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Ribonucleic Acid Synthesis in Animal Cells in the Presence of Actinomycin*

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ABSTRACT: The double-stranded ribonucleic acid synthesized in uninfected animal cells in the presence of actinomycin had many of the characteristics of the replicative form of viral ribonucleic acid; however, its synthesis was not inhibited by antiviral agents such as homologous interferon, the homopolymer duplex polyriboinosinic acid:polyribocytidylic acids or guanidine hydrochloride. The double-stranded ribonucleic acid in normal animal cells was a potent inducer of the antiviral agent interferon. After thermal denaturation the double-stranded ribonucleic acid could be hybridized with cellular deoxyribonucleic acid.

The BD-cellulose[1] column (Gillam *et al.*, 1967) is used primarily for the chromatographic resolution of tRNAs. In the course of examining the efficacy of this resin in resolving total RNA of animal cells, RNA was observed which coeluted with the double-stranded replicative form of an RNA virus. The presence of a double-stranded RNA in uninfected cells was unexpected. If this RNA indeed represented a double-stranded RNA then two possibilities had to be considered: (1) an RNA virus infection in the cell line with the replicative form of the virus present as the double-stranded RNA; or (2) a unique and heretofore undescribed form of RNA present in uninfected animal cells.

Double-stranded RNAs had previously been thought to occur in nature only in the replicative cycle of RNA viruses, in a few RNA virus genomes, and in several mycophages (Ellis and Kleinschmidt, 1967; Banks *et al.*, 1969; Lemke and Ness, 1970). Recently it has been suggested that double-stranded RNAs may occur in normal (Montagnier, 1968; Colby and Duesberg, 1969; Stern and Friedman, 1970; Stollar and Stollar, 1970) and DNA virus-infected (Colby and Duesberg, 1969; Stollar and Stollar, 1970) animal cells, as well as in DNA phage-infected bacteria (Jurale *et al.*, 1970). Double-stranded RNAs have evoked considerable interest. They are potent inducers of the antiviral agent interferon (Lampson *et al.*, 1967; Colby and Duesberg, 1969). Antibodies directed against double-stranded RNAs occur in the serum of patients with systemic lupus erythematosus (Steinberg *et al.*, 1969; Schur and Monroe, 1969; Koffler *et al.*, 1969) and in the serum of New Zealand black mice (Steinberg *et al.*, 1969), animals which display a propensity toward developing spontaneous autoimmune disease. Enzymatic activity directed against double-stranded RNAs is present in uninfected bacteria (Robertson *et al.*, 1968) in bull semen (Libonati and Floridi, 1969) and in normal animal serum (Stern, 1970a,b).

A partial description of this apparently double-stranded RNA has been published (Stern and Friedman, 1970). It is synthesized in the presence of high doses (5 μg/ml) of actinomycin D, is polydispose on sucrose gradient centrifugation (4–14 S), and represents 2–3% of the RNA synthesized in the absence of the drug. It is found in all tissues and animal cells examined, has a high degree of secondary structure, and is 60% resistant to digestion by ribonucleases A and T_1. The 4S ribonuclease-resistant core of this RNA had many of the attributes of a double-stranded structure. However it is not entirely denatured by heat and organic solvents and it does not have a base composition compatible with a simple base-paired structure.

The purpose of this communication is to characterize this RNA further. The present experiments revealed that this RNA was not the 5S rRNA form, and was unmethylated. The

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[1] Abbreviations used are: BD-cellulose, benzoylated diethylaminoethylcellulose; poly(A)·poly(U), homopolymer duplex formed between polyriboadenylic acid and polyribouridylic acid; poly(I)·poly(C), homopolymer duplex formed between polyriboinosinic acid and polyribocytidylic acid; SSC, 15 mM sodium citrate and 0.15 M sodium chloride; SFV, Semlike forest virus, a group A arbovirus; RI, replicative intermediate in the replicative cycle of an RNA-containing virus; RF, replicative form in the replicative cycle of an RNA-containing virus: SDS, sodium dodecyl sulfate; RSV, Rous sarcoma virus; RNase A, pancreatic ribonuclease; RNase T, ribonuclease T_1, the ribonuclease extracted from Taka-Diastase.

ribonuclease-resistant core of this RNA was a single peak on sucrose gradient centrifugation, and on BD-cellulose and molecular sieve column chromatography; however, on polyacrylamide gel electrophoresis, multiple components were observed. Synthesis of this RNA, though it had many of the attributes of RNA virus replication, was not inhibited by antiviral agents such as interferon, poly(I):poly(C) or guanidine·HCl. The ribonuclease-resistant core of the RNA synthesized in chick fibroblasts both in the presence and the absence of actinomycin hybridized to chicken DNA to the extent of 7–12%. The ribonuclease-resistant core of the RNA from rat liver cytoplasm was a potent inducer of interferon.

Experimental Section

Materials

Actinomycin D was the generous gift of Merck Sharp and Dohme.[2] BD-cellulose was the product of Schwarz BioResearch. Media were obtained from the NIH Media Unit and fetal bovine serum was from Flow Laboratories. The commercial enzyme preparations, ribonucleases A, T_1, and deoxyribonuclease were obtained from the Worthington Biochemical Corp., pronase was the product of California Corp. for Biochemical Research, and subtilisin was obtained from Mann Research Laboratories. Poly(I)·poly(C) was the product of Miles Laboratories and urea was from the Baker Chemical Co.

[^{32}P]Phosphoric acid, carrier free, was purchased from the New England Nuclear Corp. [^{3}H]5-Orotic acid (14.4 Ci/mmole), [^{3}H]methylmethionine (2.6 Ci/mmole), [^{3}H]methylthymidine (6 Ci/mmole), and [^{3}H]5-uridine (20 Ci/mmole) were the products of Schwarz–Mann.

N,*N*^{1},*N*^{1},*N*^{1}-Tetramethylethylenediamine, acrylamide, and *N*,*N*^{1}-methylenebisacrylamide were purchased from Eastman Organic Chemicals. They were partially purified by the method of Loening (1967). Agarose (Seakem) was obtained from Bausch & Lomb.

Methods

Culture Conditions. Burkitt lymphoma cells AL 1 (Rabson *et al.*, 1966), a line of human lymphoblasts derived from a malignant lymphoma of the jaw were cultured at 37° in medium RPM1 1640 using 20% fetal bovine serum, and penicillin and streptomycin (100 U/ml and 100 μg/ml, respectively). Cell density was 1.2×10^6/ml and reached 2.4×10^6/ml by 36 hr. Cells were divided twice weekly. Experiments were performed routinely 4 hr after cells had been divided.

The methods used in preparing monolayers of chick fibroblasts in primary culture and the methods for obtaining pools of SFV for the preparation of the RF of virus have been previously described (Friedman *et al.*, 1966).

Labeling and Extraction. Lymphoma cells were routinely incubated with actinomycin at 5 μg/ml for 40 min. Cells were concentrated fivefold and then labeled with [^{3}H]5-uridine at 2 or 20 μCi per ml or with [^{3}H]methylmethionine at 20 μCi/ml. For the latter, for RNA methylation experiments, cells were incubated in medium containing 0.05 the usual concentration of amino acids plus 1 mM sodium formate.

Rat tissues were obtained from animals following the intraperitoneal injections of actinomycin and [^{3}H]5-orotic acid using the protocol of Revel and Hiatt (1964).

RNA was extracted twice (Cooper and Kay, 1969) with phenol in the presence of bentonite and in the presence or absence of SDS. Unlabeled cytoplasmic RNA prepared from rat liver (Hiatt, 1962) was added to the aqueous phase after the first extraction. RNA was washed twice and dissolved in the appropriate buffer.

For the hybridization experiments, highly labeled RNA was prepared from primary cultures of chick embryo fibroblasts. The monolayers in 10-cm plates were preincubated in low phosphate medium with 2% serum for 30 min at 37°. Actinomycin was added at 2.5 μg/ml and incubation continued for 45 min. Carrier-free [^{32}P]phosphoric acid (0.2 mCi/plate) was added for 4 hr. Plates were washed with cold Earle's salt solution. Cells were scraped, suspended, washed three times, and extraction carried out as described under *Extraction Procedures*. No carrier RNA was used. For polyacrylamide gel electrophoresis, chick fibroblasts were labeled with [^{3}H]uridine (20 μCi/ml) in medium RPM1 1640 for 1 hr in the presence and absence of a 40-min preincubation with actinomycin (2.5 μg/ml). Plates were washed with cold Earle's salt solution, and extraction carried out as described above.

Chick liver RNA used in experiments on the induction of inteferon and in the spectrophotometric studies was obtained from 1-day-old chick liver cytoplasm by the same procedure used to obtain the marker rat liver cytoplasmic RNA (Hiatt, 1962).

