JOURNAL OF VIROLOGY, May 1973, p. 761-774

Vol. 11, No. 5
Printed in U.S.A.

Quantitative Determination and Location of Newly Synthesized Virus-Specific Ribonucleic Acid in Chicken Cells Infected with Rous Sarcoma Virus

J. THOMAS PARSONS, JOHN M. COFFIN, RICHARD K. HAROZ, PETER A. BROMLEY,[1] AND CHARLES WEISSMANN

Institut für Molekularbiologie, Universität Zürich, Zürich, Switzerland

Received for publication 8 January 1973

A sensitive and quantitative nucleic acid hybridization assay for the detection of radioactively labeled avian tumor virus-specific RNA in infected chicken cells has been developed. In our experiments we made use of the fact that DNA synthesized by virions of avian myeloblastosis virus in the presence of actinomycin D (AMV DNA) is complementary to at least 35% of the sequences of 70*S* RNA from the Schmidt-Ruppin strain (SRV) of Rous sarcoma virus. Annealing of radioactive RNA (either SRV RNA or RNA extensively purified from SRV-infected chicken cells) with AMV DNA followed by ribonuclease digestion and Sephadex chromatography yielded products which were characterized as avian tumor virus-specific RNA-DNA hybrids by hybridization competition with unlabeled 70*S* AMV RNA, equilibrium density-gradient centrifugation in Cs_2SO_4 gradients, and by analysis of their ribonucleotide composition. The amount of viral RNA synthesized during pulse labeling with ^{3}H-uridine could be quantitated by the addition of an internal standard consisting of ^{32}P-labeled SRV RNA prior to purification and hybridization. This quantitative assay was used to determine that, in SRV-infected chicken cells labeled for increasing lengths of time with ^{3}H-uridine, labeled viral RNA appeared first in a nuclear fraction, then in a cytoplasmic fraction, and still later in mature virions. This observation is consistent with the hypothesis that RNA tumor virus RNA is synthesized in the nucleus of infected cells.

Production of RNA tumor viruses by permissive host cells is sensitive to inhibition by actinomycin D at all times following infection (3, 46). The sensitivity of virus production to this antibiotic has suggested that viral progeny RNA is transcribed from a viral DNA template (47). Although a considerable number of biological experiments support this model (for references see review by Temin, reference 48), direct biochemical evidence is lacking. The investigation of viral RNA replication in infected cells requires a method for the direct measurement of newly synthesized viral RNA. A number of techniques have been reported recently to measure overall levels of virus-specific RNA within tumor virus-infected cells or cellular fractions (10, 22, 29). These techniques are based on the conversion of radioactive virus-specific DNA, prepared in vitro with viral RNA-directed DNA polymerase (2, 49), into an RNA-DNA hybrid by viral RNA. However, these methods do not allow the detection and characterization of newly synthesized pulse-labeled viral RNA within the cell. We report here the development of an RNA-DNA hybridization method that allows the detection and quantitation of newly synthesized virus-specific RNA in chicken embryo fibroblasts infected with Rous sarcoma virus. The method has been used to determine the distribution of labeled viral RNA in Rous sarcoma virus-infected chicken cells following pulse-labeling with ^{3}H-uridine. Radioactive viral RNA was found initially in a nuclear fraction and only subsequently in a cytoplasmic fraction. The time course of appearance of viral RNA in the

[1] Present address: Institut de Biologie Moléculaire, Université de Genève, Genève, Switzerland.

nuclear and cytoplasmic fractions is consistent with the proposal (48) that viral RNA is synthesized within the nucleus and later transported to the cytoplasm.

MATERIALS AND METHODS

Cells and viruses. Primary cultures of chicken embryo fibroblasts were prepared by trypsin treatment of 10- to 11-day-old White Leghorn embryos (Haager Brutzentrale, Haag, Switzerland). Cultures were transferred every 5 to 7 days. After treatment with 0.08% trypsin (National Biochemicals Co., Cleveland, Ohio) 0.01 M sodium phosphate (pH 7.0) and 0.15 M NaCl (PBS) for 5 min at 37 C, the cells were removed from the dishes into an equal volume of modified Eagle medium (obtained as a $\times 10$ concentrate from Flow Laboratories, Inc., Rockville, Md.) with 10% tryptose phosphate broth (ET medium) and 5% calf serum (Flow Laboratories, Inc.). The cells were centrifuged, resuspended in 1 ml per culture of ET medium with 5% serum, and plated at a concentration of 2×10^6 cells per 100-mm culture dish (A/S Nunc, Roskilde, Denmark) in 10 ml of ET medium. The next day, 0.5 ml of calf serum was added to all cultures. The medium (ET medium with 5% calf serum) was replaced every 3 to 5 days. All cells were grown at 37 C in a 5% CO_2 atmosphere. Secondary cultures from all embryos were tested for resistance to Rous sarcoma virus strains of subgroups A, B, C, and D (15). Only cultures susceptible to all these subgroups were used for further experiments.

Cultures of chicken cells transformed by Schmidt-Ruppin strain of Rous sarcoma virus were prepared as follows. Primary cultures (or subsequent passages) were treated with trypsin as above, and transferred to ET medium. Schmidt-Ruppin virus (SRV) was added at a multiplicity of approximately 0.1 focus-forming unit per cell. The cells were immediately plated at a concentration of 2×10^6 cells per 100-mm culture dish in 10 ml of ET medium, and 0.5 ml of calf serum was added to each culture the following day. Within 3 days after infection, the majority of cells in SRV-infected cultures appeared transformed, as judged by their altered morphology. The medium on transformed cultures was changed to ET medium with 5% calf serum every 2 to 4 days. Confluent transformed cultures were maintained in ET medium with 0.5% calf serum. SRV-transformed chicken cells were subcultured every 5 to 7 days after dissociation by trypsin and plated at a concentration of 2×10^6 cells per 100-mm culture dish in 10 ml of ET medium with 5% calf serum. Confluent cultures of transformed cells were used for further experiments 7 or more days after infection. Where stated, parallel infected and uninfected cells were prepared by infecting one-half of the cultures at the time of transfer and performing all further transfers and medium changes at the same time for both types of cells.

In all experiments reported here, SRV of subgroup D (1) was used. Stocks of this virus did not contain an excess of non-transforming virus capable of interfering with SRV in an end-point interference assay (41). SRV was harvested daily from confluent infected cultures, starting about 7 days after infection. SRV was concentrated from tissue culture fluids by differential centrifugation (9). For the preparation of RNA, concentrated SRV was used without further purification.

Avian myelobastosis virus, BAI strain A (AMV) was originally obtained from J. Beard. White Leghorn chickens were infected at 1 day of age (6), and plasma was harvested from diseased birds at 14 to 21 days. Pooled plasma was clarified by centrifugation at $10,000 \times g$, and the virus was concentrated by centrifugation at $78,000 \times g$ for 30 min in a Beckman type 65 rotor. Concentrated AMV was purified by equilibrium centrifugation in 15 to 65% sucrose density gradients (9). To obtain ^{32}P-labeled AMV, chicken embryo fibroblast cultures were infected with 0.5 ml of AMV-containing plasma diluted 1:40 in ET medium as described for SRV-infected cultures; these were transferred once before labeling.

Isolation of high-molecular-weight viral RNA. All steps in the isolation of nucleic acid were carried out by using nuclease-free glassware, heated 4 h at 140 C, and autoclaved buffers. Concentrated AMV from approximately 30 ml of plasma was suspended in 5.0 ml of 0.1 M NaCl, 50 mM Tris-hydrochloride (pH 7.5), and 10 mM EDTA (virus suspension buffer). Sodium dodecyl sulfate (SDS) was added to a final concentration of 0.5%, and the solution was extracted with an equal volume of water-saturated phenol for 5 min at room temperature. The phases were separated by centrifugation at $3,000 \times g$ at room temperature, and the phenol was reextracted with an equal volume of $0.1 \times$ SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). The RNA was precipitated from the combined aqueous phases with 2.5 volumes of cold ethanol, collected by centrifugation at $17,000 \times g$, and dissolved in 0.1 ml of $0.1 \times$ SSC. To isolate high-molecular-weight viral RNA, the RNA was centrifuged at 4 C in a 5 to 23% sucrose gradient (in 50 mM Tris-hydrochloride, 5 mM EDTA, pH 7.5) for 70 min at 55,000 rpm in a Beckman SW 65 rotor. Under these conditions, the high-molecular-weight AMV RNA sedimented 2.2 times as far as Qβ RNA added as an internal standard (30S; reference 18), and will be designated as 70S viral RNA. The RNA in the 70S peak was precipitated with 2 volumes of ethanol and 1/20 volume of 20% potassium acetate (pH 5.4). The RNA was collected by centrifugation and dissolved in $0.1 \times$ SSC. Virus from 30 ml of infected chicken plasma yielded approximately 10 to 15 μg of 70S RNA. ^{32}P- or ^{3}H-labeled RNA was prepared by a procedure similar to that described above with the exception that, after phenol extraction and ethanol precipitation, low-molecular-weight material was removed by chromatography on a column (10×0.5 cm) of Sephadex G-50 (Pharmacia AB, Uppsala, Sweden) in $0.1 \times$ SSC. The excluded RNA was then centrifuged in a 5 to 23% sucrose gradient as described above. Qβ RNA was prepared as described (51).

