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Virus-Specific Ribonucleic Acid in Cells Producing Rous Sarcoma Virus: Detection and Characterization

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Cells producing Rous sarcoma virus contain virus-specific ribonucleic acid (RNA) which can be identified by hybridization to single-stranded deoxyribonucleic acid (DNA) synthesized with RNA-directed DNA polymerase. Hybridization was detected by either fractionation on hydroxyapatite or hydrolysis with single strand-specific nucleases. Similar results were obtained with both procedures. The hybrids formed between enzymatically synthesized DNA and viral RNA have a high order of thermal stability, with only minor evidence of mismatched nucleotide sequences. Virus-specific RNA is present in both nuclei and cytoplasm of infected cells. This RNA is remarkably heterogeneous in size, including molecules which are probably restricted to the nucleus and which sediment in their native state more rapidly than the viral genome. The nature of the RNA found in cytoplasmic fractions varies from preparation to preparation, but heterogeneous RNA (ca. 4-50S), smaller than the viral genome, is always present in substantial amounts.

The ribonucleic acid (RNA) tumor viruses cannot replicate in the presence of actinomycin D (2, 28). This fact prohibits use of the antibiotic to eliminate host RNA synthesis during the course of virus growth and has impeded biochemical analysis of the replication of these viruses (29). Discovery of RNA-directed deoxyribonucleic acid (DNA) polymerase within the virions of RNA tumor viruses (3, 30) has provided a new approach to this problem. DNA synthesized by the polymerase constitutes an extremely sensitive annealing probe for virus-specific sequences of RNA and has been used to detect and measure viral RNA in cells infected with avian (17) and murine (18) RNA tumor viruses. The present communication describes the localization and further characterization of viral RNA in cells producing the Schmidt-Ruppin strain of Rous sarcoma virus (RSV). Hybridization of virus-specific radiolabeled DNA to RNA was detected by two procedures: fractionation on hydroxyapatite (17, 18) and a newly developed technique which employs single strand-specific nucleases (those of *Neurospora crassa* and *Aspergillus oryzae*). [A test for DNA-RNA hybridization using single strand-specific nuclease has also been developed by Fan and Baltimore (31), although there are differences of detail between their assay and ours.] The nuclease assay was judged to be generally superior to the use of hydroxyapatite, with the *Aspergillus* enzyme offering several advantages over the *Neurospora* nuclease. The techniques and results presented here should provide a basis for subsequent detailed analysis of the mechanism by which RNA tumor viruses replicate.

MATERIALS AND METHODS

Materials. The sources of most reagents have been described (13, 15). Conidia of *N. crassa* were purchased from Miles Laboratories, Inc.; dimethylsulfoxide from Matheson, Coleman and Bell; Takadiastase (Sanzyme) was a gift from Sankyo Ltd., Tokyo, Japan. Diastase powder obtained from Sigma also contains S-1 nuclease, but the data in the present communication pertain only to the Sankyo material. All cell cultures were prepared from embryos known to be free from carrier infection with avian leukosis virus (embryonated eggs obtained from Kimber Farms, Berkeley, Calif.). Electrophoretically purified deoxyribonuclease was purchased from Worthington Biochemicals and treated with iodoacetate by the method of Zimmerman and Sandeen (34) to inactivate traces of contaminating ribonuclease. The alkylated preparations were tested for ribonuclease with ^{32}P-labeled 70S RSV RNA, which was denatured (11, 12) after exposure to the enzyme and was analyzed by rate-zonal centrifugation.

Cells and virus. The propagation and purification of the Schmidt-Ruppin strain of RSV have been described previously (6).

Synthesis of virus-specific DNA. The conditions for synthesis of DNA in vitro with detergent-activated RSV have been reported (13, 15). ^{3}H-thymidine triphosphate (10–20 Ci/mmole) was used at a concentration of 4×10^{-6} M. Reactions were generally carried out for 2 hr, at which time approximately 50% of the enzymatic product is double-stranded DNA. The reaction mixture was extracted with sodium dodecyl sulfate (SDS)-Pronase-phenol (15), then treated with ribonuclease to disrupt RNA-DNA hybrids, and fractionated on hydroxyapatite (13). Single-stranded DNA prepared in this manner can be completely hybridized to the 70*S* RNA of RSV (16, 17), and therefore constitutes a highly specific probe for viral RNA.