Chick liver DNA was prepared by Marmur's procedure (1961).

Nuclease Digestions. For digestion of RNA preparations with various nuclease preparations the washed RNA precipitate was dissolved in 10 ml of 15 mM Tris-HCl buffer (pH 7.4) containing 50 mM NaCl and 2 mM $MgCl_2$. DNase was added (20 μg/ml) and incubated for 30 min at 23°. The NaCl concentration was increased to 0.3 M and ribonucleases A (12 μg/ml) and T_1 (2 μg/ml) added for 60 min at 23° unless otherwise indicated. At the end of this time pronase which had been preincubated (5 mg/ml for 2 hr at 37°) was added to a final concentration of 1 mg/ml. Incubation was continued for 2 hr at 37°. The preparation was then extracted with phenol at 37° and precipitated in 2% potassium acetate in 70% ethanol.

For experiments on the induction of interferon RNA from ^{32}P-labeled chick embryo fibroblasts was used. Following extraction the RNA was dissolved in 10 ml of 20 mM potassium phosphate (pH 6.8) containing 0.2 M NaCl and 10 mM $MgCl_2$ and chromatographed on a BD-cellulose column as described under *Column Chromatography*. The peak fractions of acid-precipitable [^{32}P]RNA corresponded in elution position to the entirely base-paired synthetic homopolymer duplex poly(I)·poly(C).

Column Chromatography. Sephadex G-100 column (90 × 1.5 cm i.d.) chromatography was performed using a buffer containing 0.2 M NaCl, 20 mM Tris-HCl (pH 7.4), and 2 mM EDTA. In each case, the total RNA extracted from a 3-ml aliquot of cytoplasmic rat liver extract was placed on the column in 1 ml of the same buffer. Fractions of 3 ml were collected. Using a reservoir head of pressure of 20 cm, a flow rate of 0.5 ml/min was obtained.

Chromatography on the BD-cellulose column has been described (Stern and Friedman, 1969). Following precipitation and washing in 2% potassium acetate–70% ethanol, samples were dissolved in 10 ml of 25 mM potassium phosphate buffer (pH 6.8) containing 0.2 M NaCl and 10 mM $MgCl_2$ and

[2] We have used several sources of actinomycin and have observed that many commercial preparations have no activity or are unstable despite precautions such as storage of either the powder or solutions of the drug, in the dark at −70°. Only the Merck Sharp and Dohme product had consistently stable activity.

placed on the column. Following a 50 ml wash with the same buffer two 260-ml sequential gradients were applied to the column; (a) a gradient from 0.2 to 1.8 M of NaCl in 25 mM potassium phosphate (pH 6.8) containing 10 mM $MgCl_2$, and (b) a gradient formed by a solution containing 25 mM potassium phosphate buffer (pH 6.8) 0.2 M NaCl, and 0.1 M sodium formate buffer (pH 3.4), 8 M urea, and 1.6 M NaCl. Fractions of 4.0 ml were collected, at a flow rate of 1 ml/min.

Measurement of Acid-Precipitable Radioactive Material. Solutions to be counted were chilled to 0° and one-tenth volume of 50% trichloroacetic acid added. This was incubated for 10 min at 0° and then poured on to a Millipore filter and suction supplied. The filter was washed three times with 10-ml aliquots of 5% trichloroacetic acid at 0°. The filter was dried in an oven at 55° for 30 min and placed in counting vials containing 4 ml of toluene containing 4% Liquifluor (Nuclear-Chicago Corp.) and counted in a Packard Tri-Carb liquid scintillation counter.

Direct Assay of RNA Synthesis in Cells. The following procedure was used for the estimation of RNA synthesis in Burkitt lymphoma cells following a period of labeling, without preliminary extraction of the RNA. Cells in 4-ml vials were washed with saline at 0°, washed twice with 5% trichloroacetic acid at 0°, solubilized with 0.4 ml of NCS (Nuclear-Chicago solubilizer), heated for 15 min at 60°, and 4 ml of toluene containing 4% Liquifluor was added. These vials were placed within scintillation vials and counted directly.

Polyacrylamide Gel Electrophoresis. Preparation of 2.2% polyacrylamide–0.5% agarose gels (8 × 0.6 cm i.d.) was carried out by combining 15 ml of melted 1% agarose (w/v) with 15 μl of N,N^1,N^1,N^1-tetramethylethylenediamine, and 15 ml of the following mixture: 0.72 ml of H_2O, 5.28 ml of a stock solution containing 15% acrylamide (w/v) and 0.75% N,N^1-methylenebisacrylamide (w/v), and 12 ml of gel buffer (40 mM Tris, 60 mM sodium acetate, and 3 mM EDTA adjusted to pH 7.2 with glacial acetic acid). Reagents were mixed at 45°. Following evacuation of air from the mixture, 0.3 ml of freshly prepared 10% ammonium persulfate (w/v) was added and transferred to the glass tubes. The electrophoresis buffer containing 0.5% SDS has been described (Bishop *et al.*, 1967). The samples containing dissolved crystals of sucrose were applied to the gel and electrophoresis performed at 23° for 4 hr at constant current, at 6 mA/gel. Following electrophoresis gels were transferred to test tubes containing 5% trichloroacetic acid at 4°. Gels were washed twice with 5% trichloroacetic acid and sliced into 1.2-mm segments (Chrambach, 1966). Each slice was incubated overnight at 55° with 50 μl of 30% hydrogen peroxide in scintillation vials. Toluene–Liquifluor scintillation fluid (10 ml) containing Triton X-100 was added and the samples were counted.

Hybridization. Hybridization of [^{32}P]phosphoric acid labeled chick fibroblast RNA with ^{3}H-labeled chicken liver DNA was performed according to the method of Gillespie and Spiegelman (1965). Minced chicken liver was incubated with [^{3}H]thymidine at 5 μCi/ml for 6 hr. The DNA was extracted and prepared by the procedure of Marmur (1961) and treated with ribonuclease A, subtilisin, and pronase. DNA was then dissolved in 0.1× SSC at a concentration of 0.5 mg/ml. For the preparation of the filters, DNA was denatured with 0.3 N NaOH for 10 min, and then neutralized with 1 N HCl and 2 μg of this DNA was placed on the washed Millipore filters. The amount of DNA which adhered to the membrane was monitored with the ^{3}H label. The DNA filters were incubated for 16 hr at 80° in 0.2 ml of 4× SSC together with [^{32}P]phosphoric acid labeled double-stranded RNA. Denaturation of the ribonuclease-resistant RNA was accomplished by dissolving the RNA precipitate in 0.1× SSC and boiling for 5 min, rapid cooling, and adding sufficient 20× SSC to yield a solution containing 4× SSC. Following the hybridization the filters were washed with 2× SSC, treated with 20 μg of ribonuclease A in 1 ml of 2× SSC for 1 hr, washed, dried at 65°, and counted. Experiments were done in duplicate. Simultaneous control reactions using blank filters and filters containing 2 μg of *E. coli* DNA were performed at each experimental point.

Interferon. Human interferon was prepared from human lymphocytes stimulated *in vitro* by Sendai virus (Guggenheim *et al.*, 1969). After dialysis, acid treatment and centrifugation the interferon titer was 500 U/ml.

Interferon Assay. Interferon was assayed by the Sindbis virus hemagglutinin inhibition in MA-308 cells, a line derived from a human maxillary sinus polyp. MA-308 cells were grown in Eagle's medium with nonessential amino acids, sodium pyruvate, and 10% fetal calf serum. Cells were treated with dilutions of an interferon preparation for 24 hr, washed five times, and infected at a multiplicity of 100 plaque-forming units of virus per cell. After 24 hr the cultures were frozen and thawed and the fluids tested for Sindbis virus hemagglutination inhibition in a microtiter assay. Gander red blood cells were used as an indicator of hemagglutination production (Clarke and Casals, 1955). One unit of interferon was that dilution which caused a fourfold inhibition of Sindbis virus hemagglutinin production. Interferon titers are reported as titer per milliliter. In this laboratory, the new international human interferon standard (69-16) gave a titer of 12,590 units/ml.

Results

Initial experiments were performed to establish optimal conditions for the use of actinomycin in Burkitt lymphoma cells. The experiments of Figure 1 indicated that the action of actinomycin at 5 μg/ml was complete within 30 min. All subsequent experiments were performed 40 min after addition of actinomycin. The drug was not removed at this point but was present during the remainder of each experiment.