Labeling of cell cultures with ^{32}P-phosphate or ^{3}H-uridine. The medium on confluent cultures was replaced by 10 ml (per 100-mm culture dish) of phosphate-free medium 199 (Wellcome Reagents

Ltd., Beckenham, England) containing 5% dialyzed calf serum and 0.5% dimethylsulfoxide (14). Approximately 12 h later the medium was replaced by 6 ml of the same medium containing 50 μCi of ^{32}P-phosphate (carrier free; Eidg. Institut für Reaktorforschung, Würenlingen, Switzerland) per ml. ^{32}P-labeled cells were harvested after trypsin treatment and 24 h of labeling, washed twice with PBS, and stored frozen. For the preparation of ^{32}P-labeled virus (AMV or SRV), virus-infected cultures were labeled as above; the medium was collected after 24 h, replaced with 6 ml of the same ^{32}P-phosphate-containing medium and harvested once more after another 24 h of labeling. Virus was recovered from the combined supernatant extracts as described above. RNA from virus labeled under such conditions had a specific activity of about 4×10^5 counts per min per μg. Viral RNA of higher specific activity ($> 10^6$ counts per min per μg) was prepared from virus grown by the same procedure with 200 μCi of carrier-free ^{32}P-phosphate per ml of medium. Virus labeled with ^{3}H-uridine was obtained from infected cells grown in 5 ml of Eagle medium containing 5% calf serum and 250 μCi of [5-^3H] uridine per ml (29 Ci/mmol; Amersham, Buckinghamshire, England). The medium on such cultures was changed every 24 h for 1 to 3 days, and virus was prepared from the pooled harvests. The specific activity of the RNA was $> 10^6$ counts per min per μg.

For the preparation of ^{3}H-uridine-labeled cell fractions, cultures were incubated for the times indicated with 3 ml per 100-mm culture dish of Eagle medium containing 5% calf serum and 0.4 mCi of [5-^3H]uridine per ml. Labeling was stopped by washing the cultures twice with cold PBS, the cells were suspended after trypsin treatment, counted, and washed with 1 ml of PBS per culture.

Fractionation into "nuclear" and "cytoplasmic" fractions. Trypsin-treated, washed cells were suspended in a solution containing 10 mM Tris-hydrochloride (pH 7.4), 10 mM NaCl, and 1.5 mM $MgCl_2$ (RSB), at a concentration of 2.5×10^7 cells per ml. After 10 min at 4 C, the cells were centrifuged and resuspended in 0.2 ml of RSB per culture (approximately 10^7 cells). A step gradient was prepared in a centrifuge tube (7 by 1 cm) by layering successively (i) 0.5 ml of 65% sucrose in RSB, (ii) 2.0 ml of 10% sucrose in 10 mM Tris-hydrochloride (pH 7.4), 0.2 M NaCl, and 1.5 mM $MgCl_2$, and (iii) 0.2 ml of 1% Triton X-100 in 0.2 M NaCl, 10 mM EDTA. The cell suspension was layered onto the gradient. After 10 min at 4 C the gradient was centrifuged at $7,000 \times g$ for 20 min at 4 C in a Sorvall HB-4 rotor. The upper layers down to 2 mm above the 65% sucrose layer were collected in one portion, and the particulate fraction sedimenting to the interface of the 10% sucrose and the 65% sucrose layers was drawn off with a Pasteur pipette.

The interface material consisted of whole and disrupted nuclei with less than 1% intact cells and will be referred to as the "nuclear" fraction. The supernatant material will be called the "cytoplasmic" fraction.

Purification of cellular RNA. Cells (5×10^6 to 10×10^6) were suspended in 1.5 ml of 0.1 $\times$ SSC, and SDS and Pronase (grade B, Calbiochem, Los Angeles, Calif.; predigested for 90 min at 37 C) were added to final concentrations of 0.5% and 0.01%, respectively. The suspension was incubated at 37 C for 30 min and then extracted at room temperature with two volumes of water-saturated phenol. The phenol phase was re-extracted with 0.5 volume of 0.1 $\times$ SSC; 0.5% SDS and the nucleic acids were precipitated with 2.5 volumes of ethanol. The precipitate was dissolved in 0.2 ml of 0.1 $\times$ SSC and chromatographed on a Sephadex G-100 column (0.5×10 cm) in 0.1 $\times$ SSC to remove low-molecular-weight material.

The fractions containing the excluded nucleic acid (0.5 to 1 ml) were adjusted to 0.5 M NaCl, 50 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Tris-hydrochloride (pH 7.4; HS buffer; reference 37). Electrophoretically purified DNase (Worthington Biochemical Corp., Freehold, N.J.) was added to a final concentration of 50 μg per ml, and the mixture was incubated at 37 C for 15 min. The solution was then extracted with phenol, and the nucleic acid was precipitated with ethanol, redissolved, and chromatographed on Sephadex G-100 as described above. The excluded nucleic acid (0.5 ml) was heated in a sealed tube at 100 C for 5 min to denature any RNA-DNA hybrids present, cooled in ice, adjusted to the salt concentration of HS buffer, and treated again with 50 μg of DNase per ml for 30 min at 37 C. Phenol extraction, ethanol precipitation, and chromatography on Sephadex G-100 were repeated as described above. The excluded nucleic acid (0.5 ml, 20–40 μg per ml) was adjusted to 2 $\times$ SSC and annealed for 16 h at 66 C to convert any complementary RNA strands present into a double-helical form. To separate single- from double-stranded RNA, the annealed nucleic acid was adjusted to 35% ethanol and applied to a 5-ml cellulose column (Whatman CF 11, W.R. Balstone, Ltd., Maidstone, England; reference 19). The column was washed with 20 ml of 35% ethanol in 50 mM Tris-hydrochloride (pH 7.4), 0.10 M NaCl, and 10 mM EDTA (STE). Single-stranded RNA was eluted with 10 ml of 15% ethanol in STE, and double-stranded RNA was eluted with 10 ml of STE. The 15% ethanol fractions which contained 90 to 95% of the input nucleic acid were pooled, and the nucleic acid was precipitated with ethanol. The precipitate was collected by centrifugation and dissolved in 0.1 $\times$ SSC, and the specific radioactivity was determined. Approximately 50% of labeled cell RNA was recovered by this procedure. The purified RNA obtained by this procedure was analyzed by hybridization with viral DNA as described below. RNA was prepared from cell fractions by the same procedure except that all fractions were adjusted to 2.5 ml RSB before the initial phenol extraction.

For quantitation of ^{3}H-uridine-labeled RNA in the course of purification, 25-μliter samples were spotted on Whatman 541 filter paper which had been pretreated with 25 μliters of 0.1 M sodium pyrophosphate. The filters were washed once in cold 10% trichloroacetic acid containing 1% sodium pyrophosphate for 10 min, twice in cold 5% trichloroacetic acid for 5 min, and twice in cold 96% ethanol for 5 min. Dried filters were counted by a modification of the method of Birnbaum (4). The filters were treated for

30 min at room temperature with 0.5 ml of NCS solubilizer (Amersham-Searle Corp., Arlington Heights, Ill.) diluted 1 to 3 in scintillation fluid. Five milliliters of scintillation fluid containing 0.1% glacial acetic acid were added, and the samples were counted. This procedure solubilized ^{3}H-labeled RNA almost completely, as indicated by the fact that more than 90% of the radioactivity remained in the vial after removing the filter. Counts were corrected for relative quenching by recounting the samples after the addition of approximately 1.5×10^5 counts per min of ^{3}H-uridine as an internal standard. ^{3}H counting efficiency did not vary by more than 10% between samples in one experiment.

Synthesis of virus-specific DNA. Virus-specific DNA was prepared by using endogenous RNA-directed DNA polymerase from purified AMV. Reaction mixtures contained in 1 ml: 40 μmol of Tris-hydrochloride (pH 8.0), 5 μmol of $MgCl_2$, 0.1 μmol each of dATP, dGTP, and dCTP, 30 μmol of NaCl, 5 μmol of dithiothreitol, 0.1 μmol of labeled dTTP (either [5^3*H*]dTTP, Amersham, or α-^{32}P-dTTP; specific activities, 2,000–50,000 counts per min per nmol), 0.1% Triton X-100, 25 μg of actinomycin D (Merck, Sharpe and Dohme Research Lab., West Point, Pa.), and purified AMV (1.0–1.5 mg of viral protein; reference 20). The incorporation of labeled dTMP was linear for 40 to 60 min at 37 C. DNA synthesis was routinely carried out for 60 min, and the reaction was stopped by the addition of SDS to a final concentration of 0.5%. The reaction mixture was treated with 250 μg of Pronase per ml for 15 min at 37 C, and then extracted with an equal volume of water-saturated phenol. The phenol phase was re-extracted with one volume of 0.1 × SSC and the combined aqueous phases were mixed with 2.5 volumes of ethanol. The precipitated nucleic acids were collected by centrifugation, dissolved in 0.1 × SSC, and chromatographed on a Sephadex G-50 column (10 × 0.5 cm) in 0.1 × SSC to remove unreacted substrates. The excluded nucleic acid was precipitated with ethanol and dissolved in 0.1 ml of 0.1 N KOH. After 20 to 24 h at room temperature the solution was neutralized with HCl and chromatographed on a Sephadex G-100 column (10 × 0.5 cm) in 0.1 × SSC. The labeled DNA from the excluded region of the column was pooled for use in hybridization experiments. Virus purified from about 40 ml of infected chicken plasma, containing approximately 25 mg of virus protein, yielded about 8 to 10 μg of labeled DNA. The virus-specific nature of this DNA was established by the following experiment. ^{32}P-labeled AMV DNA (2,000 counts per min, specific activity 300,000 counts per min per nmol) was annealed for 2 h with either 1.0 μg of 70*S* AMV RNA or 1.4 μg of Qβ RNA. The samples were centrifuged to equilibrium in Cs_2SO_4 density gradients. More than 88% of the labeled DNA which had been annealed with AMV RNA banded at a density greater than 1.53 g per cm^3 (10, 13, 40), whereas 90 to 95% of the DNA annealed with Qβ RNA banded at a density lower than 1.51 g per cm^3. These data, in conjunction with the known ability of actinomycin D to block double-stranded DNA synthesis by tumor virus RNA-directed DNA polymerase (32, 33), support the conclusion that the preparation consisted largely of AMV minus-strand DNA.

