*S* [^{32}P]RNA was approximately 0.5×10^6 to 1.0×10^6 counts/min per μg.

Labeled cellular RNA was prepared according to the procedure of Coffin et al. (2). Cells were suspended in 0.05 M Tris-hydrochloride (pH 7.5)-0.005 M EDTA, disrupted with 1% sodium dodecyl sulfate, and digested for 10 min at 38 C with 100 μg of self-digested Pronase per ml. Approximately 20,000 to 30,000 counts/min of RAV-49 70*S* [^{32}P]RNA were then added as an internal standard. The preparation was extracted with phenol and precipitated with ethanol in the presence of 1% potassium acetate. The nucleic acids were collected by centrifugation, suspended in 0.02 M Tris-hydrochloride (pH 7.5), 0.015 M NaCl, and 0.01 M $MgCl_2$, treated with DNase I, and extracted with phenol, and the aqueous phase was passed through a Sephadex column (G-100). The excluded peak fractions of labeled RNA were pooled and precipitated with ethanol in the presence of 1% potassium acetate. The specific activity of the [^{3}H]RNA prepared with the labeling conditions described above was approximately 0.5×10^5 to 1.0×10^5 counts/min per μg. Approximately 2 to 5 counts/min of [^{3}H]RNA per cell were recovered. In all experiments described here, more than 85% of the [^{3}H]RNA was recovered, and in no experiment was the internal standard virus RNA preferentially lost during purification.

Synthesis and elongation of virus-specific DNA. AMV-specific DNA was prepared in an endogenous RNA-directed DNA polymerase reaction in the presence of actinomycin D with purified AMV as previously described (2). The DNA product was synthesized in the presence of [^{32}P]TTP so that the specific activity of the DNA was approximately 1,000 counts/

min per μg. Purified AMV DNA was elongated with poly(dC) as described by Coffin et al. (2), according to the procedure of Bollum (1). Such AMV [^{32}P]DNA was shown to contain approximately 50 dCMP residues per chain (2). Approximately 10 to 12 μg of AMV poly(dC)-DNA was recovered from 5 mg of purified AMV.

RNA-DNA hybridization. The method used was described in detail by Coffin et al. (2). AMV poly(dC)-DNA and whole-cell [^{3}H]RNA were hybridized in 50-μl volumes in 0.5 M NaCl and 0.01 M Tris-hydrochloride (pH 7.5) in the presence of 1 μg of oligo-(C) at 66 C for 3 to 5 h. In general, hybridizations contained 1 to 10 μg of [^{3}H]RNA and 0.05 to 0.1 μg of AMV poly(dC)-DNA. All hybridizations contained 2,000 to 3,000 counts/min of RAV-49 70*S* [^{32}P]RNA as an internal standard for the reasons previously discussed (2). Background hybridization was determined by including 2 to 3 μg of unlabeled AMV 70*S* RNA in a parallel hybridization. After annealing, the reaction was diluted with 50 μg of poly(U) (Miles Laboratories, Elkhardt, Ind.), and the hybridization was analyzed by poly(I)-Sephadex G-10 chromatography. Hybrid molecules bound to the poly(I)-Sephadex were treated with 20 μg of RNase per ml at 37 C for 30 min, washed with 0.5 M NaCl and 0.05 M Tris-hydrochloride (pH 7.5), and eluted with 90% formamide, 0.5% sodium dodecyl sulfate, and 0.01 M Tris-hydrochloride (pH 7.5). The hybrid molecules were precipitated with 5% trichloroacetic acid at 4 C and collected on membrane filters (Millipore HAWP; Millipore Corp., Bedford, Mass.), and the amount of radioactivity present was determined by scintillation counting.

Scintillation counting. Membrane filters (Millipore Corp.) were counted in 10 ml of toluene solution containing 4 g of butyl-PBD (Ciba AG, Basel) and 100 mg of 1,4-bis-(5-phenyloxazolyl)-benzene (Fluka AG, Buchs) per liter of toluene. Alkaline aqueous samples of 1-ml volume were counted in 10 ml of a mixture of 2 volumes of toluene scintillation fluid, 1 volume of Triton X-100, and 0.4% acetic acid.