Experiments to establish a concentration-dependence curve (Figure 2) revealed that the level of inhibition of RNA synthesis in the Burkitt lymphoma cells reached a plateau at approximately 5 μg/ml when 98% of RNA synthesis had been inhibited. In subsequent experiments cells were incubated routinely at 5 μg/ml. As was confirmed here lymphoid cells are more resistant to actinomycin, and require higher concentrations of actinomycin to achieve comparable inhibition of RNA synthesis (Cooper, 1968) than other animal cells (Perry, 1963).

Molecular Sieve Chromatography. The chromatographic properties of the RNA synthesized in the presence of actinomycin were investigated. The profile of the RNA from Burkitt lymphoma cells was examined on a Sephadex G-100 column following different lengths of labeling with [^{3}H]uridine. Cells labeled for 15, 30, and 45 min were extracted at 62° with SDS and the RNA placed on a column together with marker RNA from rat liver cytoplasm. The latter is separated into three peaks of material on such a column; these are peaks of 4S RNA, 5S rRNA, and in the excluded volume a single peak containing 18S and 28S rRNA. At 15 min (Figure 3a) all Burkitt RNA was present in the excluded volume. At 30 min (Figure 3b) RNA was present in all three regions of the chromatogram. With longer periods of labeling the proportion of

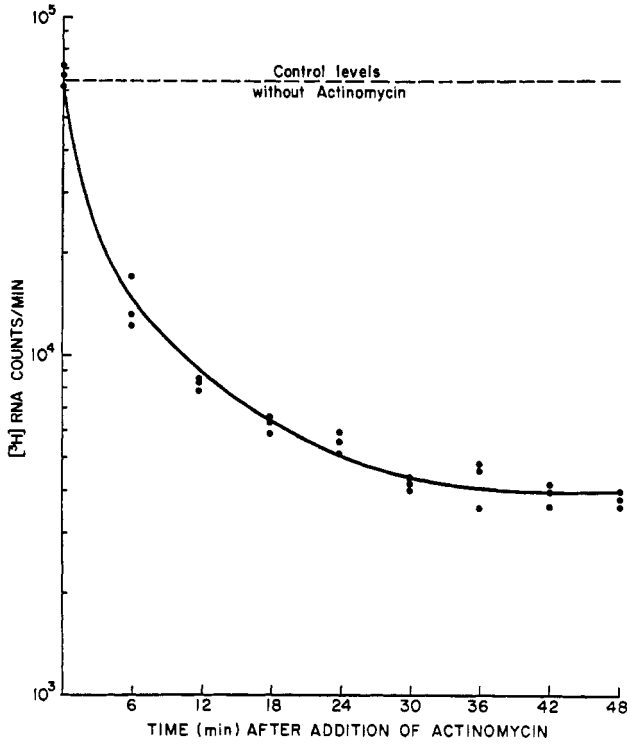

FIGURE 1: Time course of action of actinomycin on the incorporation of [^{3}H]uridine into the acid-precipitable RNA of Burkitt lymphoma cells. Actinomycin (5 μg/ml) was added to 2-ml aliquots of cells in suspension culture in triplicate and [^{3}H]uridine (5 μCi/ml) was added for 6-min intervals. Cells were then chilled and processed as described under Methods.

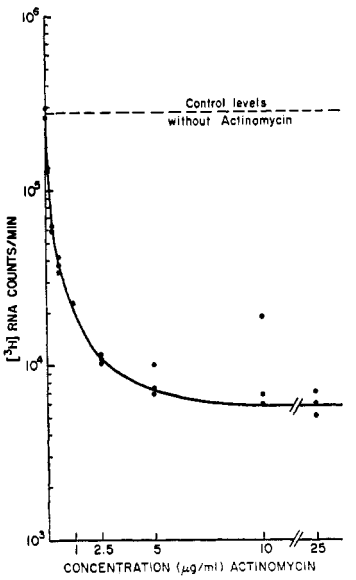

FIGURE 2: RNA synthesis in Burkitt lymphoma cells as a function of actinomycin concentration as measured by incorporation of [^{3}H]uridine into acid-precipitable RNA. Actinomycin, at the indicated concentration, was added to 2-ml aliquots of cell suspension cultures. After 40 min [^{3}H]uridine (5 μCi/ml) was added to each vial and the incubation continued for 40 min. At the end of the incubation, cells were processed as described under Methods.

RNA in the 4S region increased until at 45 min (Figure 3c) this was the predominant peak of RNA.

These experiments suggest that the RNA synthesized in the presence of actinomycin undergoes a processing procedure. The first radioactive label was incorporated into a high molecular weight form, in the void volume of a Sephadex G-100 column, and was subsequently found in a form which coeluted with marker 4S RNA. The longer the labeling period, the greater was the proportion of material which appeared in the 4S region. These data are compatible with RNA associated with a high molecular weight RNA template or with the RNA synthesized as a high molecular weight precursor which is subsequently shortened to achieve the mature form comparable to the processing of the precursor to rRNAs (Perry, 1967).

An RNA synthesized in the presence of actinomycin similar to that found in Burkitt lymphoma cells has been described previously in rat liver (Revel and Hiatt, 1964; Moulé and Landin, 1965). Rat liver RNA was prepared as described (Revel and Hiatt, 1964) using intraperitoneal pretreatment with actinomycin followed by [^{3}H]5-orotic acid. The extracted rat liver RNA was examined on a Sephadex G-100 column (Figure 4a). Three peaks of RNA were obtained with a predominant 4S peak. Similar profiles were obtained from RNA extracted from other rat organs including kidney and spleen. These profiles suggest a similarity between the RNAs synthesized in the presence of actinomycin in the Burkitt cells in continuous culture and synthesized *in vivo* in rat liver.

The RNase-resistant core of the RNA synthesized in the presence of actinomycin in Burkitt lymphoma cells was examined on the Sephadex G-100 column. A single peak of RNA corresponding to the 4S marker was observed. A similar peak was obtained from the rat liver RNA. This RNA synthesized in the presence of actinomycin following ribonucleases A and T_1 digestion pronase treatment and reextraction with phenol, was placed on the Sephadex G-100 column together with *E. coli* B tRNA (Figure 4b).

When RNA extracted from Burkitt lymphoma cells labeled with [^{3}H]uridine without actinomycin and then digested with ribonucleases A and T_1, a core of RNA was obtained representing 1.2% of the labeled RNA. This material on a Sephadex G-100 column had a profile identical with the RNase-resistant core of the RNA synthesized in the presence of the drug. This result suggested that the RNA synthesized in the presence of the drug had a ribonuclease-resistant core similar to that of an RNA synthesized in the absence of the drug. This experiment suggested that the RNA synthesized in the presence of actinomycin was not an artifact which could be attributed to the presence of actinomycin.

When the RNA fractions from the Sephadex G-100 column were examined for susceptibility to ribonuclease digestion following rather than preceding column chromatography, ribonuclease-resistant material was found in the first and last peaks from the column corresponding to the void volume and 4S RNA markers. No ribonuclease-resistant RNA was obtained from the 5S region of the chromatogram.

Methylation. The methylated base content of the RNA synthesized in the presence of actinomycin was examined. Burkitt lymphoma cells pretreated with actinomycin were labeled with [^{3}H]methylmethionine for 30 min and placed on a Sephadex G-100 column. All label was present in a single peak which corresponded to the tRNA region of the chromatogram (Figure 5). However when an identical preparation was treated with ribonucleases A and T_1 (Table I, expt 1), no acid-precipitable material remained.