RNA-DNA hybridization. RNA-DNA hybridization was routinely carried out in 50 μliters of 2 × SSC at 66 C for 14 to 16 h. After annealing, the samples were adjusted to 100 μliters with 2 × SSC and 25 μg of *Escherichia coli* RNA; 4 μg of RNase A (Worthington Biochemical Corp.; heated at 90 C for 10 min) and 2 μg of RNase T_1 (40 units) were added. The samples were incubated for 30 min at 37 C, and SDS was added to a final concentration of 0.2%. The samples were passed through Sephadex G-100 columns (10 × 0.5 cm) equilibrated with 2 × SSC containing 0.2% SDS. Fractions (0.1 ml each) were collected, and acid-insoluble radioactivity was determined. Twenty micrograms of yeast RNA and 0.1 ml of 60% trichloroacetic acid were added to each fraction. After 10 min at 0 C, the samples were collected on membrane filters (Millipore Corp.), washed with 6% trichloroacetic acid, dried, and counted in a toluene-2,5-diphenyloxazole-1,4-bis-2-(5 phenyloxazoly) benzene scintillation fluid. Where necessary, a correction for spillover of ^{32}P in the ^{3}H channel (2.9%) was subtracted. Recovery of labeled DNA from the hybridization assays was 80% or greater.

Cs_2SO_4 density gradient centrifugation. Cs_2SO_4 (British Drug House Ltd., Poole, England) was recrystallized from boiling water before use. Samples were centrifuged in 3 ml of 10 mM Tris-hydrochloride (pH 7.5) and 1 mM EDTA with Cs_2SO_4 added to a density of 1.54 g per cm^3.

After centrifugation in an SW65 rotor for 60 h at 38,000 rpm at 15 C, the gradients were fractionated from the bottom. The density of each fraction was determined by weighing a 100-μliter sample; absorbance was measured at 260 nm. After the addition of 20 μg of yeast RNA, the nucleic acid was precipitated with trichloroacetic acid and counted. Recovery of labeled RNA and DNA from such gradients was routinely 70 to 90%.

Base analysis. Samples of ^{32}P-labeled RNA or ^{32}P-labeled RNA-DNA hybrid were mixed with 100 μg of Qβ RNA as carrier and hydrolyzed in 1 ml of 10% piperidine for 90 min at 100 C in a sealed tube (5). The hydrolysate was evaporated to dryness, and the residue was taken up in 50 μliters of water. The hydrolysate was spotted on Whatman 3MM paper and 2′,3′-nucleoside monophosphates were separated by electrophoresis at 3,000 V for 1 h in 5% acetic acid, 0.5% pyridine, and 0.5 mM EDTA (pH 3.5). The spots corresponding to the four ribonucleoside monophosphates were located with a UV lamp, cut out, and counted in a liquid scintillation counter. Blank areas of the paper were also cut out and counted.

Preparation of α-^{32}P-TTP. A mixture of α-^{32}P-5′- and 3-TMP was synthesized chemically by using the procedure of Symons (44). α-^{32}P-5′-TMP was converted to α-^{32}P-5′-TTP with a crude mononucleotide kinase preparation from *E. coli* (step 2 preparation in the purification of Qβ replicase as described by Eoyang and August, reference 17). α-^{32}P-5′-TTP was separated from mono- and dinucleotides by chromatography on a Dowex-1-formate column (10 × 0.5 cm), by using an ammonium formate gradient from 1 M formic acid, 0.45 M ammonium formate to 3 M formic acid, and 1.35 M ammonium formate. Fractions containing α-^{32}P-5′-TTP were passed through a Dowex-50-H^+ column and evaporated to dryness. The re-

sultant α-^{32}P-5'-TTP was neutralized with NH_4OH and adjusted to the desired concentration. Routinely, α-^{32}P-5'-TTP with a specific activity of 30×10^6 to 35×10^6 counts per min per nmol was prepared; 25 to 30% of the ^{32}P-phosphate was recovered in the triphosphate.

RESULTS

Hybridization of RNA to short DNA molecules (such as the product of tumor virus RNA-directed DNA polymerase, references 13, 33) entails a number of problems. Short DNAs are neither bound nor retained quantitatively (21, 34) on cellulose nitrate filters, as required for hybridization procedures such as those described by Gillespie and Spiegelman (21). Similarily, RNA-DNA hybrids formed in solution (36) cannot be quantitatively retained on cellulose nitrate filters unless the hybrid contains overhanging single-stranded DNA regions (23, 43). Therefore most studies which utilized RNA tumor virus-specific DNA in hybridization experiments (10, 13) have used hybridization in solution followed by a determination of acid-insoluble radioactivity after RNase digestion. This procedure, however, gives relatively high backgrounds, since RNase digestion under conditions necessary to preserve RNA-DNA hybrids leaves as much as 0.2% of a single-stranded viral RNA such as Qβ or R17 RNA (18), or 5% of SRV RNA (10, 13) acid insoluble.

To detect low levels of labeled virus-specific RNA in preparations of labeled RNA from infected cells, it was necessary to develop a sensitive and quantitative hybridization assay. Labeled RNA is annealed with virus-specific minus-strand DNA prepared in vitro with virion RNA-directed DNA polymerase in the presence of actinomycin D (32, 33). The annealed mixture is digested with RNase A and T_1, and the labeled hybrid is separated from degraded RNA by chromatography on Sephadex G-100. Since most of the RNase-resistant, nonhybridized RNA is too small to be excluded on Sephadex G-100, the chromatographic analysis gives backgrounds 5- to 10-fold lower than those obtained by acid precipitation. The sensitivity of the assay is thereby increased accordingly. Quantitation is attained by the use of an internal standard, as described below.

In all the studies reported in this paper we have utilized SRV, a biologically well-defined virus (1, 15). Since the hybridization assays required relatively large amounts of minus-strand DNA (0.03–0.1 μg per assay), it was desirable to use the DNA synthesized by the endogenous RNA-directed DNA polymerase of AMV, a virus that can be obtained in large quantity. We, therefore, first examined the extent of homology between AMV DNA and SRV RNA.

^{32}P-labeled 70S SRV RNA was annealed with a >10-fold excess of ^{3}H-labeled AMV DNA, digested with RNase, and chromatographed through Sephadex G-100. As shown in the elution profiles of Fig. 1A, the ^{3}H-AMV DNA and part of the ^{32}P-RNA emerged as a peak in a position similar to that of high-molecular-weight ^{3}H-labeled chicken cell DNA (Fig. 1B) that was well removed from the peak of ^{32}P-labeled almost completely acid-soluble digestion products. In the absence of AMV DNA, no

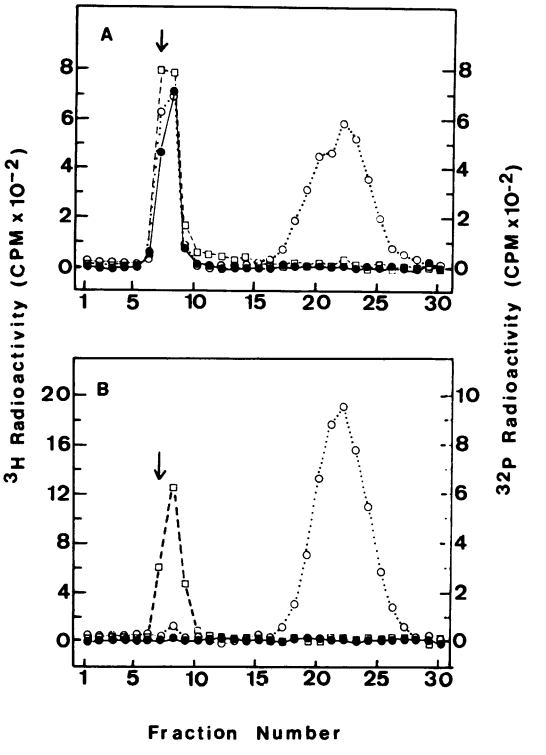

FIG. 1. *Hybridization of 70S ^{32}P-SRV RNA to ^{3}H-AMV DNA: detection of hybrids by Sephadex chromatography. Hybridization conditions were described in Materials and Methods. 70S ^{32}P-SRV RNA (4,000 counts/min; specific activity >10^6 counts/min/μg) was annealed (A) with 0.04 μg of ^{3}H-AMV DNA (specific activity 45,000 counts/min/μg) or (B) with no added DNA. All samples were treated with RNase and analyzed by chromatography on Sephadex G-100. Total ^{32}P (○) and ^{3}H (□) radioactivity and acid-insoluble ^{32}P-radioactivity (●) of each fraction are plotted. In sample B, 2,500 counts/min of ^{3}H-labeled chicken cell DNA were added as marker just prior to chromatography. The arrows indicate the position of the peak fraction of Dextran blue 2000 (Pharmacia Fine Chemicals, Inc.), chromatographed separately on the same columns, under the same conditions as above.*

acid-insoluble ^{32}P-RNA (less than 0.5% of input) was detected in the excluded column volume (Fig. 1B). The elution profile of ^{3}H-AMV DNA was not significantly altered by hybridization with ^{32}P-SRV RNA (data not shown).