Extraction of RNA. The 70*S* RNA of RSV was extracted from purified virus with SDS-phenol (6) and purified by velocity sedimentation in density gradients of sucrose. Chick embryo fibroblasts, either uninfected or infected with and fully transformed by RSV, were removed from culture dishes by scraping, and trypsinized (0.05%) in 0.14 M NaCl-0.005 M KCl-0.0055 M glucose-0.005 M Na_2PO_4-0.02 M tris(hydroxymethyl)-aminomethane (Tris)-hydrochloride, *p*H 7.4, for 5 min at a concentration of ca. 1×10^7 cells/ml. The cells were then washed twice with the same buffer and either extracted immediately with 0.5% SDS and phenol at room temperature or disrupted by Dounce homogenization in 0.001 M NaCl-0.00015 M $MgCl_2$-0.001 M Tris-hydrochloride, *p*H 7.4. The cell homogenates were centrifuged at 2,000 rev/min for 5 min to sediment nuclei and debris. The supernatant cytoplasm was then extracted with 0.5% SDS-phenol at room temperature. The nuclei were washed with detergents and extracted with phenol and chloroform as described by Penman (23) at either 60 or 40 C. Nucleic acids were precipitated with ethanol, and the precipitate was washed with cold ethanol prior to use. Residual DNA was hydrolyzed with alkylated preparations of deoxyribonuclease.

RNA prepared from whole cells by SDS-phenol extraction at room temperature is contaminated with large amounts of DNA. Attempts to remove DNA by digestion with commercially available electrophoretically purified deoxyribonuclease were repeatedly thwarted by the presence of trace amounts of ribonuclease in the deoxyribonuclease (this portion of the work was undertaken before introduction of the alkylation procedure). Consequently, deoxyribonuclease treatment was abandoned, and RNA was freed of DNA by fractionation with 1 M NaCl (5, 6). This results in a precipitate which contains virtually all of the high-molecular-weight cellular and viral RNA species (ca. > 5*S*) and is free of all but trace amounts of DNA. RNA obtained in the above manner could be denatured with dimethylsulfoxide without loss of the 28*S* and 18*S* ribosomal RNA species (as judged by centrifugation in sucrose gradients), and was therefore considered free of chain breaks.

Denaturation of RNA in dimethylsulfoxide. Denaturation of RNA and disaggregation prior to subsequent analysis were carried out as described previously (M. Best, B. Evans, and J. M. Bishop, Virology, *in press*).

Rate-zonal centrifugation. RNA was analyzed by centrifugation in gradients of 15 to 30% sucrose containing 0.1 M NaCl-0.001 M ethylenediaminetetraacetic acid (EDTA)-0.02 M Tris-hydrochloride, *p*H 7.4. Fractionation of the gradients through a Gilford recording spectrophotometer and preparation of samples for measurement of radioactivity have been described (5, 15).

Nucleic acid hybridization. Enzymatically synthesized single-stranded DNA (5,000–7,500 counts per min of ^{3}H-thymidine monophosphate per 0.001 μg) was prepared as described above. The purified DNA was treated with 0.6 N NaOH for 1 hr at 37 C to destroy contaminating RNA and nucleases. Hybridization of 500 to 1,000 counts/min of DNA with varying amounts of RNA (20–10,000-fold excess over DNA) was carried out in 0.3 M NaCl-0.001 M EDTA-0.02 M Tris-hydrochloride, *p*H 7.4, at 68 C for 4 hr unless otherwise stated.