The argument could be invoked that the RNA synthesized in the presence of actinomycin is normally methylated but

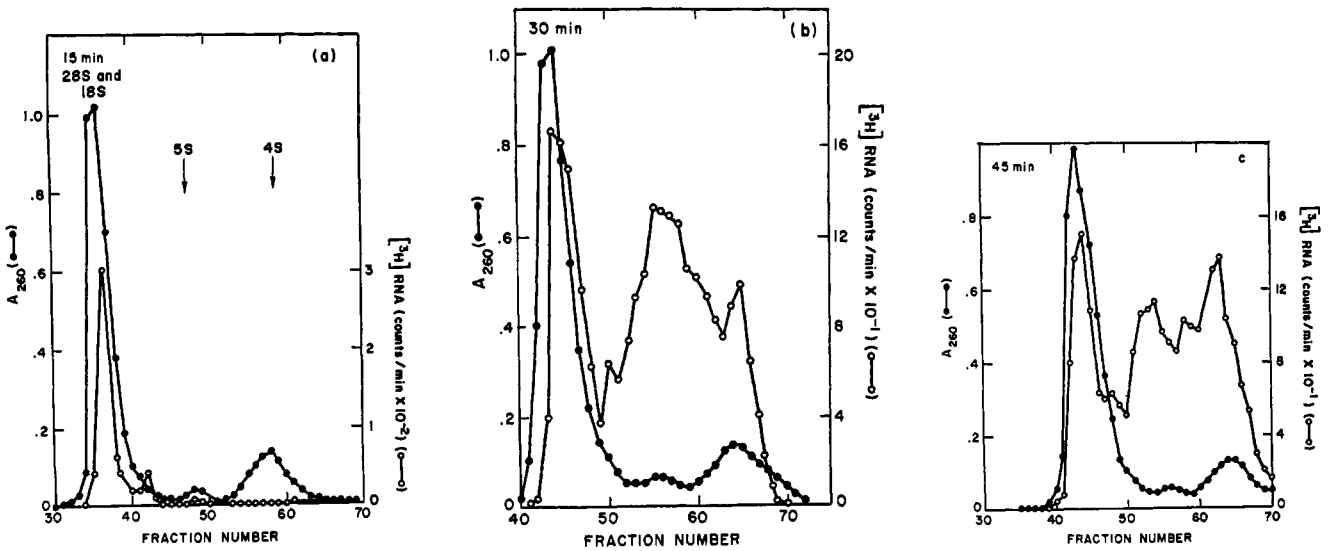

FIGURE 3: Sephadex G-100 column chromatography of the RNA synthesized in Burkitt lymphoma cells in the presence of actinomycin following 15-, 30-, and 45-min labeling with [^{3}H]uridine. Actinomycin (5 μg/ml) was added followed after 40 min by [^{3}H]uridine at 2 μCi/ml. Aliquots of the culture were removed at 15, 30, and 45 min and extracted as described under Methods. A_{260} and the level of acid-precipitable ^{3}H-labeled material were determined for each fraction as described under Methods. A_{260} (●) and [^{3}H]RNA counts per minute (○). The arrows indicate the position of the marker RNAs.

that actinomycin inhibits the methylation reaction of the RNA synthesized during the period of exposure to the drug. This possibility was tested by labeling Burkitt lymphoma cells with [^{3}H]methylmethionine for 60 min in the absence of actinomycin. When RNA extracted from such a preparation was treated with ribonucleases A and T_1, no acid-precipitable radioactively labeled material remained (Table I, expt 2). We concluded that RNA synthesized in the presence of actinomycin was unmethylated, and that a putative methylation reaction was not inhibited by actinomycin. Since tRNA is a highly methylated polynucleotide it is unlikely that the RNA synthesized in the presence of actinomycin was tRNA. The 4S RNA synthesized in rat liver in the presence of high doses of actinomycin (Revel and Hiatt, 1964; Moulé and Landin, 1965) is assumed to be tRNA. However it was unlikely that the analogous RNA synthesized in the presence of actinomycin in the present experiments was tRNA. Base composition experiments confirmed that such material was not due to the turnover of 3′-terminal cytidine and adenine residues (Preiss *et al.*, 1961; Daniel and Littauer, 1965).

BD-cellulose Column Chromatography. The RNA synthesized in the presence of actinomycin could be attributed to RNA virus infection in animal cells. Partial ribonuclease resistance is also reminiscent of the components of RNA virus replication. For this reason, the RNA synthesized in the presence of actinomycin was examined on a BD-cellulose column. This column resolves RNAs on the basis of secondary structure (Gillam *et al.*, 1967, 1968; Stern and Friedman, 1969). In a previous communication (Stern and Friedman, 1969) we demonstrated that among the components of the replicative cycle of the RNA virus SFV, the entirely ribonuclease-resistant RF (base-paired RNA template) and the partially ribonuclease-resistant RI (viral template with attached nascent single-stranded RNA strands) are eluted from the BD-cellulose column with a NaCl gradient. However the ribonuclease sensitive single-stranded viral RNAs require a gradient of urea for their elution. RNA from [^{3}H]uridine-labeled Burkitt lymphoma cells was chromatographed on a BD-cellulose column. Two major components were eluted (Figure 6A); one by the NaCl gradient and a second by the gradient of urea. This is similar to the chromatographic pattern obtained from the components of RNA virus replication (Stern and Friedman, 1969). Furthermore when fractions from the NaCl and the urea gradients were dialyzed, and treated with pancreatic RNase, 65% of the radioactively labeled RNA remained in acid-precipitable form from the NaCl gradient, while all of the labeled RNA from the urea gradient became acid soluble. The material from the NaCl gradient was reapplied to the BD-cellulose column. The RNA now eluted together with the entirely base-paired RF from ^{32}P-labeled SFV (Figure 6B). This suggested that the ribonuclease-resistant material from the NaCl gradient had a structure similar to that of the entirely base-paired RF of an RNA virus. These experiments were compatible with the RNA synthesized in the presence of

TABLE I: Methylation of RNA Synthesized in Burkitt Lymphoma Cells in the Presence and Absence of Actinomycin.[a]

Labeling Condn	Treatment of RNA	Acid-Precipitable Material Remaining (cpm)
1. Actinomycin and [^{3}H]methylmethionine	None	2750
	Ribonuclease digestion	0
2. [^{3}H]methylmethionine	None	2200
	Ribonuclease digestion	0

[a] Burkitt lymphoma cells were labeled and RNA extracted with phenol and SDS at 64° as described under Methods. Digestion with ribonucleases A and T_1 and precipitation with trichloroacetic acid are described under Methods.

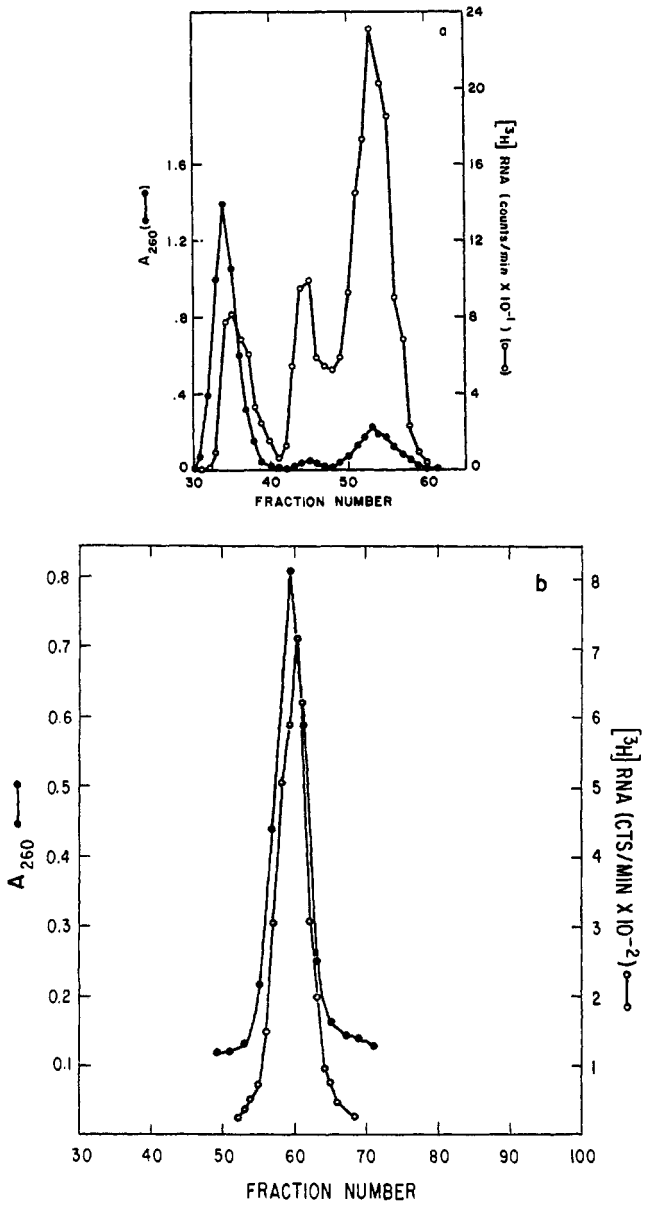

FIGURE 4: Sephadex G-100 column chromatography of (a) the RNA synthesized in the presence of actinomycin in *in vivo* [^{3}H]orotic acid labeled rat liver cytoplasm and (b) the same RNA chromatographed after preliminary digestion with ribonucleases A and T_1. The rat liver was excised 2 hr following intraperitoneal injection of 500 μCi of [^{3}H]5-orotic acid and 24 hr after the injection of actinomycin, 8 mg/kg. Chromatography carried out as described under Methods. (b) An aliquot of the same preparation of RNA described in (a) was digested with ribonucleases A and T_1 digested with pronase and reextracted with phenol as described under Methods. This RNA was placed on a Sephadex G-100 column together with 0.5 mg of *E. coli* B tRNA. A_{260} (●) and [^{3}H]RNA counts per minute (○).