Characterization of the hybridization of RAV-49 70S [^{32}P]RNA to AMV poly(dC)-DNA. The experiments presented below utilize the heterologous hybridization between AMV poly(dC)-DNA and RAV-49 [^{3}H]RNA to study the synthesis of the virus-specific RNA in synchronized RAV-49-infected chicken embryo fibroblasts. To determine what fraction of the RAV-49 genome was detected in this hybridization, different amounts of AMV poly(dC)-DNA were hybridized with a constant amount of RAV-49 70*S* [^{32}P]RNA and AMV 70*S* [^{3}H]RNA, and the hybridizations were analyzed by poly(I)-Sephadex columns and RNase treatment. The results (Fig. 1) demonstrate that approximately 35% of the RAV-49 genome was resistant to RNase treatment after hybridization with 0.05 to 0.08 μg of AMV poly(dC)-DNA. In the homologous hybridization, the same amount of AMV poly(dC)-DNA protected approximately 55% of the AMV [^{3}H]RNA from RNase digestion. These results are similar to those previously obtained with AMV DNA and RNA from the Schmidt-Ruppin strain of RSV (2). All the hybridization data presented below are taken from experiments in which 0.05 to 0.1 μg of AMV poly(dC)-DNA was hybridized to cellular [^{3}H]RNA. The data presented in Fig. 1 demonstrate that approximately one-third of the RAV-49 sequences labeled with [^{3}H]uridine would be detected in these experiments. In hybridizations including 2.5 μg of unlabeled AMV 70*S* RNA in addition to the RAV-49 70*S* [^{32}P]RNA, 0.05 μg of AMV poly(dC)-DNA protected only 1% of the RAV-49 70*S* [^{32}P]RNA from RNase digestion (Fig. 1).

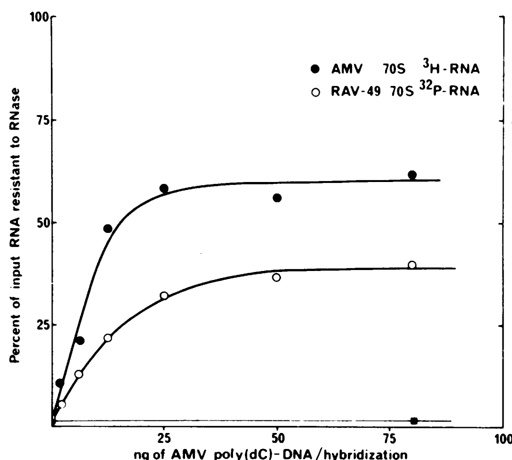

FIG. 1. *Hybridization of AMV 70S [^{3}H]RNA and RAV-49 70S [^{32}P]RNA with increasing amounts of AMV poly(dC)-DNA. A mixture of AMV 70S [^{3}H]RNA (●, 2,200 counts/min, specific activity, approximately 1 × 10^{6} counts/min per μg) and RAV-49 70S [^{32}P]RNA (○, 1,000 counts/min, specific activity, approximately 1 × 10^{6} counts/min per μg) was annealed with increasing amounts of AMV poly(dC)-DNA (labeled with ^{32}P, specific activity 1,000 counts/min per μg) and analyzed for RNase-resistant RNA with poly(I)-Sephadex chromatography and RNase treatment as described in the text. In all cases, the amount of RAV-49 70S [^{32}P]RNA hybridized has been corrected for the amount of AMV poly(dC)-DNA present in the reaction. In a control reaction, 80 ng of AMV poly(dC)-DNA was hybridized to both AMV 70S [^{3}H]RNA and RAV-49 70S [^{32}P]RNA in the presence of 2.5 μg of unlabeled AMV 70S RNA (■).*

RESULTS

"Virus-specific" RNA synthesis in synchronized uninfected chicken embryo fibroblasts. It has previously been demonstrated that uninfected chicken embryo fibroblasts contain low levels of RNA that will hybridize with [^{3}H]cDNA synthesized in an endogenous ALV DNA polymerase reaction (4). To determine to what extent such RNA would interfere with the determination of virus-specific RNA synthesis in infected cells, the rate of

"virus-specific" RNA synthesis was measured at different times in the cell cycle of synchronized uninfected chicken embryo fibroblasts.