Fractionation of nucleic acids on hydroxyapatite. Nucleic acid solutions were diluted to 3 ml with 0.01 M sodium phosphate, *p*H 6.8, adsorbed to hydroxyapatite at room temperature, and eluted with successive washes of 0.16 M and 0.4 M sodium phosphate at 60 C. All operations were performed in centrifuge tubes (13). Eluates were precipitated with 5% trichloroacetic acid after addition of 80 μg of calf thymus DNA, and the precipitates were prepared for scintillation counting as described previously (13). The use of 0.16 M sodium phosphate to elute single-stranded DNA results in slight contamination (ca. 10–15%) of the 0.4 M eluate with single strands, but was used in the present experiments because it provides material compatible with the optimal conditions for detection of DNA-RNA hybrids (see below). On those occasions when purified double-stranded DNA was required, the elution sequence was 0.18, 0.4 M sodium phosphate. All single-stranded DNA used in the present experiments was eluted with 0.16 M sodium phosphate.

Preparation of nucleic acid standards. The 70*S* DNA-RNA hybrid synthesized by virion-associated DNA polymerase was purified from the product of a 2-hr reaction by rate-zonal centrifugation (15). ^{3}H-labeled DNA species of phage lambda and fd were a gift from H. Boyer. Circularity of the fd DNA was confirmed by centrifugation in alkaline CsCl (10).

Digestion of nucleic acids with single strand-specific nucleases. The single strand-specific nuclease described by Rabin et al. (24) was purified from conidia of *N. crassa* with his procedure, omitting the gel filtration and electrophoresis. Reaction mixtures contained approximately 0.10 units of nuclease/ml, 0.1 M Tris-hydrochloride, *p*H 7.4, 0.01 M $MgCl_2$, denatured, unlabeled calf thymus DNA (10 μg/ml), radioactive test DNA in quantities below 1 μg/ml, and less than 0.05 M monovalent cation. Digestions were carried out at 37 C for 2 hr, followed by addition of 80 μg of calf thymus DNA and precipitation with 5% trichloroacetic acid. The data to be presented all represent the results of duplicate assays which never varied more than $\pm$ 10%.

S-1 nuclease (1) was purified from Takadiastase powder as described by Sutton (27). These preparations are contaminated with T-1 ribonuclease (1), and tests for hydribidization are therefore carried out in a relatively high concentration of salt (0.3 M NaCl). This is both feasible and desirable because, in contrast to the *Neurospora* enzyme, S-1 nuclease is active under these conditions and has a greater degree of substrate specificity than at low concentrations of NaCl (see below; Tables 2 and 6). Standard reaction mixtures contained 1,600 units of nuclease/ml, 0.03 M sodium acetate buffer, *p*H 4.5, 1.8 × 10^{-3} M $ZnCl_2$, 0.3 M NaCl, denatured calf thymus DNA (10 μg/ml), and radioactive test DNA. Incubations and acid precipitation were carried out as for the *Neurospora* nuclease. Both the *Neurospora* and *Aspergillus* enzymes were stored at 4 C in their respective preparative buffers with no detectable loss of activity over a period of 3 months.

RESULTS

Detection of hybridization with hydroxyapatite. In a preliminary report (17), we described the manner by which fractionation on hydroxyapatite can be used to detect hybridization of radioactive single-stranded DNA to viral and cellular RNA species. This procedure permits rapid and convenient analysis of large numbers of samples. Its characteristics are illustrated in Table 1. The bulk of single-stranded DNA can be eluted from hydroxyapatite in 0.16 M sodium phosphate. Additional single-stranded DNA can be eluted with 0.18 or 0.2 M phosphate (13), but these concentrations reduce the sensitivity of the procedure for detection of hybrid formation (*unpublished observations*). By contrast, both 70*S* viral RNA and enzymatically synthesized 70*S* RNA-DNA hybrids are retained on hydroxyapatite at 0.16 M phosphate and elute in the 0.4 M wash. The same is true of 70*S* viral RNA which has been dissociated into its constituent subunits (11, 12) by denaturation with dimethylsulfoxide or heat, and of single-stranded DNA which has been hybridized to a vast excess (ca. 1,000:1) of viral RNA. The extent to which viral RNA and DNA-RNA hybrid are retained in 0.16 M sodium phosphate is superior to that reported previously (17), and is a consequence of more careful standardization of buffer concentrations. (The sodium phosphate concentration in the previous report was erroneously listed as 0.125 M.) High-molecular-weight cellular RNA fractionates in a manner identical to that of viral RNA, and transfer RNA also elutes mainly in 0.4 M phosphate. Consequently, hybridization of single-stranded DNA to RNA extracted from infected cells shifts that DNA into the 0.4 M phosphate eluate. The specificity of these observations is demonstrated by the failure of test DNA to react with either poliovirus RNA or HeLa cell RNA.