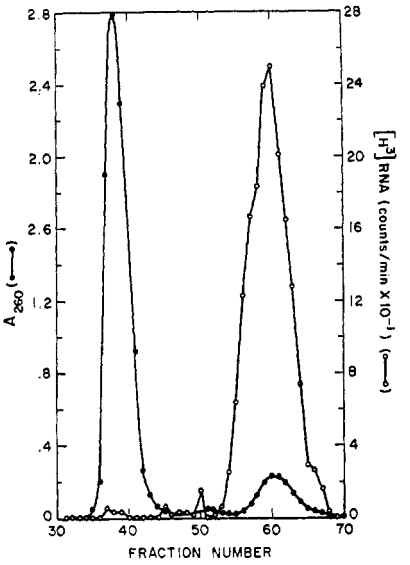

FIGURE 5: Sephadex G-100 column chromatography of [^{3}H]methylmethionine-labeled RNA synthesized in Burkitt lymphoma cells in the presence of actinomycin. Cells were incubated with actinomycin and then with [^{3}H]methylmethionine for 40 min. RNA was extracted with phenol at 23° without SDS. The RNA was chromatographed together with rat liver cytoplasmic RNA as described under Methods. A_{260} (●) and [^{3}H]RNA cpm (○).

actinomycin having structures like the intermediates in RNA virus replication.

From BD-cellulose column profiles additional evidence was obtained that the RNA synthesized in the presence of actinomycin was neither tRNA nor 5S rRNA. Commercial *E. coli* B tRNA and 5S rRNA taken from Sephadex G-100 column chromatography of normal [^{14}C]orotic acid labeled rat liver cytoplasmic RNA were applied together to a BD-cellulose column. The profile of these two RNAs (Figure 7) did not resemble the elution pattern of the RNA synthesized in the presence of actinomycin (Figures 6A,B).

Double-stranded RNAs including synthetic duplexes such as poly(I):poly(C) are potent inducers of the antiviral agent interferon (Lampson *et al.*, 1967; Tytell *et al.*, 1967; Field *et al.*, 1967). The synthesis of interferon is one of the primary responses of organisms to viral infection. The BD-cellulose column chromatographic profile of the synthetic homopolymer duplex poly(I):poly(C) and the ribonuclease-resistant core of the RNA synthesized in Burkitt lymphoma cells in the presence of actinomycin were identical. A similar profile was obtained for the ribonuclease-resistant RNA synthesized *in vivo* in rat liver in the presence of actinomycin. All of these were identical with the profile shown in Figure 6B.

Induction of Interferon. Because of the similarity in the BD-cellulose column elution profiles between the two RNA preparations known to induce interferon and the present RNA, the ability of the ribonuclease-resistant RNA from chicken

TABLE II: Stimulation of Interferon Production by a Synthetic and a Naturally Occurring Double-Stranded RNA.[a]

Source of RNA	Amount Used (μg/ml)	Interferon Produced (units/ml)
Ribonuclease-resistant RNA from chick liver	40	300
Poly(I):poly(C)	40	94

[a] RSTC-2 cells in monolayers were treated with DEAE-dextran (25 μg/ml) and then with the RNA preparations. Interferon synthesis was measured by the inhibition of virus hemagglutinin production as described under Methods.

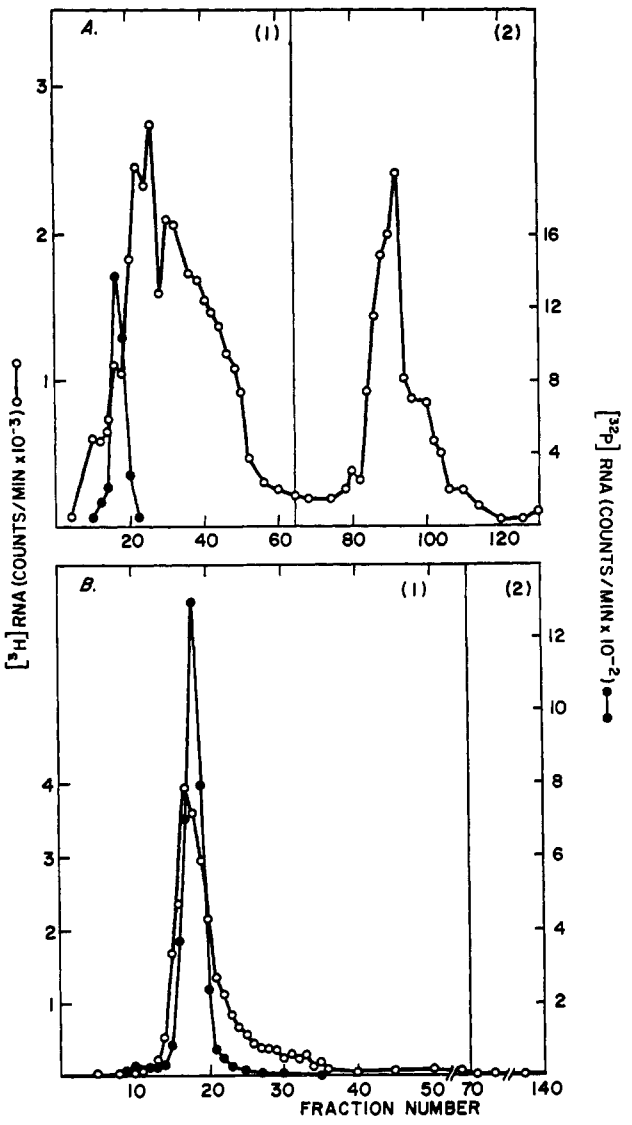

FIGURE 6: BD-cellulose column chromatography of the RNA synthesized in Burkitt lymphoma cells in the presence of actinomycin before and after treatment with ribonucleases A and T_1. RNA was prepared and chromatography carried out as described under Methods. (A) RNA synthesized in the presence of actinomycin together with ^{32}P-labeled RF from the SFV. (B) RNA synthesized in the presence of actinomycin, and treated with ribonucleases A and T_1, reextracted with phenol, and placed on the column together with ^{32}P-labeled RF from the SFV. ^{32}P-Labeled SFV RF marker RNA counts per minute (●) and [^{3}H]RNA counts per minute (○).

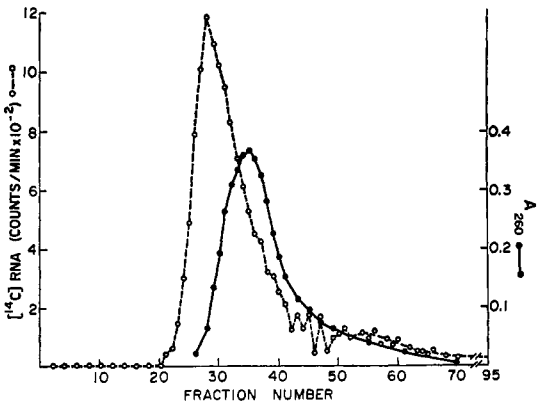

FIGURE 7: BD-cellulose column chromatography of *E. coli* B tRNA and 5S rRNA from rat liver cytoplasm. *E. coli* B tRNA and 5S RNA obtained from [^{14}C]orotic acid labeled rat liver cytoplasmic extract which had been chromatographed on a Sephadex G-100 column similar to the one illustrated in Figure 2 were placed on the BD-cellulose column. Fractions from the 5S region of the chromatogram, comparable to fractions 47–49 in Figure 3a, were pooled. An aliquot of these pooled fractions together with 0.5 mg of *E. coli* B tRNA were placed on the column. Chromatography was carried out using the gradient of NaCl described under Methods. [^{14}C]RNA counts per minute (○) and *E. coli* B tRNA A_{260} (●). The contribution of the 5S rRNA to the total absorption was negligible.

liver to induce interferon was examined. An RNA preparation from chicken liver cytoplasm was used, obtained by DNase and ribonucleases A and T_1 digestion followed by pronase treatment and reextraction with phenol, as described under Methods. This RNA was applied to a BD-cellulose column and the peak tubes from a profile similar to that shown in Figure 8 were used directly in the assay. A line of normal human diploid fibroblasts, RSTC-2, was used for interferon induction. The RNA was applied and interferon production was measured by the inhibition of Sindbis virus hemagglutinin production as described under Methods. Table II demonstrates that the RNA was three times as potent inducer of interferon, as the synthetic polymer poly(I)·poly(C). This suggested that there exists in presumably normal uninfected animal tissue a form of RNA which is able to induce interferon.