The hybridization efficiency of ^{32}P-AMV RNA and ^{3}H-SRV RNA to AMV DNA was compared by annealing the three components together and determining RNase-resistant RNA as above. The AMV DNA was, for technical reasons, also ^{32}P-labeled, however at a 10^4-fold lower specific activity than the ^{32}P-AMV RNA, so that it contributed no significant radioactivity. In Fig. 2, the proportion of labeled RNA recovered in an RNase-resistant form is plotted against the amount of input AMV DNA. It is apparent that with the first increments of DNA, the proportion of viral RNA converted into hybrid rose steeply up to a value of about 20% for SRV RNA and 50% for AMV RNA, and then increased with a 30-fold reduced slope. These results suggest that the major part of AMV DNA is complementary to about 50% of the AMV RNA and 20% of the SRV RNA sequences, whereas only a small fraction of the DNA is complementary to the remainder of the RNA. By the criterion of this assay SRV RNA formed stable hybrids with AMV minus-strand DNA at somewhat less than half the efficiency of AMV RNA. Hybridization of 70S SRV RNA purified from a clonal isolate of SRV gave a similar extent of hybridization (data not given), showing that the homology observed was, in all likelihood, not due to a contamination of the SRV stock by a leukosis virus. In control experiments, no hybridization (less than 0.1%) of either AMV RNA or SRV RNA was observed when T_4 DNA was substituted for AMV DNA (Fig. 2). In another experiment less than 0.1% of labeled Qβ RNA hybridized with AMV DNA (data not shown).

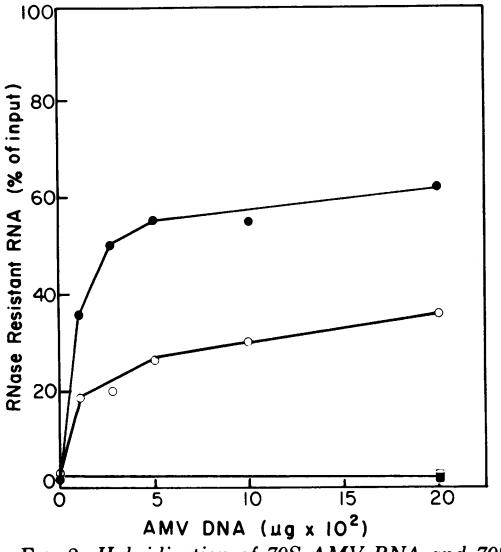

FIG. 2. *Hybridization of 70S AMV RNA and 70S SRV RNA to increasing amounts of AMV DNA. 70S ^{32}P-AMV RNA (●, 3,400 counts/min; specific activity > 1 × 10^6 counts/min/μg) and 70S ^{3}H-SRV RNA (○, 770 counts/min; specific activity > 1 × 10^6 counts/min/μg) were mixed and annealed with increasing amounts of AMV DNA (labeled with ^{32}P, specific activity 60 counts/min/μg). All samples were analyzed as described in the legend to Fig. 1, and the amount of acid-insoluble labeled RNA eluting in the excluded volume (0.3–0.6 column volumes corresponding to fractions 6–10 in Fig. 1) was determined. In each case the small amount of ^{32}P-radioactivity contributed by the AMV DNA has been subtracted. As a control hybridization, a mixture of ^{32}P-AMV RNA (■) and ^{3}H-SRV RNA (□) was annealed with 0.15 μg of sonicated denatured T_4 DNA.*

To further substantiate the conclusion that a specific hybrid was being formed between SRV RNA and AMV DNA, a hybridization-competition experiment was performed. A mixture of ^{3}H-labeled SRV 70S RNA and ^{32}P-labeled AMV 70S RNA was annealed with a constant amount of AMV minus-strand DNA in the presence of increasing amounts of unlabeled AMV 70S RNA. Both labeled RNAs were diluted out of the hybrid to the same extent within the limits of experimental accuracy (Fig. 3). No dilution effect was obtained with unlabeled Qβ RNA. These experiments show that AMV DNA was hybridizing to SRV RNA sequences common to both SRV and AMV RNA.

The RNase-resistant product obtained by annealing ^{32}P-SRV RNA with AMV DNA was further characterized as an RNA-DNA hybrid by equilibrium centrifugation in a Cs_2SO_4 density gradient. The ^{32}P-radioactivity banded at 1.51 g per cm^3, a buoyant density typical of an RNA-DNA hybrid (See Fig. 6B; references 8, 45). ^{32}P-labeled SRV RNA mixed, but not annealed, with AMV DNA banded at approximately 1.65 g per cm^3 (See Fig. 6C), as did the unlabeled Qβ RNA added to all gradients as marker. The ^{32}P-moiety of the hybrid had a ribonucleotide composition similar to that of total SRV RNA, showing that the hybridized sequences were not due to an odd fraction of the viral RNA (Table 1).

Detection of labeled SRV RNA in SRV-infected chicken embryo cells. The hybridization assay developed above was used to search for radioactive SRV RNA in normal and SRV-infected chicken cells.

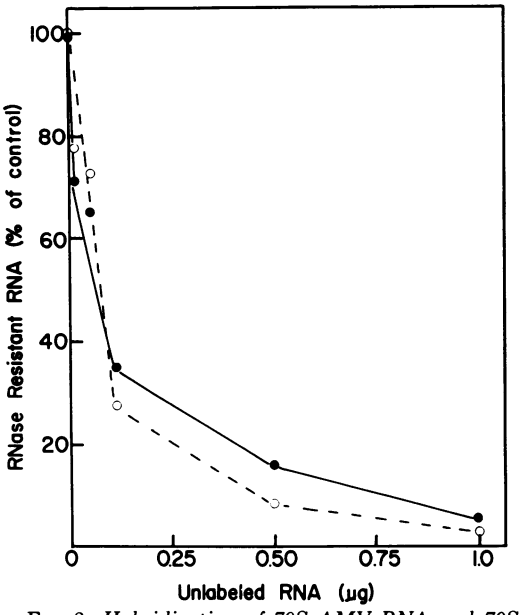

FIG. 3. *Hybridization of 70S AMV RNA and 70S SRV RNA to AMV DNA: competition with unlabeled 70S AMV RNA. 70S ^{32}P-AMV RNA (●, 1,200 counts/min; specific activity > 1 × 10^6 counts/min/μg) and 70S ^{3}H-SRV RNA (○, 2,200 counts/min; specific activity > 1 × 10^6 counts/min/μg) were mixed and annealed with 0.025 μg of ^{32}P-AMV DNA (16 counts/min/μg) and increasing amounts of unlabeled 70S AMV RNA. Hybrid formation was determined as in Fig. 2. All values are given in percent of the controls obtained by hybridization in the absence of unlabeled AMV RNA (17% for ^{3}H-SRV RNA and 24% for ^{32}P-AMV RNA; these values are lower than in Fig. 3 because 10-fold less AMV DNA was used for the hybridization).*

Nucleic acids were extracted from cells labeled for 24 h with ^{32}P-phosphate. The preparations were treated with DNase, and degraded DNA was separated from intact nucleic acid by Sephadex chromatography. To destroy residual DNA and RNA-DNA hybrids, the nucleic acid was heat-denatured, treated once more with DNase and purified by Sephadex chromatography. To remove double-stranded RNA known to occur in chicken cells (11, 35), the preparation was chromatographed on cellulose by the procedure of Franklin (19). It will be shown below that this purification procedure did not entail specific losses of either labeled host or viral RNA. Hybridization of the ^{32}P-labeled RNA preparations was carried out with increasing amounts of AMV DNA. Figure 4 shows the results obtained with two independent RNA preparations from infected cells, and with one from uninfected cells. At the highest level of input DNA, 0.08% and 0.09%, respectively, of the radioactive RNA from infected cells was hybridized. The corresponding value for the RNA from uninfected cells, 0.004% above a background of 0.007%, did not significantly differ from zero. Since a clear saturation plateau was not reached (Fig. 4), these hybridization values must be considered as minimal values.

Before proceeding to render the assay for virus-specific RNA quantitative (see following section), it was necessary to demonstrate that the material we were measuring indeed was a virus-specific RNA-DNA hybrid. Figure 5 shows a competition-hybridization experiment in which a mixture of ^{3}H-labeled RNA from SRV-infected chicken cells and ^{32}P-70S SRV RNA was annealed with AMV DNA in the presence of increasing amounts of unlabeled 70S AMV RNA. The extent of hybridization of both RNAs was reduced to about half by the addition of 0.05 μg, and to the background value by 1 μg of unlabeled 70S AMV RNA. No inhibition of hybrid formation resulted upon addition of 1 μg of unlabeled Qβ RNA. In a further experiment, ^{32}P-labeled infected-cell RNA was annealed with AMV DNA and the RNase-resistant material was purified and centrifuged to equilibrium in a Cs_2SO_4 density gradient. The major part of the ^{32}P radioactivity was found in a band with a mean buoyant density of 1.51 to 1.52 g

TABLE 1. *^{32}P-ribonucleotide composition of ^{32}P-labeled RNA from SRV, and of the products obtained by annealing AMV DNA with ^{32}P-labeled RNA from SRV or SRV-infected cells*[a]

Source of RNA	Mol % ^{32}P in			
	2',3' CMP	2',3' AMP	2',3' GMP	2',3' UMP
SRV 70S[b]	22.4	25.5	27.9	24.1
SRV RNA annealed with AMV DNA[c]	20.1	25.7	29.0	25.2
RNA from SRV-infected cells annealed with AMV DNA[c]	23.9	26.7	27.2	22.2

[a] The preparations were digested with piperidine and analyzed by paper electrophoresis as described in Materials and Methods. More than 80% of the radioactivity of the starting material was recovered in nucleoside monophosphates. Less than 5% of the radioactivity applied to the paper was located at the origin of the electropherograms. Each analysis was carried out on 1,400 counts/min or more.

[b] Prepared as described in Materials and Methods.

[c] Prepared as described in the legend of Fig. 6.