Stationary uninfected chicken embryo fibroblasts were prepared by allowing the cells to deplete growth medium of serum growth factors as described above. The stationary cells were then stimulated with serum and labeled at different times with [^{3}H]uridine. The total cellular RNA was purified and hybridized to AMV poly(dC)-DNA, and the hybridization reactions were analyzed by chromatography on poly(I)-Sephadex. The synchronized cells were also examined at different times after addition of serum for the number of cells per culture dish, the rate of [^{3}H]thymidine incorporation, and the percentage of the nuclei labeled with [^{3}H]thymidine during a 1-h pulse.

The results (Fig. 2) demonstrate that, by allowing cells to deplete growth medium containing a low concentration of serum, the majority of the cells become stationary (6, 11). After addition of serum, approximately 85% of the cells underwent synchronous division with an average time of 18 h. Two major peaks in the rate of DNA synthesis were detected at 10 to 14 and 28 to 34 h after addition of serum. The proportion of nuclei labeled with [^{3}H]-thymidine during a 1-h pulse reached a maximum of 25% during the first period of DNA synthesis at approximately 12 h after addition of serum. In similar experiments, a 4-h pulse of [^{3}H]thymidine from 10 to 14 h after addition of serum labeled approximately 80 to 85% of the nuclei (data not shown). Thus the majority of the stationary cells respond to stimulation by serum with a relatively synchronous cell cycle.

The RNA from cells labeled with [^{3}H]uridine at 0, 6, 12, 20, and 50 h after addition of serum was hybridized with AMV poly(dC)-DNA. The results (Fig. 2 and Table 1) demonstrate the detection of low levels of "virus-specific" RNA synthesis in these cells. The levels detected range from 0.02 to 0.07% of the total cellular RNA labeled during the 2-h pulse and are in general agreement with previously reported values (4, 8). However, the number of counts in hybrid (Table 1) was too low to determine whether any significant variation in the rate of "virus-specific" RNA synthesis occurred in these cells.

Virus-specific RNA synthesis in synchronized ALV-infected chicken embryo fibroblasts. Leong et al. (7) have reported that the amount of virus-specific RNA detected in synchronized ASV-infected chicken embryo fibroblasts varied during the cell cycle. In hybridizing ASV [^{3}H]DNA to total cell RNA, approximately 10-fold more virus-specific RNA was detected in RNA from cells in early S than in RNA from cells in G_1 or late S or in RNA from unsynchronized cells (7). To examine this observation with a hybridization technique capa-

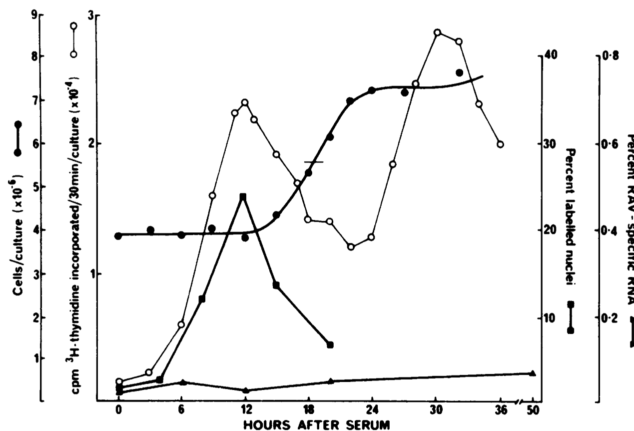

FIG. 2. *Synthesis of virus-specific RNA in synchronized uninfected chicken embryo fibroblasts. Stationary uninfected chicken embryo fibroblasts were prepared by depletion of growth medium containing 1.2% newborn calf serum and then stimulated with ET medium containing 10% newborn calf serum as described in the text. At different times after addition of serum, the number of cells per culture dish (●), the rate of DNA synthesis (○), and the percentage of nuclei labeled with [^{3}H]thymidine (■) were determined. At 0, 5, 11, 19, and 49 h after addition of serum, cells were labeled with 500 μCi of [^{3}H]uridine/ml for 2 h, the RNA was purified and hybridized with AMV poly(dC)-DNA, and the hybridizations were analyzed by poly(I)-Sephadex chromatography. (See Table 1 for hybridization data.) The percentage of virus-specific RNA is plotted at the midpoint of the labeling period (▲).*