Spectrophotometry. The profile of the ribonuclease-resistant RNA from chick fibroblasts was examined. An aliquot of the same fraction used in the interferon induction experiments (Table III) and a solution of *E. coli* B tRNA were compared using a Cary 14 recording spectrophotometer. In Figure 8, it can be seen that the two profiles are similar, with a spectrum typical of pure RNA and an A_{260}/A_{280} ratio of 2.1. At 310–330 nm the absorbany contributed by the thiolated bases of tRNA was observed (Lipsett, 1965) which was absent in the spectrum of the double-stranded RNA. This was further evidence that the 4S RNA synthesized in the presence of actinomycin was not tRNA. The tRNA had an absorption maximum at a 0.5 nm higher wavelength. The trough at 230 nm for the double-stranded RNA was greater than that of tRNA relative to the

TABLE III: Effect of Various Agents on the Biosynthesis of RNA in the Presence of Actinomycin in Burkitt Lymphoma Cells.

Expt	Agent	Concn in Culture Medium	Length of Incubn[a] (hr)	Amount of RNA Synthesis (cpm)
1	Human interferon	10 U/ml	16	5720
	None			5017
2	Poly(I):poly(C)	10 μg/ml	16	4782
	None			5450
3	Guanidine·HCl	5 mg/ml	2	7200
				6714

[a] With agent preceding exposure to actinomycin and [^{3}H]uridine.

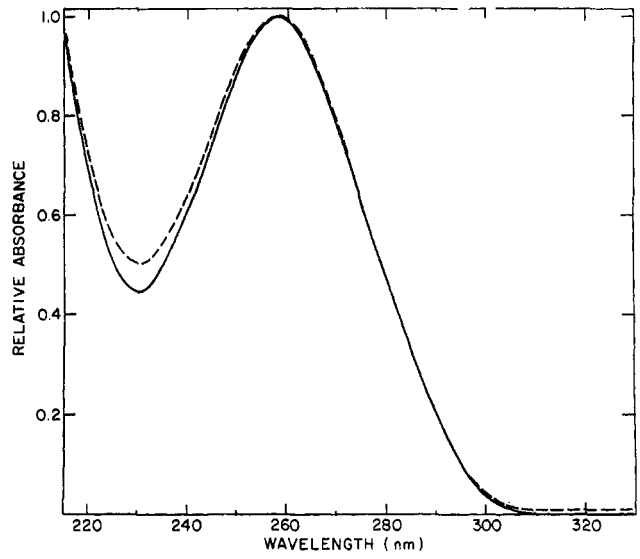

FIGURE 8: Relative absorption profiles of solutions of ribonuclease-resistant RNA from chick liver cytoplasm and *E. coli* B tRNA using a Cary 14 recording spectrophotometer. Base-line absorption was obtained from the chromatographic buffer (25 mM potassium phosphate (pH 6.8), containing 10 mM $MgCl_2$ and 0.2 M NaCl). The ribonuclease-resistant RNA was extracted from livers of 2-day-old chicks, digested with DNase, ribonucleases A and T_1, and pronase, and reextracted with phenol as described under Methods. The RNA was then chromatographed on a BD-cellulose column and the peak fraction from the profile similar to the one illustrated in Figure 6B was used for the spectrophotometric study. A_{260} was 0.82 for this fraction. tRNA was dissolved in the same buffer at a similar concentration. Absorbancy profiles were redrawn to give the same relative absorption of 1.0 at the A_{258} maximum. Absorption of *E. coli* B tRNA (---) and absorption of ribonuclease-resistant RNA (—).

absorption maximum at 258 nm. However no major differences in the spectra were found.

Polyacrylamide Gel Electrophoresis. The [^{3}H]uridine-labeled ribonuclease-resistant RNAs synthesized in Burkitt lymphoma cells in the presence and absence of actinomycin were analyzed on 2.2% polyacrylamide gels in the presence of SDS. These RNAs were analyzed together with ^{32}P-labeled chick cell cytoplasmic RNAs containing predominantly 28S, 18S, and 4S markers. The ribonuclease-resistant core of the RNAs synthesized in the presence and absence of actinomycin was examined on polyacrylamide gel electrophoresis (Figure 9a,b). Both of these preparations had multiple peaks of RNA. Such RNAs had a single peak on Sephadex G-100 (Figure 6B), BD-cellulose column chromatography (Figure 8) and on sucrose density gradient centrifugation (Stern and Friedman, 1970). In addition, the profile of the RNA synthesized in the presence of actinomycin differed from the profile of the RNA synthesized in the absence of the drug. One peak in each preparation corresponded to the 4S marker RNA. The RNA from actinomycin-treated cells (Figure 9a) contained a peak which migrated more rapidly than the corresponding peak from untreated cells (Figure 9b).

Thermal Denaturation. ^{3}H-Labeled Burkitt cell RNA synthesized in the presence of actinomycin was digested by ribonucleases A and T_1 as described under Methods. The thermal denaturation of the resistant core was examined. This RNA, which represented 60% of the original RNA, following precipitation in NaCl–ethanol was dissolved in 0.1× SSC. The RNA was heated to the indicated temperature (Figure 10) for 5 min rapidly cooled, one-tenth volume of 20× SSC added, and digested for 30 min with ribonucleases A and T_1. Acid-precipitable ^{3}H-labeled RNA was then determined. A sharp transition was observed at 70° consistent with a molecule with a high degree of structure. However, only 60% of the molecule was made sensitive to ribonuclease following thermal denaturation.

Solvent denaturation with Me_2SO as described by Katz and Penman (1966) was performed. RNA synthesized in the presence of actinomycin was extracted, digested with ribonuclease A for 1 hr at 37°, and then incubated in 90% Me_2SO for 30 min at 37°. The preparation was dialyzed overnight and again treated with ribonuclease A. The same ribonuclease-resistant core observed following thermal denaturation was again noted.

Intermolecular and intramolecular disulfide bonds are present in some tRNAs (Lipsett, 1967). Such structures could be invoked in the structure of the ribonuclease-resistant RNA core to explain the present data. However, treatment with 0.1 M 2-mercaptoethanol failed to make this resistant RNA susceptible to further hydrolysis. Susceptibility to ribonuclease A in the presence of low salt concentrations (0.1× SSC) and resistance in high salt is used as a criterion for a double-stranded RNA structure (Shatkin, 1965; Borst and Weissman, 1965). The ribonclease-resistant RNA was hydrolyzed completely by ribonuclease A in 0.1× SSC by KOH (0.3 M for 16 hr at 37°), suggesting that all of the ^{3}H-labeled material was actually RNA.

Inhibition Studies. RNA synthesis in the presence of actinomycin had many of the properties of the replication of an RNA virus. For this reason the response of actinomycin-resistant RNA synthesis to inhibitors of viral RNA synthesis was examined. Interferon is an antiviral protein synthesized in response to viral infection. The response of the RNA synthesized in the presence of actinomycin to homologous interferon was examined. Human interferon (10 U/ml) was incubated with Burkitt lymphoma cells for 16 hr. Actinomycin was then added and the [^{3}H]uridine-labeled RNA extracted. No difference was observed between this and the RNA extracted from a cell culture incubated without interferon (Table III, expt 1).

Poly(I)·poly(C) is synthetic homopolymer duplex which induces the synthesis of interferon in normal uninfected cells. Preincubation of Burkitt lymphoma cells with poly(I)·poly(C) (10 μg/ml) for 16 hr had no effect on the level of subsequent RNA synthesis in the presence of actinomycin (Table III, expt 2).

Guanidine hydrochloride inhibits the replication of certain RNA viruses (Caliguiri and Tamm, 1968). However, preincubation of Burkitt lymphoma cells for 2 hr with guanidine·HCl (5 mg/ml), 50 times the level which will effectively inhibit polio virus RNA replication, had no effect on the level of RNA synthesized in the presence of actinomycin (Table III, expt 3).

Guanidine hydrochloride is reported to enhance the synthesis of certain RNA species synthesized in the presence of actinomycin in baby hamster kideny cells (Martin and Brown, 1967). However no such effect was observed in the present experiments.