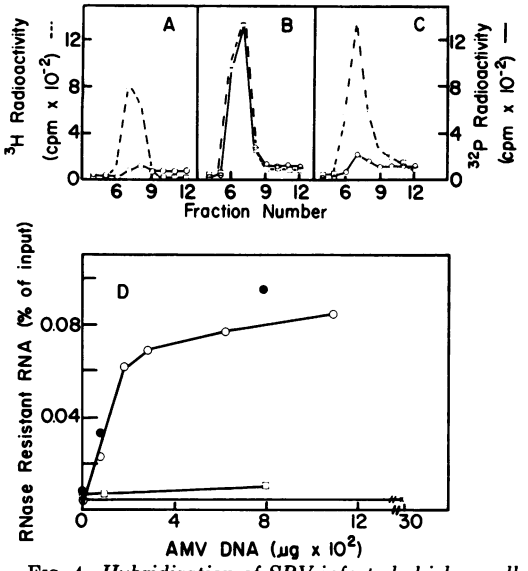

FIG. 4. *Hybridization of SRV-infected chicken cell RNA to AMV DNA. ^{32}P-labeled RNA was isolated from SRV-infected chicken cells and uninfected cells labeled in parallel for 24 h. A constant amount of ^{32}P-RNA was annealed with increasing amounts of ^{3}H-AMV DNA, or T_4 DNA as a control. All experimental details are as described in the Methods section and legend to Fig. 2. (A), (B), and (C) show acid-insoluble radioactivities in fractions from the Sephadex G-100 columns. The annealing mixture contained (A) no DNA (1,700 counts/min of ^{3}H-chicken embryo fibroblast DNA was added to the hybridization mixture after annealing, as a marker) and ^{32}P-RNA from SRV-infected chicken cells (2.9 × 10^6 counts/min, 4.1 μg); (B) 0.08 μg ^{3}H-AMV DNA (2,800 counts/min) and ^{32}P-RNA from SRV-infected chicken cells (2.9 × 10^6 counts/min, 4.1 μg); and (C) 0.08 μg AMV DNA (2,800 counts/min) and ^{32}P-RNA from uninfected chicken cells (5.2 × 10^6 counts/min, 17 μg). ^{32}P-RNA (○); ^{3}H-DNA (□). (D) The fraction of ^{32}P-RNA converted into hybrid as function of the DNA concentration in the annealing reaction. The assay was carried out as above using the ^{32}P-RNA purified from uninfected chicken cells (□) or SRV-infected chicken cells (●) described above, or a separate preparation of ^{32}P-RNA from SRV-infected chicken cells (○). A control annealing experiment was carried out with ^{32}P-RNA purified from SRV-infected cells and 0.3 μg of T_4 DNA (×).*

per cm^3 (Fig. 6A), the same position in which the ^{32}P-SRV RNA-AMV DNA hybrid was found (Fig. 6B). The small amount of ^{32}P label banding at a density around 1.63 g per cm^3 (Fig. 6A) could be due to residual double-stranded host cell RNA.

The base composition of the ^{32}P-RNA moiety of the hybrid isolated after annealing ^{32}P-labeled RNA from SRV-infected cells with AMV DNA was similar to that of the hybrid obtained after annealing ^{32}P-SRV RNA and AMV DNA (Table 1) as well as to that of 70S SRV RNA itself. This excludes the possibility that the RNase-resistant RNA consists of poly A sequences such as those which are known to occur in eukaryotic mRNA and RNA tumor virus RNA (12, 25, 27, 28).

Quantitative analysis of SRV RNA synthesis in SRV-infected cells. The hybridization experiments presented so far (Fig. 2, Fig. 4) suggest that a plateau value for hybrid formation could only have been reached at a very large excess of virus-specific minus-strand DNA over viral RNA. Furthermore, the amount of radioactive virus-specific RNA synthesized under certain experimental conditions may be small compared to the pre-existent, unlabeled

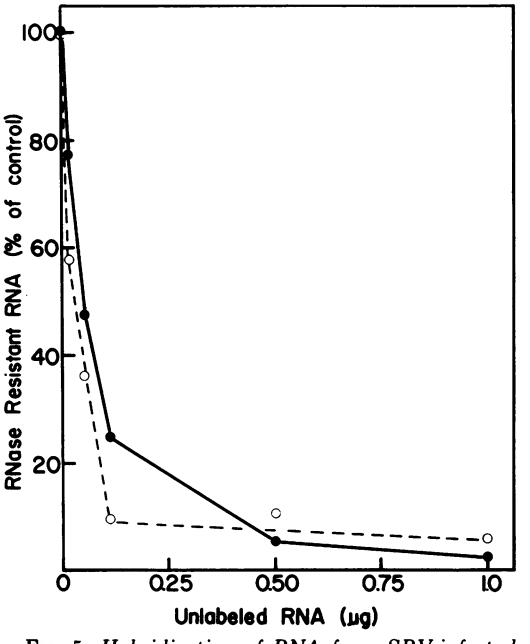

FIG. 5. *Hybridization of RNA from SRV-infected chicken cells to AMV DNA; competition with unlabeled 70S AMV RNA. A mixture of ^{3}H-labeled RNA from SRV-infected chicken cells (3.7 × 10^5 counts/min; 4.5 μg) and ^{32}P-labeled 70S SRV RNA (3.2 × 10^3 counts/min; specific activity > 1 × 10^6 counts/min/μg) was annealed with 0.05 μg of ^{32}P-labeled AMV DNA (specific activity 16 counts/min/μg) and increasing amounts of unlabeled 70S AMV RNA. The amount of ^{3}H (○) and ^{32}P (●) hybrid formed was determined. Background values obtained by annealing the RNA mixture with T_4 DNA instead of AMV DNA (60 counts/min of ^{3}H; 1 count/min of ^{32}P) were subtracted. The amounts of radioactive ^{3}H- and ^{32}P-hybrid (370 and 1,700 counts/min, respectively) in the sample with no added AMV RNA were taken as 100%. A hybridization carried out in the presence of 1.4 μg of Qβ RNA gave 95% of the control.*

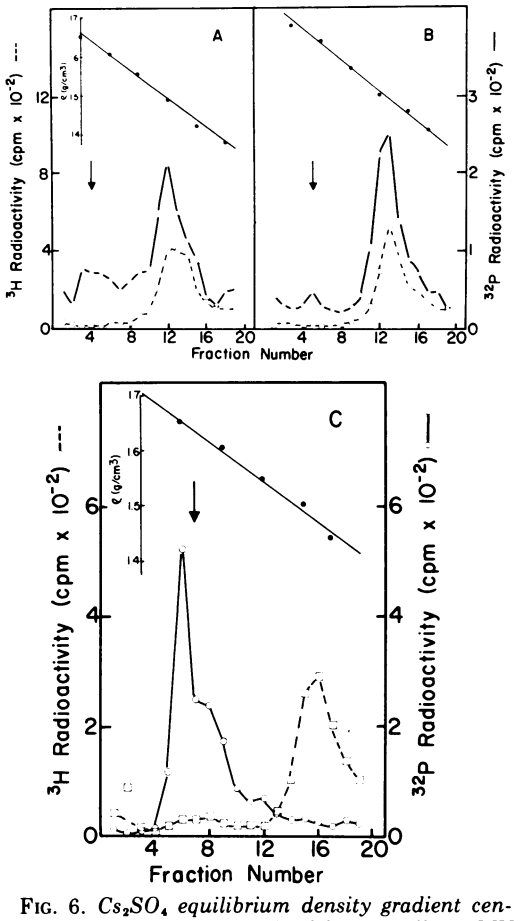

FIG. 6. *Cs_2SO_4 equilibrium density gradient centrifugation of products obtained by annealing AMV DNA with SRV RNA or SRV-infected chicken cell RNA. (A) ^{32}P-labeled RNA from SRV-infected chicken cells (9 × 10^6 counts/min, 24 μg) was annealed with 0.1 μg of ^{3}H-AMV DNA (35,000 counts/min/μg) and treated as described in the legend to Fig. 2. The RNase-resistant material was isolated by chromatography on Sephadex G-100, Qβ RNA was added as carrier and marker, and the nucleic acids were extracted with phenol and precipitated with ethanol. The purified nucleic acids (1,600 counts/min of ^{32}P, 3,500 counts/min of ^{3}H), were centrifuged in a Cs_2SO_4 gradient as described in Materials and Methods. (B) Centrifugation of a product (1,600 counts/min of ^{32}P, 3,000 counts/min of ^{3}H) obtained by annealing 70S ^{32}P-SRV RNA (70,000 counts/min, 400,000 counts/min/μg) and ^{3}H-AMV DNA (0.1 μg 35,000 counts/min/μg), was carried out as described under (A). (C) 70S ^{32}P-SRV RNA (2,000 counts/min) and ^{3}H-AMV DNA (1,800 counts/min) were mixed, but not annealed, and centrifuged as described above. Acid-insoluble radioactivity and absorbance at 260 nm were determined for all fractions. Recovery of ^{32}P-RNA and ^{3}H-DNA was, in all cases, greater than 80%. Arrows indicate the position of the Qβ RNA marker. Symbols: ○, ^{32}P-RNA; □, ^{3}H-AMV DNA.*

viral RNA in the infected cell. Thus, one cannot be certain that any particular amount of viral DNA added to an annealing assay is, in fact, in the required excess unless a saturation experiment is carried out for each unknown sample.

This problem can be overcome by adding an internal standard to the assay, i.e., a small quantity of SRV RNA labeled with an isotope different from that present in the sample. The proportion of internal standard converted into hybrid is a measure of the overall efficiency of hybridization and the values obtained for the unknown can thus be corrected accordingly. The assumptions underlying this use of an internal standard are set forth in the discussion (cf. also reference 51 for a detailed discussion of double-isotope hybridization assays).