TABLE 1. *Fractionation of nucleic acids on hydroxyapatite*

Nucleic acids[a]	Conc of sodium phosphate (%)	
	0.16 M	0.4 M
Single-stranded DNA	80	20
70*S* viral RNA	5	95
Denatured 70*S* RNA	10	90
Ribosomal RNA	8	92
Transfer RNA	25	75
70*S* DNA-RNA hybrid	15	85
Hybridized ss DNA (70*S* RNA)	18	82
Hybridized ss DNA (infected cell RNA)	15	85
Hybridized ss DNA (HeLa cell RNA)	83	17
Hybridized ss DNA (polio RNA)	84	16

[a] The preparation of single-stranded enzymatic product and the various RNA species is described in Materials and Methods. Each analysis employed ca. 1,000 counts of radioactive nucleic acid per min. Hybridization was performed with RNA in vast excess, under conditions previously shown to allow complete hybridization of test DNA (16, 17). Analysis in equilibrium gradients of Cs_2SO_4 (16, 25, 26) confirmed this for the present experiments. Results are expressed as proportion of total radioactivity recovered in each wash of sodium phosphate. Recoveries from hydroxyapatite were usually 100%. ss, single-stranded.

The hydroxyapatite assay has two major limitations: (i) formation of low-molecular-weight hybrids (<100,000) cannot be reliably detected (*unpublished observation*); this requires that there be minimal breakdown of RNA during the annealing procedure; and (ii) sequences of DNA which are not complementary to the test RNA will score as having been hybridized if they are covalently linked to sequences which are complementary to the RNA. To obviate these problems, we have employed an alternate assay for hybridization which utilizes nucleases specific for single-stranded nucleic acids. This procedure derives from the work of Manly et al. (21) who demonstrated the resistance of DNA-RNA hybrids to digestion by the single strand-specific nuclease of *Neurospora crassa* (21). Our initial studies were carried out with the same enzyme. More recently we have used S-1 nuclease obtained from extracts of *A. oryzae* (1, 27). In the following section, we compare the properties and general utility of these two nucleases as agents for the detection of RNA-DNA hybrids in which the DNA is radioactive.

General properties of the nucleases; detection of hybridization. The effect of monovalent cation on hydrolysis of single-stranded substrate by the *Neurospora* and S-1 nucleases is illustrated in Table 2. The *Neurospora* enzyme is partially inhibited by sodium chloride concentrations as low as 0.05 M, whereas the S-1 nuclease is completely active at salt concentrations in excess of 0.2 M. This difference alone makes the S-1 nuclease a more convenient reagent for the detection of hybridization because standard conditions of annealing generally involve NaCl concentrations of at least 0.3 M. The S-1 enzyme is active over a broad range of Zn^{2+} concentrations (Table 3; other divalent cations such as Mg^{2+} and Mn^{2+} are less effective—see reference 1). We routinely use $ZnCl_2$ at a concentration substantially higher than that recommended by Ando (1). This eliminates the need for individual adjustments in reaction conditions to allow for different amounts of EDTA present in buffers used for hybridization and velocity sedimentation.