Hybridization Studies. An attempt was made to determine whether the ribonuclease-resistant RNA synthesized in both the presence and absence of actinomycin was transcribed from the DNA genome by hybridization with cellular DNA. Table IV demonstrates that both RNAs from chick embryo fibroblasts could be hybridized with chick liver DNA. Early hybridization experiments were performed at 60° and failed to

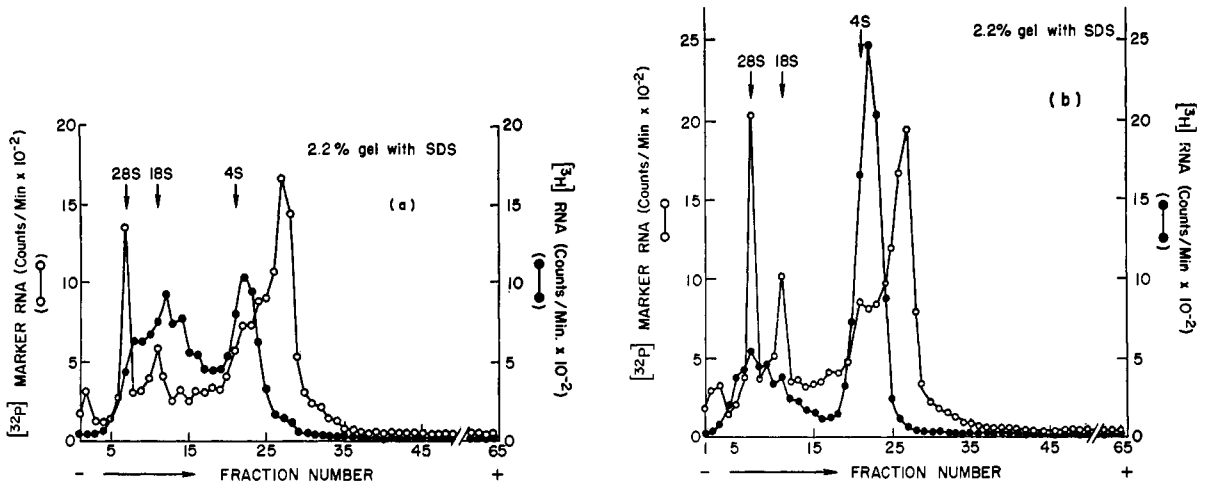

FIGURE 9: Polyacrylamide gel electrophoresis of the ribonuclease-resistant RNA from chick fibroblasts synthesized in the (a) presence and (b) absence of actinomycin. ^{3}H-Labeled RNA was obtained from monolayers of chick embryo fibroblasts and digested with ribonucleases A and T_1 as described under Methods. Aliquots (25 μl) of the resulting RNA preparations were electrophoresed together with 10 μl of marker ^{32}P-labeled whole RNA extracted from monolayers of chick embryo fibroblasts. Electrophoresis was carried out as described under Methods. [^{32}P]RNA marker RNA counts per minute (○) and [^{3}H]RNA counts per minute (●).

TABLE IV: Hybridization Experiments; Hybridization of Heat-Denatured Ribonuclease-Resistant Chick Embryo Fibroblast ^{32}P-Labeled RNA, Synthesized in the Presence and Absence of Actinomycin, to ^{3}H-Labeled Chick Liver DNA.[a]

	[^{32}P]RNA Input (cpm)	DNA on the Filter: None	*E. coli* DNA (2 μg)	Chick Liver DNA (2 μg)	% of Input Annealed
Ribonuclease-resistant RNA synthesized in the presence of actinomycin	75,000	3,995	1,850	5,124	4.4
	100,000	5,769	2,196	7,985	5.8
	200,000	13,479	4,620	18,404	6.9
Ribonuclease-resistant RNA synthesized in the absence of actinomycin	100,000	7,093	3,376	11,136	7.7
	200,000	19,105	7,181	31,000	11.9

[a] Preparation of the RNA, DNA, and the filters, and the hybridization were carried out as described under Methods. Control filters contained either no DNA or 2 μg of *E. coli* DNA. The per cent annealing was calculated by subtracting levels observed with *E. coli* DNA-containing filters from the experimental values. Each point was performed in duplicate and the average is presented.

yield hybridization. When the temperature was elevated to 78°, significant hybridization was observed. Early experiments were characterized also by high levels of nonspecific binding to filter without DNA. This was eliminated by prolonged treatment of the RNA with pronase following DNase and ribonucleases A and T_1 digestion and by a third reextraction with phenol. Background binding was markedly decreased by this procedure. However, the binding to blank filters continued to be higher than to filters containing 2 μg of *E. coli* DNA. Hybridization was calculated by subtracting values obtained from *E. coli* DNA-containing filters.

Discussion

The present experiments continue the description (Stern and Friedman, 1970) of an RNA-replicating system in animal cells which was not inhibited by high doses of actinomycin D and whose product is partially resistant to ribonuclease digestion. This RNA is found in a large number of animal cells in normal and neoplastic cells, cells in primary and in continuous culture in rapidly growing as well as in cells which are not undergoing DNA synthesis and mitosis (Stern and Friedman, 1970). In the present experiments such RNA was also found in normal rat kidney and spleen as well as in liver. The ribonuclease-resistant core of this RNA which eluted as a single peak on molecular sieve and BD-cellulose chromatography and on sucrose density gradient centrifugation had multiple components on polyacrylamide gel electrophoresis. Total RNA made in the presence of actinomycin was not examined by these techniques because there was no way of excluding products of those reactions which convert nucleosides to acid-insoluble forms and which are resistant to actinomycin, such as chain extension of preexisting polynucleotides (Daniel and Littauer, 1965; Wilkies and Smellie, 1968).

All of the radioactively labeled material became acid soluble after treatment with KOH, compatible with all of the label being in RNA. That a significant portion of the RNA remained resistant to RNase after heat dissociation or denatura-

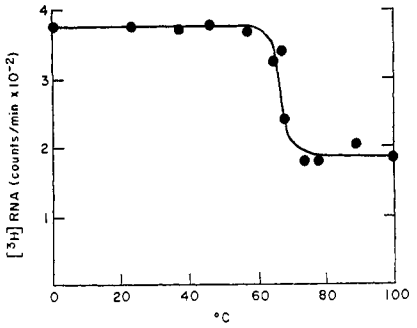

FIGURE 10: Thermal denaturation curve of ribonuclease-resistant RNA synthesized in the presence of actinomycin in Burkitt lymphoma cells. Each experimental point contained 830 cpm of [^{3}H]-RNA initially. RNA was dissolved in 2 ml of 0.1× SSC, incubated for 4 min at the indicated temperature, chilled rapidly, and buffer was added to 2× SSC, followed by 24 μg/ml of ribonuclease A for 30 min at 37°. The level of acid-precipitable [H^3]RNA was determined by chilling to 0°, precipitating with 10% trichloroacetic acid, washing on to a Millipore filter, drying, and placing the filter in 4 ml of toluene–liquifluor scintillation fluid.

tion with Me_2SO suggested that this RNA did not have a simple base-paired structure. This was confirmed by base analysis in a previous communication (Stern and Friedman, 1970). There are two other reports of the retention of ribonuclease resistance in RNAs after denaturation; in the RF of Mengovirus (Faras *et al.*, 1970), a picornovirus, and in a nuclear RNA fraction of murine sarcoma-leukemia virus transformed cells which hybridized to viral RNA (Biswal and Benyesh-Melnick, 1970).

The RNA described in the present communication synthesized in the presence of actinomycin was unmethylated. This RNA might be an unmethylated cytoplasmic precursor to tRNA. However synthesis of such an RNA has been described and is sensitive to actinomycin (Lal and Burdon, 1967; Burdon *et al.*, 1967; Kay and Cooper, 1969).

The observation that this RNA when partially denatured could be hybridized with the DNA of the cell suggests that transcription from a DNA template is the origin of this RNA. However the resistance to inhibition by actinomycin, the partial resistance to ribonuclease, and the BD-cellulose chromatographic evidence of a viral-like RNA form remains unexplained. Transcription which has both an RNA and a DNA phase, as is present in the replication of RNA tumor viruses (Baltimore, 1970; Temin and Mizutani, 1970; Gallo *et al.*, 1970; Scolnick *et al.*, 1971) is a possibility which is being examined.

A double-stranded RNA–RNA complex between mRNA and derepressor RNA molecules in animal cells has been postulated by Frenster (1969). This model stems from the observation that mRNA and derepressor RNA at a given gene focus are each complementary to one of two DNA strands at that locus and thus complementary to each other.

An additional explanation for the origin of the RNA of the present experiments is that they represent DNA–RNA hybrids, nascent RNA strands on DNA templates transcribed up to the first actinomycin attachment site. However no label is associated with the present RNA when cells are labeled with [^{3}H]-thymidine (Stern and Friedman, 1970). Also, DNA–RNA hybrids would be expected to be present in the interphase in RNA extractions using phenol (Scherrer and Darnell, 1962). A major argument against the DNA–RNA hybrid hypothesis is that these molecules, unlike the RNA of the present experiments, do not induce interferon (Colby *et al.*, 1971).