We shall discuss the use of the internal standard in the quantitative determination of SRV RNA by considering part of the more complex experiment of Table 2. Chicken cells, either uninfected or infected with SRV, were labeled with ^{3}H-uridine for 60 min. The cells were collected after trypsin treatment, lysed with SDS, and digested with Pronase. ^{32}P-labeled SRV RNA was added prior to the purification of the cell RNA and thus served to monitor the recovery of viral RNA throughout the preparative procedure and the hybridization assay. As shown in Table 2 (lines 4 and 10) the yields of ^{3}H and ^{32}P radioactivity were similar, indicating that no preferential loss of either RNA had occurred during purification. Hybridization of the sample from infected cells with AMV DNA yielded 26% of the ^{32}P-labeled SRV RNA and 0.13% of the ^{3}H-RNA as hybrid (after subtracting blanks of 0.1% and 0.04% respectively, obtained by annealing a sample with T_4 DNA). Correcting for the difference in recovery of ^{3}H- and ^{32}P-RNA during purification, and taking into account the efficiency of the annealing reaction as indicated by the hybridization of the ^{32}P-RNA, the proportion of ^{3}H radioactivity present in virus-specific RNA is: 100 × (0.13 × 53/26 × 55) = 0.46%. The corresponding (corrected) number for uninfected cells was 0.02%, but the measured hybridization value was, in fact, not significantly higher than the blank value which had been subtracted from it (cf. line 10 of Table 2).

Distribution of labeled SRV RNA between nuclear and cytoplasmic fractions after short and long labeling periods. It has been proposed that RNA of RNA tumor viruses is synthesized from a template of virus DNA integrated into the host-cell DNA (47, 48). If this hypothesis is correct, then SRV RNA in

TABLE 2. *Distribution of total and SRV-specific ^3H-labeled RNA between "nuclear" and "cytoplasmic" fractions after different times of labeling with ^3H-uridine*[a]

Labeling time (min)	Number of cells $\times 10^{-6}$	Fraction	^3H-Uridine incorporated (counts/min /cell)	Recovery after purification (% of input)		Hybridization input[b]		Radioactivity in hybrid (% of input)		Virus-specific ^3H-RNA (corrected %)[e]
				^3H	^{32}P	^3H (counts /min $\times 10^{-5}$)	^{32}P (counts /min $\times 10^{-3}$)	^3H[c]	^{32}P[d]	
15	8.3	Total	0.92	51	49	1.1	6.0	0.17 (0.10)	46 (0.3)	0.37
		Nuclear	0.60	43	28	2.6	5.1	0.20 (0.09)	53 (0.6)	0.58
		Cytoplasmic	0.042	35	49	0.14	7.4	0 (1.6)	39 (0.4)	0[f]
60	11	Total	4.5	53	55	10	8.7	0.13 (0.04)	26 (0.1)	0.46
		Nuclear	2.9	33	39	17	7.9	0.17 (0.03)	33 (0.1)	0.42
		Cytoplasmic	0.67	44	35	4.1	5.5	0.16 (0.11)	33 (0.1)	0.60
240	9.4	Total	13	40	43	11	9.6	0.17 (0.01)	43 (0.1)	0.36
		Nuclear	3.6	24	25	5.5	5.2	0.23 (0.05)	47 (0.1)	0.46
		Cytoplasmic	4.3	47	43	11	7.2	0.13 (0.03)	40 (0.1)	0.36
60 (uninfected)	4.7	Total	6.5	53	55	16	7.1	0.0097 (0.016)	46 (0.1)	0.02

[a] Parallel cultures of uninfected or SRV-infected chicken cells were incubated with 400 μCi of ^3H-uridine per ml under the labeling conditions described in the Materials and Methods section. At the times indicated a culture was treated with trypsin, and the cell number was determined (column 2). One sample (about $\frac{3}{4}$ of the material) was fractionated into "nuclear" and "cytoplasmic" fractions, the rest was processed directly ("total"). After treatment with SDS and Pronase, approximately 60,000 counts/min of ^{32}P-labeled 70S SRV RNA (specific activity $> 1 \times 10^6$ counts/min/μg) were added to each sample as internal standard. Acid-insoluble ^3H (column 4) and ^{32}P radioactivity to each fraction was measured, and the RNA was extracted and purified. The recoveries of acid-insoluble ^{32}P- and ^3H-labeled RNA were determined (column 5). Experimental details are given in the Methods section.

[b] A sample of each preparation of purified RNA, containing the radioactivities indicated in column 6 was annealed with 0.13 μg of AMV DNA (specific ^{32}P radioactivity, 1,200 counts/min/μg) or with 0.15 μg of T_4 DNA (control), and the radioactive RNase-resistant RNA was measured after Sephadex chromatography.

[c] After subtraction of the background ^3H radioactivity determined in a control hybridization with T_4 DNA (given in parentheses) and correction for 2.9% spillover from the ^{32}P channel.

[d] After subtraction of background ^{32}P radioactivity determined by hybridization with T_4 DNA (given in parentheses) and subtraction of ^{32}P radioactivity due to labeled input DNA.

[e] [^3H hybridized (%) $\times$ ^3H recovery (%)/^{32}P hybridized (%) $\times$ ^{32}P recovery (%)] 100.

[f] 50 counts/min over a background of 220 counts/min would have given a value of 0.63%.

infected cells would be synthesized in the nucleus and subsequently transferred to the cytoplasm. One would, therefore, expect that after a sufficiently short pulse of a radioactive RNA precursor all of the radioactive SRV RNA would be found in the nucleus and that with successively longer labeling times increasing amounts of radioactive viral RNA would be found in the cytoplasm. If, on the other hand, viral RNA synthesis occurred in the cytoplasm then, after a short labeling period, all of the radioactive viral RNA in the cell would be found in the cytoplasmic fraction.

To determine the distribution of SRV RNA between nucleus and cytoplasm, SRV-infected chicken cells were labeled with ^3H-uridine for 15, 60, and 240 min, respectively. Each preparation of labeled cells was divided into two portions. RNA was purified from one portion directly. The other portion was fractionated into a "nuclear" and a "cytoplasmic" fraction prior to extraction of the RNA. The fractionation was carried out by disrupting the suspended cells with Triton X-100 and centrifuging the lysate through a preformed two-step sucrose gradient for 20 min at 7,000 $\times$ g. The particulate material that sedimented to the boundary between the sucrose layers contained more than 90% of the DNA, but little of the RNA and the protein (cf. Table 3), and is referred to as "nuclear" fraction. The upper part of the gradient which contained no detectable DNA, but most of the RNA and the protein, was pooled to give the "cytoplasmic" fraction. ^{32}P-labeled 70S SRV RNA was added to each preparation as internal standard, and purification of RNA was car-

TABLE 3. *Distribution of nucleic acids and protein between "nuclear" and "cytoplasmic" fractions*[a]

Determination	RNA[b]	DNA[c]	Protein[d]
Experiment I[e]			
"Nuclear" fraction	100 (20%)	80 (>90%)	220 (9%)
"Cytoplasmic" fraction	402 (80%)	0[f] (<10%)	2260 (91%)
Experiment II			
"Nuclear" fraction	35 (15%)	60 (>90%)	360 (19%)
"Cytoplasmic" fraction	202 (85%)	0[f] (<10%)	1570 (81%)
Experiment III			
"Nuclear" fraction	48 (21%)	55 (>90%)	421 (26%)
"Cytoplasmic" fraction	228 (79%)	0[f] (<10%)	1185 (74%)

[a] Chicken embryo fibroblasts, infected or uninfected, were grown, harvested and fractionated on a two-step sucrose gradient as described in Materials and Methods. The "nuclear" fraction was recovered from the interface of the two sucrose layers; the remaining contents of the tube were pooled as "cytoplasmic" fraction. Thus, all of the DNA, RNA, and protein was contained in the two fractions. Each fraction was diluted to 4 ml, and the protein was determined on a 0.4-ml sample. The remainder of each fraction was precipitated with 0.5 N perchloric acid, and RNA and DNA were determined in the precipitate. Results are given in μg per total fraction and (in parentheses) as percent of the sum of the two fractions.

[b] Determined as described by Shatkin (42).

[c] Determined by the method of Burton (7).

[d] Determined by the method of Lowry et al. (31).

[e] Experiment I was carried out with 15×10^6 to 20×10^6 SRV-infected chicken cells, and experiments II and III were carried out with 10^7 to 5×10^7 uninfected chicken cells.