As reported previously (27), the S-1 nuclease is relatively inactive in the presence of low concentrations of substrate (DNA) when hydrolysis is carried out at a low concentration of NaCl (Table 4). The same is true of the *Neurospora* nuclease (Table 4). Double-stranded DNA serves equally well to facilitate the hydrolysis of trace amounts of single-stranded DNA by both nucleases (*unpublished observation*). Addition of NaCl to 0.3 M eliminates the requirement of S-1 nuclease for a critical amount of substrate (Table 4). The inhibition of *Neurospora* nuclease by high concentrations of either DNA (Table 4) or RNA (Table 5) is probably due to the limited amounts of available enzyme protein. The fact that RNA as well as DNA is a substrate for these enzymes and can therefore retard enzymatic activity when present in excess must be considered in the design of hybridization experiments.

Within the limits of the standard assay, both enzymes are specific for single-stranded DNA (Table 6). Our preparations do convert RF I DNA of fd phage to RF II without further degradation. Traces of endonuclease active on double-stranded DNA are therefore present. The hydrolysis of circular single-stranded DNA (fd genome) indicates that the enzymes have at least a modicum of endonuclease activity (Table 6). As reported previously (27), the substrate specificity of S-1 nuclease is substantially diminished at low concentrations of NaCl (Table 6), but this problem is readily circumvented by virtue of the enzyme's activity at 0.3 M NaCl.

The kinetics of substrate hydrolysis by the

TABLE 2. *Effect of monovalent cation on single strand-specific nucleases*

NaCl (moles/liter)	Resistance[a] (%)	
	Neurospora	S-1
0.01	5	3
0.05	28	2
0.15		3
0.20	100	2
0.30	100	5
0.50	100	10

[a] Trace amounts (ca. 1,000 counts/min) of denatured lambda ^{3}H-DNA were incubated with either *Neurospora* or S-1 nuclease under standard conditions, except that the concentration of NaCl was varied as indicated. Results are expressed as the percentage of radioactive test DNA remaining acid-precipitable after a 2-hr incubation.

TABLE 3. *Requirement of S-1 nuclease for $ZnCl_2$*

$ZnCl_2$ (moles/liter)	Resistance[a] (%)
0	100
3×10^{-5}	8
9×10^{-5}	6
3×10^{-4}	4
1.5×10^{-3}	6
3×10^{-3}	5
1.5×10^{-2}	42

[a] Trace amounts (1,000 counts/min) of denatured lambda ^{3}H-DNA were incubated with S-1 nuclease under standard conditions, except that the concentration of $ZnCl_2$ was varied as indicated. Results are expressed as in Table 2.

TABLE 4. *Effect of DNA concentration on single strand-specific nucleases.*

Concentration of DNA[a] (μg/ml)	Resistance (%)		
	Neurospora	S-1 Nuclease	
	0.01 M NaCl	0.3 M NaCl	0.01 M NaCl
0	85	4	
0.1	70	4	35
1.0	25	6	
10	9	3	4
100	100	4	

[a] Trace amounts (ca. 1,000 counts/min) of radiolabeled test DNA (denatured lambda DNA; similar results were also obtained with single-stranded enzymatic product) were incubated with either *Neurospora* or S-1 nuclease under standard conditions, except that the total DNA (denatured calf thymus) and NaCl concentrations were varied as indicated. Results are expressed as the proportion of input radioactivity remaining acid-precipitable after a 2-hr incubation period.

TABLE 5. *Effect of RNA concentration on single strand-specific nucleases*

Concentration of RNA[a] (μg/ml)	Resistance (%)	
	Neurospora	S-1
1	3	3
10	10	2
20	16	2
50	20	3
100	67	3

[a] Trace amounts (ca. 1,000 counts/min) of radiolabeled denatured lambda DNA were incubated with either *Neurospora* or S-1 nuclease under standard conditions (including denatured calf thymus DNA at 10 μg/ml). The concentration of RNA was varied as indicated by the addition of unlabeled ribosomal RNA of HeLa cells. Results are expressed as in Table 4.