TABLE 1. *Determination of RAV-specific RNA synthesis at different times in synchronized uninfected chicken embryo fibroblasts*

Cell RNA in hybridization (HAS)[a]	Hybridization input RNA[b] (counts/min)		RNA hybridized to AMV poly(dC)-DNA[c] (counts/min)		RAV-specific RNA (% of total cell RNA)[d]
	Cell [^{3}H]RNA	RAV-49 70*S* [^{32}P]-RNA	Cell [^{3}H]-RNA	RAV-49 70*S* [^{32}P]-RNA	
0	461,000	1,500	29 (44)	470 (8)	0.02
6	406,000	1,440	35 (26)	240 (3)	0.05
12	335,000	1,500	17 (80)	475 (2)	0.02
20	163,000	1,500	25 (41)	485 (3)	0.05
50	102,000	2,100	9 (4)	265 (2)	0.07

[a] Synchronized uninfected chicken embryo fibroblasts were prepared, stimulated with serum, and labeled with [^{3}H]uridine at different times as described in the legend to Fig. 2. The RNA was purified and hybridized with AMV poly(dC)-DNA as described in the text. The hours after serum (HAS) is the midpoint of the 2-hr [^{3}H]uridine labeling period for the particular sample of cellular RNA hybridized (see the legend to Fig. 2).

[b] The input counts per minute of cellular [^{3}H]RNA (specific activity, approximately 1×10^6 counts/min per μg) and the input counts per minute of RAV-49 70*S* [^{32}P]RNA (specific activity, approximately 1×10^6 counts/min per μg) were determined by precipitation of duplicate samples of one fourth volume of the actual input of the RNA mixture.

[c] The counts per minute of cellular [^{3}H]RNA or of the internal standard RAV-49 70*S* [^{32}P]RNA bound to poly(I)-Sephadex and resistant to RNase treatment after annealing with 0.05 μg of AMV poly(dC)-DNA. The value is an average of duplicate hybridizations and has been corrected for both machine background (^{3}H, 10 counts/min; ^{32}P, 15 counts/min) and nonspecific hybridization determined by annealing the same amount of input RNA mixture with 0.05 μg of AMV poly(dC)-DNA in the presence of 2.5 μg of unlabeled AMV 70*S* RNA. The value for the nonspecific hybridization is the average of duplicate determinations and is given in parentheses. The specific activity of the AMV poly(dC)-[^{32}P]DNA was approximately 9 counts/min per μg and had no significant contribution to the ^{32}P counts per minute detected as hybrid.

[d] (%[^{3}H]hybrid/%[^{32}P]hybrid) $\times$ 100. There was no significant difference in the recovery of the [^{32}P]RNA in the different samples in this experiment and no selective loss of the RAV-49 70*S* [^{32}P]RNA internal standard during purification of the RNA.

ble of detecting pulse-labeled virus-specific RNA, synchronized RAV-49-infected chicken embryo fibroblasts were prepared in serum-depleted growth medium, stimulated with serum, and labeled at different times during the cell cycle with [^{3}H]uridine, and the RNA was purified and analyzed by hybridization. In addition, the number of cells per culture, the rate of incorporation of [^{3}H]thymidine, and the percentage of nuclei labeled with [^{3}H]thymidine were determined at different times as in the preceding section.

The results (Fig. 3) demonstrate that the cell cycle of RAV-49-infected chicken embryo fibroblasts is not significantly different from that of uninfected chicken embryo fibroblasts. Approximately 75% of the cells underwent synchronous division with an average time of 19 h. Two major peaks of DNA synthesis were observed at 12 and 30 to 32 h after addition of serum. During the first 20 h, the proportion of nuclei labeled with [^{3}H]thymidine reached a maximum of 23% at approximately 12 h after addition of serum. In this experiment, total cellular RNA prepared from cells labeled with [^{3}H]uridine at 0, 3, 6, 9, 12, 15, 19, 27, and 50 h after addition of serum was hybridized to AMV poly(dC)-DNA. The results (Fig. 3 and Table 2) demonstrate that no more than a twofold variation in the rate of virus-specific RNA synthesis was detected. The maximum rate, 0.75% of total labeled RNA synthesized, was observed in stationary cells. The rate at the other times examined ranged between 0.33 and 0.59%. In two other experiments, the maximum rate of virus RNA synthesis was observed in stationary cells and, again, it was approximately twofold greater than the lowest rate observed in the experiment (data not shown).