[f] No optical density above the blank was measurable. As little as 5 to 6 μg could be detected in this fraction.

ried out as described in the previous section. The recoveries of ^3H and ^{32}P radioactivities (Table 2, column 5) were determined and corrected for quenching and spillover. Although the yields varied between 24 and 55% from one preparation to another, recoveries of ^{32}P and ^3H radioactivity for any one preparation were similar in almost all cases. To measure the amount of virus-specific RNA, a portion of each sample was annealed with AMV DNA and with T_4 DNA, respectively, and treated with RNase. The ^3H and ^{32}P radioactivities chromatographing in the excluded region of the Sephadex G-100 columns were determined (Table 2, column 7) as before. The values obtained with T_4 DNA were subtracted from the values resulting from hybridization to AMV DNA. The proportion of ^{32}P-SRV RNA (internal standard) hybridized in the different preparations ranged from 26 to 54% and was 100-fold or more over the background. The proportion of ^3H-labeled RNA hybridized was 4 to 17 times the background at the longest labeling times and about twice the background for the shortest labeling time, except for the cytoplasmic fraction where no SRV RNA was detected. The backgrounds tended to be substantially higher after short than after long labeling periods. If, as we believe, the backgrounds were due to residual double-stranded RNA this finding would suggest that a larger proportion of labeled complementary RNA is present in chick fibroblasts after short than after long labeling periods. The total amount of ^3H-label in virus-specific RNA (on a per-cell basis) was calculated from the data of Table 2, correcting for recoveries during purification and for efficiency of hybridization, as described above. Figure 7 summarizes the results of the experiment. The incorporation of precursor into total cell RNA (Fig. 7A) was almost linear during the course of the experiment. Net incorporation of label into the "nuclear" fraction leveled off at about 60 min, whereas the rate of incorporation into the "cyto-

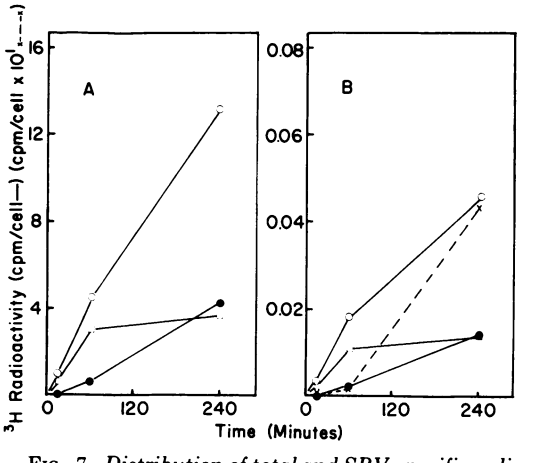

FIG. 7. *Distribution of total and SRV-specific radioactive RNA between "nuclear" and "cytoplasmic" fractions after different times of labeling. SRV-infected cells were labeled with ^3H-uridine for the times indicated. At each time point a sample was analyzed for (A) total radioactivity and (B) radioactivity in SRV-specific RNA. Assays were carried out on RNA extracted from unfractionated cells (○) as well as from "nuclear" (□) and "cytoplasmic" (●) fractions. ^3H radioactivity in virions in the supernatant medium was determined by equilibrium density gradient centrifugation with a ^{32}P-SRV marker (×). All values are expressed as counts/min/cell. The data were recalculated from Table 2, in which experimental details are described.*

plasmic" fraction was at first very low but increased substantially during the subsequent period of observation. These results are in agreement with previous, more detailed analyses (38).

Figure 7B shows that the ^{3}H-labeled SRV RNA behaved similarly to the total ^{3}H-labeled RNA: there was an initial, rapid accumulation of labeled virus-specific RNA in the "nuclear" fraction, and a delayed appearance of this RNA in the "cytoplasmic" fraction. There was an even longer delay in the appearance of radioactivity in mature virus particles. The ratio of radioactivity in SRV RNA in the "nuclear" fraction to that in the "cytoplasmic" fraction diminished from more than 30 at 15 min to 3.5 at 1 h and 1 at 4 h. These findings are compatible with the hypothesis that SRV RNA is synthesized in the "nuclear" fraction and is subsequently transferred to the "cytoplasmic" fraction and thence to viral particles; these findings are not compatible with the alternative possibility that the viral RNA is synthesized in the cytoplasmic fraction. The fact that the sums of incorporation into "nuclear" and "cytoplasmic" fractions do not, in all cases, add up exactly to the incorporation into total cell RNA does not affect the conclusions drawn from the experiment.

DISCUSSION

The investigation of tumor virus RNA biosynthesis has been hampered by the fact that only a very small fraction of the RNA synthesized in the infected cell is virus specific and that selective inhibition of host RNA synthesis has not been achieved by use of inhibitors such as actinomycin D. Furthermore, the usually sensitive hybridization assays of Nygaard and Hall (36) and Gillespie and Spiegelman (21) commonly used for the measurement of specific RNAs have not been successfully applied to quantitation of tumor virus RNA for technical reasons. In previous reports, tumor-virus-specific RNA has been determined by measuring the effect of the RNA sample on the hybridization of radioactive virus-specific DNA (10, 22, 29). This technique measures the total viral RNA content of the sample and does not allow the quantitation of viral RNA synthesized during a specified labeling period.

The hybridization assay described in this paper was designed to determine quantitatively radioactive viral RNA in the presence of a large excess of radioactive nonviral RNA. The readily available AMV-specific DNA synthesized by RNA-directed DNA polymerase in AMV virions was used for the hybridization of radioactive SRV-specific RNA, since model experiments showed that 35 to 50% of the labeled viral RNA could be converted into hybrid in this heterologous system. Background values were reduced to a low level by (i) removing most of the double-stranded RNA which normally occurs in chicken cells (11, 26, 35) by purification on a cellulose column (19) prior to the hybridization assay and (ii) isolating the product of the annealing reaction by Sephadex chromatography after RNase digestion. The labeled material obtained by this procedure, both from purified SRV RNA and from SRV-infected cell RNA was characterized as a specific RNA-DNA hybrid by equilibrium density gradient centrifugation, hybridization competition with AMV RNA and nucleotide analysis. A possible difficulty with hybridization assays with DNA synthesized by virion-RNA-directed DNA polymerase is that RNA tumor viruses may contain small amounts of DNA believed to be of host origin (30, 39). Such DNA could contaminate the in vitro DNA product and confuse the results of the hybridization assay by converting host-specific RNA into a hybrid. We have excluded this possibility by showing that conversion of in vivo labeled RNA and purified labeled viral RNA into a RNA-DNA hybrid was diminished to the same extent by addition of purified nonradioactive 70*s* viral RNA (cf. Fig. 5). The conclusion that the hybridization assay is, in fact, detecting virus-specific RNA was further reinforced by the virtually negative outcome of the assay with RNA from uninfected cells.

To quantitate the hybridization assay, a small amount of viral RNA labeled with an isotope different from that present in the sample was introduced into the assay as an internal reference for the efficiency of recovery during purification and hybridization. It was assumed that the distribution of sequences in the reference RNA and in the intracellular viral RNA is similar. This assumption is probably justified if viral RNA synthesis is asynchronous or if the time of synthesis of an RNA strand is short compared to the labeling period, or both. It was furthermore assumed that the molecular weights and, therefore, the hybridization rates of both reference and sample RNA were similar, a condition we strove to meet by purifying reference and sample together. Even if the molecular weight of intracellular viral RNA differed substantially from that of mature virion RNA, we would expect both RNAs to be of similar, reduced size after being subjected to the numerous purification steps, which include heating at 100 C. Although our quantitative determinations were carried out on RNA that

had not been labeled to constant specific activity, the proportion of labeled viral RNA found, 0.3 to 0.5%, is close to the value of 0.5 to 1% determined by Leong et al. (29) on unlabeled RNA samples from RSV-infected chicken cells.

It has been reported that cells from chickens of all strains examined contain DNA complementary to avian tumor virus DNA (50). Our failure to detect significant amounts of RNA complementary to AMV DNA in uninfected chicken cells after either 1 or 24 h of labeling means that the rate of synthesis of such RNA is either 10 to 20 times lower than in infected cells or that, if it is higher, its turnover rate is substantially less than 1 h.

The main intention of our experiments was to define the site of synthesis of virus-specific RNA. Disruption of cells by Triton X-100 and fractionation of the lysate on a two-step sucrose gradient yielded two fractions. The rapidly sedimenting material containing all of the DNA and relatively little of the cell protein and RNA was clearly derived from the nucleus. The slowly sedimenting material, containing no detectable DNA (less than 10% of the total) but the bulk of the protein and the cell RNA, is presumed to be mainly derived from the cytoplasm, although it is possible that soluble components from the nucleus may have been released into it. The distribution of radioactive virus-specific RNA between these two fractions was similar to that of the total labeled RNA at all labeling times examined from 15 to 240 min. Radioactive RNA was detected exclusively in the nuclear fraction after 15 min of labeling and later increased almost linearly in the cytoplasm while leveling off in the nucleus. The most likely conclusion is that SRV RNA is synthesized in the nucleus and is subsequently transferred to the cytoplasm.

It has been suggested (24) that RNA tumor viruses are synthesized in the mitochondria. It could be argued that in our fractionation procedure, nucleoprotein containing nascent viral RNA is released from the mitochondria and aggregates with nuclear chromatin. Although we do not consider this a likely possibility, it will be necessary to study the distribution of labeled viral RNA between isolated intact mitochondria and the cytoplasm as a function of labeling time to completely exclude the mitochondrion as a site of synthesis of viral RNA.

ACKNOWLEDGMENTS

We acknowledge the excellent technical assistance of Pamela Lewis, Ulla Jonsson, and Lydia Boller.

This investigation was supported by the Schweizerische Nationalfonds no. 3506, the Jane Coffin Childs Fund no. 243, and the Schweizerische Krebsliga. J.M.C. was a fellow of the Jane Coffin Childs Memorial Fund for Medical Research, R.K.H. was a fellow of the Damon Runyon Memorial Fund for Cancer Research. P.A.B. was a long-term fellow of the European Molecular Biology Organization.