TABLE 6. *Specificity of Neurospora and S-1 nucleases*

Nucleic acid	Resistance[a] (%)		
	Neurospora	S-1 Nuclease	
	0.01 M NaCl	0.01 M NaCl	0.3 M NaCl
Single-stranded enzymatic product	5	3	2
Double-stranded enzymatic product	100	75	100
Native lambda DNA	100	75	100
Denatured lambda DNA	3	4	3
fd Genome DNA	2	3	—

[a] Hydrolysis of DNA was carried out under standard conditions for the two nucleases. Results are expressed as the proportion of input radioactivity remaining acid-precipitable after a 2-hr incubation period. Dash indicates analysis not done.

Neurospora enzyme under standard conditions are illustrated in Fig. 1. Similar results were obtained with S-1 nuclease. Both linear (denatured lambda) and circular (fd genome) single-stranded DNA species are hydrolyzed within 60 min. An incubation period of 2 hr was chosen for routine use.

Single-stranded DNA synthesized by the virion polymerase of RSV is completely resistant to hydrolysis by either the *Neurospora* or the S-1 nuclease after hybridization with a vast excess of 70S viral RNA (Table 7). Similar results are obtained with RNA extracted from cells infected with RSV. DNA annealed in the absence of RNA, in the presence of RNA from normal

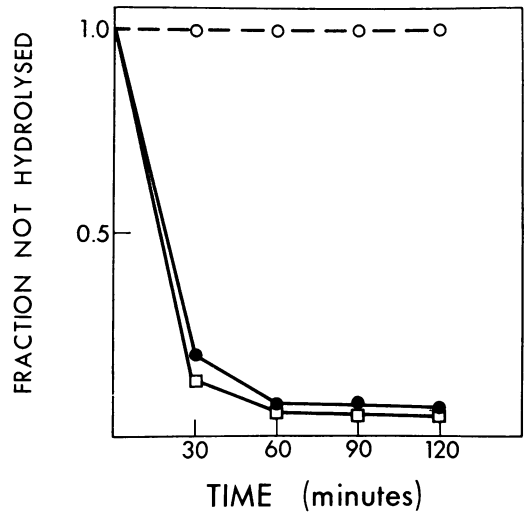

FIG. 1. *Kinetics of DNA hydrolysis by Neurospora nuclease. Substrate DNA species were prepared as described in Materials and Methods. Hydrolyses were carried out under standard conditions. Each point represents the mean of duplicate assays, expressed as the proportion of total input radioactivity remaining acid-precipitable.* ○, *Native lambda DNA;* ●, *denatured lambda DNA;* □, *fd DNA (genome).*

TABLE 7. *Detection of hybridization with single strand-specific nucleases*

Deoxyribonucleic acid annealed[a]	% Resistance	
	Neurospora	S-1
Without RNA	6	5
With 70S RSV RNA (1.5 μg)	100	102
With 35S polio RNA (1.5 μg)	4	5
With infected cell RNA (10 μg)	98	100
With avian fibroblast RNA (50 μg)	5	4
With HeLa RNA (10 μg)	3	4
With 70S RNA, then boiled	5	4

[a] Radioactive (ca. 1,000 counts/min) single-stranded enzymatic product was annealed with vast excesses (10,000:1–100,000:1) of the indicated RNA, then tested for extent of resistance to hydrolysis by either *Neurospora* or S-1 nuclease under standard conditions (achieved by diluting the annealing mixture in appropriate buffers). The cells used to prepare normal avian fibroblast RNA were free from group-specific viral antigen when tested by complement fixation (32). Results are expressed as proportion of total radioactivity remaining acid-precipitable after a 2-hr incubation period.

avian fibroblasts or in the presence of heterologous RNA (HeLa cell or poliovirus), is hydrolyzed to background levels (ca. 5% under present conditions). The two nucleases were used under conditions providing maximal activity and specificity for single-stranded substrate. The concentration of NaCl (0.3 M) in the case of S-1 nuclease offers the further advantage of preventing hydrolysis of hybrids by T-1 ribonuclease known to contaminate S-1 enzyme (1).