To determine whether the rate of total cellular RNA synthesis undergoes extensive variation in the cell cycle, the incorporation of [^{3}H]uridine into samples of the cells labeled at the different times was determined. The results (Table 2) demonstrate that the amount of [^{3}H]uridine incorporated into acid-precipitable material at different times in the cell cycle varied from 3 to 5 counts/min per cell. It seems unlikely, therefore, that either the percentage of virus-specific RNA synthesized or the total virus-specific RNA synthesized varies more than twofold during the cell cycle.

The rate of virus-specific RNA synthesis in chicken embryo fibroblasts blocked during the cell cycle. Since the experiments presented above showed no great difference in the rate of virus RNA synthesis in cells progressing through the cell cycle, the rate of virus-specific RNA synthesis was measured in cells blocked at different stages in the cell cycle. Stationary RAV-49-infected chicken embryo fibroblasts were prepared and stimulated with serum as described above. At different times, some of the cultures were treated with cytosine arabinoside

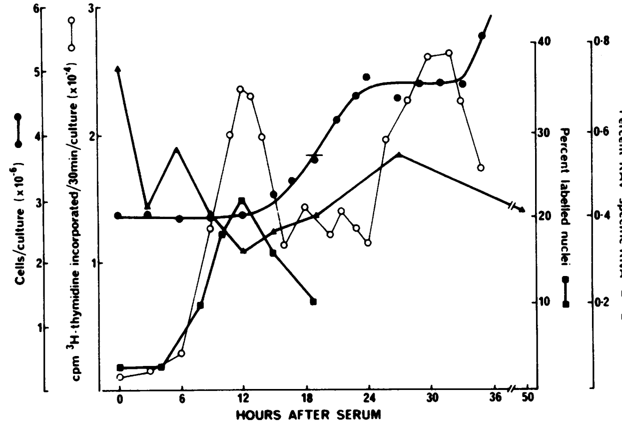

FIG. 3. *Synthesis of virus-specific RNA in synchronized RAV-49-infected chicken embryo fibroblasts. Chicken embryo fibroblasts were infected with RAV-49, grown in ET medium with 5% calf serum, transferred three times, and made stationary by depletion of growth medium containing 1.6% newborn calf serum. The stationary cells were stimulated with ET medium containing 10% newborn calf serum, and at different times after addition of serum, the number of cells per culture dish* (●), *the rate of DNA synthesis* (○), *and the percentage of nuclei labeled with [³H]thymidine* (■) *were determined. At 0, 2, 5, 8, 11, 14, 18, 26, and 49 h after addition of serum, cells were labeled with 500 μCi of [³H]uridine/ml for 2 h, the RNA was purified and hybridized with AMV poly(dC)-DNA, and the hybridizations were analyzed by poly(I)-Sephadex chromatography. (See Table 2 for hybridization data.) The percentage of virus-specific RNA is plotted at the midpoint of the labeling period* (▲).

TABLE 2. *Determination of RAV-specific RNA synthesis at different times in synchronized RAV-infected chicken embryo fibroblasts*[a]

Cell RNA in hybridization (HAS)	[³H]uridine incorporation (counts/min per cell[b])	Hybridization input RNA (counts/min)		RNA hybridized to AMV poly(dC)-DNA (counts/min)		RAV-specific RNA (% of total)
		Cell [³H]RNA	RAV-49 70S [³²P]RNA	Cell [³H]RNA	RAV-49 70S [³²P]RNA	
0	3.9	447,000	1,440	515 (13)	220 (−15)	0.754
3	3.6	283,000	1,410	250 (34)	290 (−13)	0.429
6	3.7	589,000	1,520	660 (28)	290 (1)	0.587
9	3.8	384,000	1,320	410 (14)	345 (−12)	0.409
12	3.7	505,000	1,780	290 (25)	315 (−12)	0.325
15	3.3	470,000	1,870	325 (15)	350 (−13)	0.369
19	3.7	497,000	1,710	395 (17)	340 (−13)	0.399
27	4.7	281,000	1,710	320 (12)	355 (−15)	0.549
50	ND	518,000	1,830	430 (15)	370 (−14)	0.411