LITERATURE CITED

1. Altaner, C., and H. M. Temin. 1970. Carcinogenesis by RNA sarcoma viruses. XII. A quantitative study of infection of rat cells in vitro by avian sarcoma virus. Virology **40**:118–134.
2. Baltimore, D. 1970. RNA-dependent DNA polymerase in virions of RNA tumour viruses. Nature (London) **226**:1209–1211.
3. Baluda, M. A., and D. P. Nayak. 1969. Incorporation of precursors into ribonucleic acid, protein, glycoprotein, and lipoprotein of avian myeloblastosis virions. J. Virol. **4**:554–566.
4. Birnbaum, H. C. 1970. Optimal conditions for counting of precipitated ^{3}H-RNA on glass fiber filters. Anal. Biochem. **37**:178–182.
5. Bock, R. M. 1967. Alkaline hydrolysis of RNA, p. 224–228. *In* L. Grossman and K. Moldave (ed.), Methods in enzymology, vol. XII, part A. Academic Press Inc., New York.
6. Bonar, R. A., and J. W. Beard. 1959. Virus of avian myeloblastosis. XII. Chemical constitution. J. Nat. Cancer Inst. **23**:183–197.
7. Burton, K. 1965. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. **62**:315–323.
8. Chamberlin, M., and P. Berg. 1964. Mechanism of RNA polymerase action: formation of DNA-RNA hybrids with single-stranded templates. J. Mol. Biol. **8**:297–313.
9. Coffin, J. M., and H. M. Temin. 1971. Comparison of Rous sarcoma virus-specific deoxyribonucleic acid polymerase in virions of Rous sarcoma virus and in Rous sarcoma virus-infected chicken cells. J. Virol. **7**:625–634.
10. Coffin, J. M., and H. M. Temin. 1972. Hybridization of Rous sarcoma virus deoxyribonucleic acid polymerase product and ribonucleic acids from chicken and rat cells infected with Rous sarcoma virus. J. Virol. **9**:766–775.
11. Colby, C., and P. H. Duesberg. 1969. Double-stranded RNA in vaccinia virus infected cells. Nature (London) **222**:940–944.
12. Darnell, J. E., R. Wall, and R. J. Tushinski. 1971. An adenylic acid-rich sequence in messenger RNA of HeLa cells and its possible relationship to reiterated sites in DNA. Proc. Nat. Acad. Sci. U.S.A. **68**:1321–1325.
13. Duesberg, P. H., and E. Canaani. 1970. Complementarity between Rous sarcoma virus (RSV) RNA and the in vitro synthesized DNA of the virus-associated DNA polymerase. Virology **42**:783–788.
14. Duesberg, P. H., H. L. Robinson, W. S. Robinson, R. J. Huebner, and H. C. Turner. 1968. Proteins of Rous sarcoma virus. Virology **36**:631–646.
15. Duff, R. G., and P. K. Vogt. 1969. Characteristics of two new avian virus subgroups. Virology **39**:18–30.
16. Edmonds, M., M. H. Vaughan, and H. Nakazato. 1971. Polyadenylic acid sequences in the heterogeneous nuclear RNA and rapidly-labeled polyribosomal RNA of HeLa cells: possible evidence for a precursor relationship. Proc. Nat. Acad. Sci. U.S.A. **68**:1336–1340.
17. Eoyang, L., and J. T. August. 1968. Phage Qβ RNA polymerase, p. 530–540. *In* L. Grossman and K. Moldave (ed.), Methods in enzymology, vol. XII, part B. Academic Press Inc., New York.
18. Feix, G., H. Slor, and C. Weissmann. 1967. Replication of

viral RNA. XIII. The early product of phage RNA synthesis *in vitro*. Proc. Nat. Acad. Sci. U.S.A. **57**:1401-1408.
19. Franklin, R. M. 1966. Purification and properties of the replicative intermediate of the RNA bacteriophage R17. Proc. Nat. Acad. Sci. U.S.A. **55**:1504-1511.
20. Fujinaga, K., J. T. Parsons, J. W. Beard, D. Beard, and M. Green. 1970. Mechanism of carcinogenesis by RNA tumor viruses. III. Formation of RNA-DNA complex and duplex DNA molecules by the DNA polymerase(s) of avian myeloblastosis virus. Proc. Nat. Acad. Sci. U.S.A. **67**:1432-1439.
21. Gillespie, D., and S. Spiegelman. 1965. A quantitative assay for DNA-RNA hybrids with DNA immobilized on a membrane. J. Mol. Biol. **12**:829-842.
22. Green, M., H. Rokutanda, and M. Rokutanda. 1971. Virus specific RNA in cells transformed by RNA tumor viruses. Nature N. Biol. **230**:229-232.
23. Haas, M., M. Vogt, and R. Dulbecco. 1972. Loss of simian virus 40 DNA-RNA hybrids from nitrocellulose membranes; implications for the study of virus-host DNA interactions. Proc. Nat. Acad. Sci. U.S.A. **69**:2160-2164.
24. Kara, J., O. Mach, and H. Cerna. 1971. Replication of Rous sarcoma virus and the biosynthesis of the oncogenic subviral ribonucleoprotein particles ("virosomes") in the mitochondria isolated from Rous sarcoma tissue. Biochem. Biophys. Res. Commun. **44**:162-170.
25. Kates, J. 1970. Transcription of the vaccinia virus genome and the occurrence of polyriboadenylic acid sequences in messenger RNA. Cold Spring Harbor Symp. Quant. Biol. **35**:743-752.
26. Kimball, P. C., and P. H. Duesberg. 1971. Virus interference by cellular double-stranded ribonucleic acid. J. Virol. **7**:697-706.
27. Lai, M. C., and P. H. Duesberg. 1972. Adenylic acid-rich sequence in RNA of Rous sarcoma virus and Rauscher mouse leukemia virus. Nature (London) **235**:383-386.
28. Lee, S. Y., J. Mendecki, and G. Brawerman. 1971. A polynucleotide segment rich in adenylic acid in the rapidly-labeled polyribosomal RNA component of mouse sarcoma 180 ascites cells. Proc. Nat. Acad. Sci. U.S.A. **68**:1331-1335.
29. Leong, J., A. Garapin, N. Jackson, L. Fanshier, W. Levinson, and J. M. Bishop. 1972. Virus-specific ribonucleic acid in cells producing Rous sarcoma virus: detection and characterization. J. Virol. **9**:891-902.
30. Levinson, W., J. M. Bishop, N. Quintrell, and J. Jackson. 1970. Presence of DNA in Rous sarcoma virus. Nature (London) **227**:1023-1025.
31. Lowry, H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
32. McDonnell, J. P., A. Garapin, W. E. Levinson, N. Quintrell, L. Fanshier, and J. M. Bishop. 1970. DNA polymerase of Rous sarcoma virus: delineation of two reactions with actinomycin. Nature (London) **228**:433-435.
33. Manly, K. F., D. F. Smoler, E. Bromfeld, and D. Baltimore. 1971. Forms of deoxyribonucleic acid produced by virions of the ribonucleic acid tumor viruses. J. Virol. **7**:106-111.
34. Melli, M., and J. O. Bishop. 1970. Molecular hybridization between rat liver deoxyribonucleic acid and complementary ribonucleic acid. Biochem. J. **120**:225-235.
35. Montagnier, L. M. 1968. Présence d'un acid ribonu-'cléique en double chaîne dans des cellules animales. C. R. Acad. Sci. **267**:1417-1420.
36. Nygaard, A. P., and B. H. Hall. 1964. Formation and properties of RNA-DNA complexes. J. Mol. Biol. **9**:125-142.
37. Penman, S. 1969. Preparation of purified nuclei and nucleoli from mammalian cells, p. 35-48. *In* K. Habel and N. Salzman (ed.), Fundamental techniques in virology. Academic Press Inc., New York.
38. Penman, S., C. Vesco, and M. Penman. 1968. Localization and kinetics of formation of nuclear heterodisperse RNA, cytoplasmic heterodisperse RNA and polyribosome-associated messenger RNA in HeLa cells. J. Mol. Biol. **34**:49-69.
39. Riman, J., and G. S. Beaudreau. 1970. Viral DNA-dependent DNA polymerase and the properties of thymidine labeled material in virions of an oncogenic RNA virus. Nature (London) **228**:427-430.
40. Rokutanda, M., H. Rokutanda, M. Green, K. Fujinaga, R. K. Ray, and C. Gurgo. 1970. Formation of viral RNA-DNA hybrid molecules by the DNA polymerase of sarcoma-leukaemia viruses. Nature (London) **227**:1026-1028.
41. Rubin, H. 1960. A virus in chick embryos which induces resistance in vitro to infection with Rous sarcoma virus. Proc. Nat. Acad. Sci. U.S.A. **46**:1105-1119.
42. Shatkin, A. J. 1969. Colorimetric reactions for DNA, RNA, and protein determinations, p. 231-237. *In* K. Habel and N. P. Salzman (ed.), Fundamental techniques in virology. Academic Press Inc., New York.
43. Spadari, S., and F. Ritossa. 1970. Clustered genes for ribosomal ribonucleic acids in *Escherichia coli*. J. Mol. Biol. **53**:357-367.
44. Symons, R. H. 1969. Preparation of α-^{32}P nucleoside and deoxynucleoside 5'-triphosphates from ^{32}Pi and protected and unprotected nucleosides. Biochim. Biophys. Acta **190**:548-550.
45. Szybalski, W. 1968. Use of cesium sulfate for equilibrium density gradient centrifugation, p. 330-360. *In* L. Grossman and K. Moldave (ed.), Methods in enzymology, vol. XII, part B. Academic Press Inc., New York.
46. Temin, H. M. 1963. The effects of actinomycin D on growth of Rous sarcoma virus in vitro. Virology **20**:577-582.
47. Temin, H. M. 1964. Nature of the provirus of Rous sarcoma. Nat. Cancer Inst. Monogr. **17**:557-570.
48. Temin, H. M. 1971. Mechanism of cell transformation by RNA tumor viruses. Annu. Rev. Microbiol. **25**:609-648.
49. Temin, H. M., and S. Mizutani. 1970. RNA-dependent DNA polymerase in virions of Rous sarcoma virus. Nature (London) **226**:1211-1213.
50. Varmus, H. E., R. A. Weiss, R. R. Friis, W. Levinson, and J. M. Bishop. 1972. Detection of avian tumor virus-specific nucleotide sequences in avian cell DNAs. Proc. Nat. Acad. Sci. U.S.A. **69**:20-24.
51. Weissmann, C., L. Colthart, and M. Libonati. 1968. Determination of viral "plus" and "minus" strands by an isotope dilution assay. Biochemistry **7**:865-874.