Animal cells infected with certain DNA viruses also have elevated levels of a double-stranded RNA (Colby and Duesberg, 1970) which induces interferon. The synthesis of this RNA in contrast to the present RNA is inhibited by actinomycin. The RNA in infected cells hybridizes with the viral genome. Uninfected animal cells also contain small amounts of an additional form of double-stranded RNA whose synthesis is inhibited by actinomycin (Montagnier, 1968; Harel and Montagnier, 1971). Twenty per cent of this RNA extracted from rat liver hybridizes with the cellular genome. Its relationship to the RNA described in the present experiments which also hybridizes to the cellular genome is not clear. The actinomycin-sensitive double-stranded RNA is also an inducer of interferon (De Maeyer *et al.*, 1971), has an *s* value greater than that of the RNA described in the present experiments, and has a base composition markedly different from the present RNA.

Synthesis of tRNA is generally regarded as more resistant to the action of actinomycin than the synthesis of other RNAs. Various reasons are given as the basis for this phenomenon; the small size of the genome permits escape from the action of actinomycin, or the base composition of the tRNA genome offers fewer actinomycin binding sites. However these conclusions are based on observations of a 4S RNA synthesized in the presence of actinomycin similar to the RNA described in the present experiments (Revel and Hiatt, 1964; Moulé and Landin, 1965). We have established herein that this RNA is unlikely to be tRNA. A reexamination of the question of relative resistance of tRNA synthesis to actinomycin is warranted.

Acknowledgments

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Added in Proof

A publication has appeared following submission of this manuscript which bears on the present material (Kimball and Duesberg, 1971). The biosynthesis of cellular double-stranded RNA described by these authors is inhibited by actinomycin. The relationship between the cellular double-stranded RNAs whose synthesis is resistant to and sensitive to actinomycin is unclear.

References

Baltimore, D. (1970), *Nature (London) 226*, 1209.

Banks, G. T., Buck, K. W., Chain, E. B., Darbyshire, J. E., and Himmelweit, F. (1969), *Nature (London) 222*, 89.

Bishop, D. H. L., Claybrook, J. R., and Spiegelman, S. (1967), *J. Mol. Biol. 26*, 373.

Biswal, N., and Benyesh-Melnick, M. (1970), *Virology 42*, 1072.

Borst, P., and Weissmann, C. (1965), *Proc. Nat. Acad. Sci. U. S. 54*, 982.

Burdon, R. H., Martin, B. T., and Lal, B. M. (1967), *J. Mol. Biol. 28*, 357.

Caliguiri, L. A., and Tamm, I. (1968), *Virology 35*, 408.
Chrambach, A. (1966), *Anal. Biochem. 15*, 544.
Clarke, D. H., and Casals, J. (1955), *Proc. Soc. Exp. Biol. Med. 88*, 96.
Colby, C., and Duesberg, P. H. (1969), *Nature (London) 222*, 940.
Colby, C., Stollar, B. D., and Simon, M. I. (1971), *Nature New Biol. 229*, 172.
Cooper, H. L. (1968), *J. Biol. Chem. 243*, 34.
Cooper, H. L., and Kay, J. E. (1969), *Biochim. Biophys. Acta 174*, 503.
Daniel, V., and Littauer, U. Z. (1965), *J. Mol. Biol. 11*, 692.
De Maeyer, E., De Maeyer-Guignard, J., and Montagnier, L. (1971), *Nature New Biol. 229*, 109.
Ellis, L. F., and Kleinschmidt, W. J. (1967), *Nature (London) 215*, 649.
Faras, A. J., Wild, T. F., Mazurkiewicz, J., and Erikson, R. L. (1970), *Bacteriol. Proc.*, 182.
Field, A. K., Tytell, A. A., Lampson, G. P., and Hilleman, M. R. (1967), *Proc. Nat. Acad. Sci. U. S. 58*, 1004.
Frenster, J. H. (1969), *in* Handbook of Molecular Cytology, Lima-de-Faria, A., Ed., Amsterdam, North-Holland, p 252.
Friedman, R. M., Levy, H. B., and Carter, W. B. (1966), *Proc. Nat. Acad. Sci. U. S. 56*, 440.
Gallo, R. C., Yang, S. S., and Ting, R. C. (1970), *Nature (London) 228*, 927.
Gillam, I., Blew, D., Warrington, R. C., von Tigerstrom, M., and Tener, G. M. (1968), *Biochemistry 7*, 3459.
Gillam, I., Millward, S., Blew, D., von Tigerstrom, M., Wimmer, E., and Tener, G. M. (1967), *Biochemistry 6*, 3043.
Gillespie, D., and Spiegelman, S. (1965), *J. Mol. Biol. 12*, 829.
Guggenheim, M. A., Friedman, R. M., and Rabson, A. S. (1969), *Proc. Soc. Exp. Biol. Med. 130*, 1242.
Harel, L., and Montagnier, L. (1971), *Nature New Biol. 229*, 106.
Hiatt, H. H. (1962), *J. Mol. Biol. 5*, 217.
Jurale, C., Kates, J. R., and Colby, C. (1970), *Nature (London) 226*, 1027.
Katz, L., and Penman, S. (1966), *Biochem. Biophys. Res. Commun. 23*, 557.
Kay, J. E., and Cooper, H. L. (1969), *Biochim. Biophys. Acta 186*, 62.
Kimball, P. C., and Duesberg, P. H. (1971), *Virology 7*, 697.
Koffler, D., Carr, R. I., Agnello, V., Fiezi, T., and Kunkel, H. G. (1969), *Science 166*, 1648.
Lal, B. M., and Burdon, R. H. (1967), *Nature (London) 213*, 1134.
Lampson, G. P., Tytell, A. A., Field, A. K., Nemes, M. M., and Hilleman, M. R. (1967), *Proc. Nat. Acad. Sci. U. S. 58*, 782.
Lemke, P. A., and Ness, T. M. (1970), *J. Virol. 6*, 813.
Libonati, M., and Floridi, A. (1969), *Eur. J. Biochem. 8*, 81.
Lipsett, M. N. (1965), *Biochem. Biophys. Res. Commun. 20*, 224.
Lipsett, M. N. (1967), *J. Biol. Chem. 242*, 4067.
Loening, U. E. (1967), *Biochem. J. 102*, 251.
Marmur, J. (1961), *J. Mol. Biol. 3*, 208.
Martin, S. J., and Brown, F. (1967), *Biochem. J. 105*, 979.
Montagnier, L. (1968), *C. R. Acad. Sci., Ser. D, 267*, 1417.
Moulé, Y., and Landin, R. M. (1965), *Biochem. Biophys. Res. Commun. 20*, 491.
Perry, R. (1963), *Exp. Cell. Res. 29*, 400.
Perry, R. P. (1967), *Progr. Nucl. Acid. Res. Mol. Biol. 6*, 219.
Preiss, J. M., Dieckmann, U., and Berg, P. (1961), *J. Biol. Chem. 236*, 1748.
Rabson, A. S., O'Conor, G. T., Barton, S., Whang, J. J., and Legallais, F. Y. (1966), *Int. J. Cancer*, 89.
Revel, M., and Hiatt, H. H. (1964), *Biochem. Biophys. Res. Commun. 17*, 730.
Robertson, H. D., Webster, R. E., and Zinder, N. D. (1968), *J. Biol. Chem. 243*, 82.
Scherrer, K., and Darnell, J. E. (1962), *Biochem. Biophys. Res. Commun. 7*, 486.
Schur, P. H., and Monroe, M. (1969), *Proc. Nat. Acad. Sci. U. S. 63*, 1108.
Scolnick, E. M., Aaronson, S. A., Todaro, G. J., and Parks, W. P. (1971), *Nature (London) 229*, 318.
Shatkin, A. J. (1965), *Proc. Nat. Acad. Sci. U. S. 54*, 1721.
Steinberg, A. D., Baron, S., and Talal, N. (1969), *Proc. Nat. Acad. Sci. U. S. 63*, 1102.
Stern, R. (1970a), *Fed. Proc. (Fed. Amer. Soc. Exp. Biol.) 29*, 933.
Stern, R. (1970b), *Biochem. Biophys. Res. Commun. 41*, 608.
Stern, R., and Friedman, R. M. (1969), *J. Virol. 4*, 356.
Stern, R., and Friedman, R. M. (1970), *Nature (London) 226*, 612.
Stollar, V., and Stollar, B. D. (1970), *Proc. Nat. Acad. Sci. U. S. 65*, 993.
Temin, H. M., and Mizutani, S. (1970), *Nature (London) 226*, 1211.
Tytell, A. A., Lampson, G. P., Field, A. K., and Hilleman, M. R. (1967), *Proc. Nat. Acad. Sci. U. S. 58*, 1719.
Wilkies, N. M., and Smellie, R. M. S. (1968), *Biochem. J. 109*, 229.