[a] Synchronized RAV-49-infected chicken embryo fibroblasts were prepared, stimulated with serum, and labeled with [³H]uridine at different times as described in the legend to Fig. 3. The [³H]RNA was purified as described in the text. Hours after serum (HAS) is the midpoint of the [³H]uridine labeling period for the sample of cellular RNA hybridized (see the legend to Fig. 3). Other experimental details are as described in Table 1.

[b] A sample of the labeled cells was washed and spotted on no. 541 filter paper disks, precipitated with 6% trichloroacetic acid, washed with 95% ethanol, and dried. The disks were treated for 30 min with 0.5 ml of tissue solubilizer (Nuclear Chicago) diluted 1:2 with scintillation fluid. The samples were then counted in 10 ml of scintillation fluid with 0.4% acetic acid. ND, Not determined.

or colchicine. Some of the cultures treated with cytosine arabinoside were later treated with fresh medium containing deoxycytidine. For both control cultures and cultures treated in different ways with cytosine arabinoside or colchicine, the number of cells per culture, the rate of incorporation of [³H]thymidine, and the amount of virus-specific RNA synthesis were measured at different times (Fig. 4) as described in the previous section.

RAV-49-infected chicken embryo fibroblasts were treated in four different ways to block cells at different stages in the cell cycle. Some of the cells were treated with colchicine at 12 h after

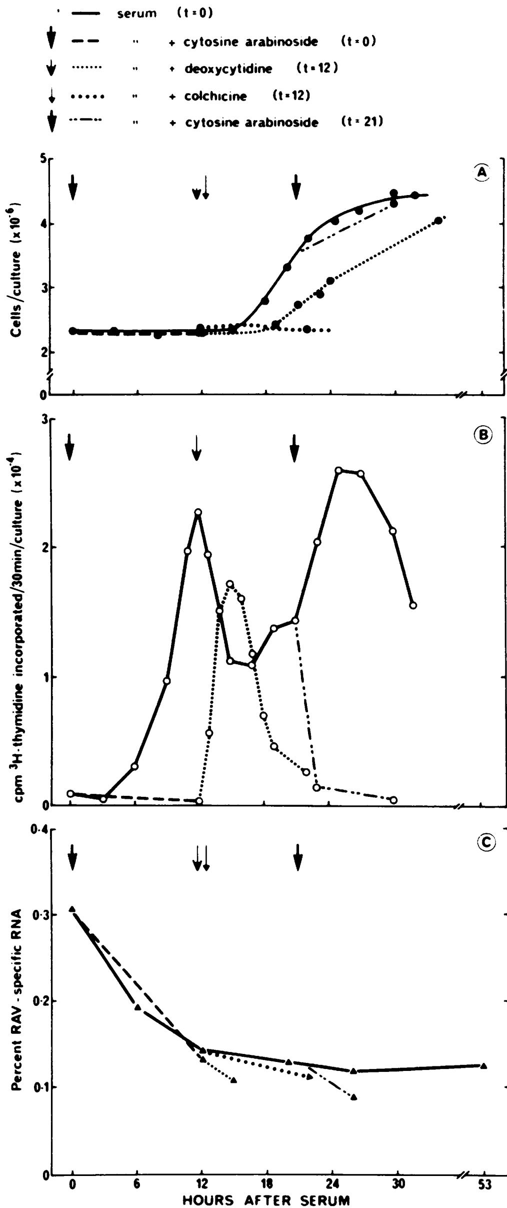

FIG. 4. *The synthesis of virus-specific RNA in RAV-49-infected chicken embryo fibroblasts treated with cytosine arabinoside or with colchicine. Stationary RAV-49-infected chicken embryo fibroblasts were prepared as described in the legend to Fig. 3. Some cultures were stimulated with ET medium containing 10% newborn calf serum only (—). Some cultures were treated with 5×10^{-5} M cytosine arabinoside at the time of addition of serum (↓ – –), and 12 h later some of the cultures exposed to serum and cytosine arabinoside were changed to medium containing serum and 10^{-4} M deoxycytidine (↓ ······). At 12 h after addition of serum, some cultures were changed to medium containing serum and 5×10^{-7} M colchicine (↓ ·····). At 21 h after addition of serum, some cultures were changed to medium containing serum and 5×10^{-5} M cytosine arabinoside (↓ –·–·–). At different times during the experiment the number of cells per culture dish (●) and the rate of DNA synthesis (○) were determined. At different times, cells were labeled with 500 μCi of [³H]uridine/ml for 2 h, the RNA was purified and hybridized with AMV poly(dC)-DNA, and the hybridizations were analyzed by poly(I)-Sephadex (see Table 3 for hybridization data). The percentage of virus-specific RNA is plotted at the midpoint of the labeling period (▲).*

addition of serum. The colchicine-treated cells continued normal DNA synthesis (data not shown) but no increase in cell number was observed (Fig. 4A), indicating the cells were blocked in mitosis.

Some cells were treated with cytosine arabinoside at the time of addition of serum to the stationary cells. They exhibited very low levels of DNA synthesis 12 h later, at which time control cells had reached the peak of S-phase DNA synthesis (Fig. 4B). Some of the cells that were exposed to serum and cytosine arabinoside for 12 h were changed to fresh medium containing serum and deoxycytidine. DNA synthesis began immediately in these cells (Fig. 4B) and after a 4-h lag, as compared with the control cultures, there was approximately 85% of the increase in cell number that was observed in cultures that had received no cytosine arabinoside treatment (Fig. 4A). Thus stationary cells treated with serum and cytosine arabinoside were blocked near the beginning of S-phase DNA synthesis. Those cells treated first for 12 h with serum and cytosine arabinoside and then with serum and deoxycytidine began DNA synthesis immediately and provided a relatively uniform population of cells in S phase.

Some cells were treated with cytosine arabinoside at 21 h after addition of serum when approximately half the cells had undergone division. The cells continued to divide and achieved approximately 95% of the increase in cell number observed in control cultures (Fig. 4A), but they did not undergo a second period of DNA synthesis (Fig. 4B), indicating the majority of the cells were blocked near the beginning of the first period of S-phase DNA synthesis after mitosis.

By using cytosine arabinoside and colchicine as described, cells producing RAV-49 were prepared at four stages in the cell cycle: (i) just before entering S-phase DNA synthesis during a cytosine arabinoside block, (ii) during S-phase

DNA synthesis after deoxycytidine release of the cytosine arabinoside block, (iii) in mitosis during a colchicine block, and (iv) just before S-phase DNA synthesis in cells treated with cytosine arabinoside just after cell division. All of these cells and the parallel untreated control cells were labeled with [^{3}H]uridine for 2 h, and the RNA was prepared and hybridized with AMV poly(dC)-DNA. The results of the analysis of the hybridizations (Fig. 4C and Table 3) show that in all the situations analyzed, including the control cells, the rate of virus RNA synthesis varied no more than two- to threefold. The overall amount of [^{3}H]uridine incorporated per cell varied from 3 to 6 counts/min. (data not shown). The greatest difference observed in the rate of RNA synthesis was between stationary cells, 0.31%, and cells blocked just before the second period of DNA synthesis, 0.09%, or approximately a threefold difference. It appears, therefore, that the presence of these inhibitors did not greatly affect the rate of virus-specific RNA synthesis nor did they provide a cell population capable of synthesizing virus-specific RNA at a rate significantly different from that observed in control cells.

DISCUSSION

The results presented above indicate that approximately 0.4 to 0.6% of the total ALV-infected cell RNA synthesized at different stages in the cell cycle is virus specific and that no significant cell cycle-dependent variation in the rate of virus-specific RNA synthesis occurs in synchronized ALV-infected chicken embryo fibroblasts. The rate of virus-specific RNA synthesis in cells treated in different ways with cytosine arabinoside or colchicine revealed no large differences in the rates of synthesis either between cells treated in different ways with these two inhibitors or between cells treated with inhibitors and the untreated controls. If the rate of virus-specific RNA synthesis during S-phase was sevenfold greater than during all the other stages of the cell cycle, our experiments should have detected it since approximately 30% of the cells underwent DNA synthesis simultaneously in a 1-h period at the peak of S phase. Less than a sevenfold difference would probably have been too small to detect. Similarly, a complete lack of virus RNA synthesis at any stage in the cell cycle would not have been detected. In all our experiments, the

TABLE 3. *Determination of RAV-specific RNA synthesis in synchronized RAV-infected chicken embryo fibroblasts treated with cytosine arabinoside and colchicine*[a]

Cell RNA in hybridization (HAS)	Chemical addition[b]	Hybridization input RNA (counts/min)		RNA hybridized to AMV poly(dC)-DNA (counts/min)		RAV-specific RNA (% of total)
		Cell [^{3}H]RNA	RAV-49 70*S* [^{32}P]RNA	Cell [^{3}H]RNA	RAV-49 70*S* [^{32}P]RNA	
0	none	632,000	871	547 (59)	232 (14)	0.312
6	none	846,000	879	436 (62)	225 (10)	0.193
12	cytosine arabinoside at 0 HAS	991,000	772	343 (106)	201 (23)	0.133
12	none	1,541,000	908	532 (92)	218 (14)	0.144
15	cytosine arabinoside at 0 HAS and deoxycytidine at 12 HAS	879,000	664	237 (88)	171 (7)	0.104
20	none	2,030,000	985	637 (132)	242 (15)	0.128
22	colchicine added at 12 HAS	689,000	900	186 (29)	210 (7)	0.116
26	none	990,000	948	268 (81)	213 (12)	0.120
26	cytosine arabinoside at 21 HAS	1,064,000	1010	223 (72)	242 (7)	0.087
53	none	1,129,000	752	377 (94)	199 (10)	0.126

[a] Synchronized RAV-49-infected chicken embryo fibroblasts were prepared, stimulated with serum, exposed to different inhibitors or not, and labeled with [^{3}H]uridine at different times as described in the legend to Fig. 4. Hours after serum (HAS) is the midpoint of the [^{3}H]uridine labeling point for the sample of cellular RNA hybridized (see the legend to Fig. 4). Other experimental details are as described in Table 1, except that 0.1 μg of AMV poly(dC)-DNA was used in the hybridizations.

[b] The medium used for stimulation of the cells without chemical addition was ET medium with 10% newborn calf serum. The different additions of cytosine arabinoside or colchicine are described in detail in the legend to Fig. 4. Hours after serum (HAS) is the time the medium with the chemical was added.

rate of virus RNA synthesis in stationary cells has been two- to threefold higher than in S-phase cells. Although this observation is reproducible, we consider such a difference too small to be significant. Our results indicate that virus RNA synthesis, once initiated after the initial division of the infected cell, continues without significant variation in stationary cells, in cells at different stages in the cell cycle, and in cells blocked in early S or in mitosis.

Low levels of RNA synthesis scored as "virus specific" were detected at different times in the cell cycle of synchronized uninfected chicken embryo fibroblasts, but the amount of hybrid detected was too low to determine whether or not a cell cycle-dependent variation in the rate of synthesis existed. In addition, it has not yet been firmly established that such synthesis represents virus-related sequences.

One possible complication in interpreting the results presented above arises if the specific activity of the ^3H precursors from which the ALV RNA is synthesized varies during the cell cycle. Previous studies on this subject have not demonstrated any significant changes in either uridine transport or uridine pools that would affect the specific activity of the virus RNA synthesized by more than two- or threefold (3, 14), but satisfactory data concerning the question involved here is not available. Nevertheless, differences in the specific activity of the precursors would only affect conclusions regarding the absolute amount of virus RNA synthesized and not the rate relative to that of total-cell RNA synthesis.

The conclusions supported by the results obtained in this study are in contrast with the findings of Leong et al. (7) who observed an increase in the cell content of virus-specific RNA during the S phase of the cell cycle. A direct comparison of our results and those of Leong et al. is not possible because of significant differences in the experimental approaches used that include (i) cell synchronization procedure and (ii) hybridization technique. It is also possible that the virus sequences represented in our AMV poly(dC)-DNA have contributed to the discrepancy between the two results. Possibly of less significance is the fact that we used an ALV, whereas Leong et al. used an ASV.

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