Denaturation of RNA-DNA hybrids. The complete resistance of hybridized DNA to hydrolysis by the *Neurospora* enzyme suggests that the DNA is entirely base-paired with complementary sequences of RNA. This matter was examined further by comparing the thermal denaturation of hybrids prepared with the annealing procedure to that of hybrids synthesized enzymatically. The course of denaturation was followed with the hydroxyapatite and nuclease assays. The thermal stabilities of the two forms of hybrid are quite similar (Fig. 2a and b), and there is little difference between the results obtained with hydroxyapatite and those with the nuclease assay (Fig. 2b). In the absence of repeated determinations, the observed differences in T_m cannot be considered significant. The configuration of the melting curves indicates a high degree of precision in base-pairing between DNA and RNA, although the hybrids formed by annealing have a shoulder in the range of 60 to 70 C which suggests a limited amount of mismatching. For comparison, the denaturation of double-stranded DNA is also illustrated.

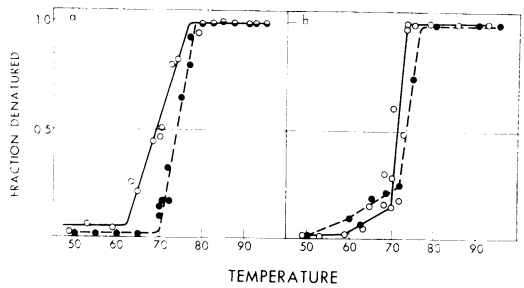

FIG. 2. *Denaturation of RNA-DNA hybrids and double-stranded DNA. ^{3}H-labeled enzymatic product was annealed to unlabeled 70S RNA of RSV. The hybridized DNA was entirely resistant to hydrolysis by Neurospora nuclease, as shown in Table 7. Enzymatically synthesized 70S DNA-RNA hybrid and double-stranded DNA were prepared with detergent-activated virions as described under Materials and Methods. Samples (ca. 1,000 counts/min) of nucleic acids in 0.01 M sodium phosphate, pH 6.8, were heated for 15 min at the indicated temperatures, quenched in ice, and assayed for extent of denaturation with either single strand-specific nuclease or hydroxyapatite. (a) Enzymatically synthesized nucleic acids. Extent of denaturation was assessed with hydroxyapatite. ○, 70S hybrid; ●, double-stranded DNA. (b) Hybrids formed by annealing. Extent of denaturation was measured with either hydroxyapatite (○) or Neurospora nuclease (●).*

Kinetics of hybridization. The kinetics of hybridization with either cellular or viral RNA in vast excess are illustrated in Fig. 3a. It is convenient to express data such as these according to the convention C_0t (concentration of nucleic acid × time of incubation) introduced by Britten and Kohne to describe the interaction of complementary polynucleotides (8). Birnstiel et al. (4) have suggested, and we have adopted, the analogous expression C_rt to describe RNA-DNA reactions with homogeneous RNA species in excess. [The reaction between RNA and DNA with RNA in vast excess should obey first-order kinetics and can be represented by double-reciprocal plots (4, 7). We have chosen to use the more familiar semilogarithmic plot popularized by Britten and Kohne (8). Nevertheless, all of the data presented were obtained under conditions approximating those required for first-order reactions. Thus, at the lowest C_rt values obtained with cellular RNA, virus-specific RNA sequences were present in at least 20-fold excess over the labeled DNA. At the half-C_rt, the ratio of virus-specific RNA-DNA was at least 200:1.] This convention permits direct comparison of the results obtained by varying either the concentration of nucleic acids or the period of incubation. The validity of this approach is illustrated in Fig. 3b, where the results of hybridization with either different concentrations of RNA (and a constant incubation period) or different periods of incubation (and constant concentrations of RNA) are compared. The minor variations illustrated do not exceed variations observed with replicate analyses under identical conditions (*unpublished observation*). A C_rt of 10^{-2} to 2×10^{-2} mole-sec/liter is required to hybridize 50% of test DNA to viral RNA. This value, or half-C_rt, is a highly reproducible figure which characterizes the interaction of viral RNA with complementary DNA, and which can be used for quantification of virus-specific nucleotide sequences in infected cells (see below).

Quantification of virus-specific RNA: a comparison of the hydroxyapatite and nuclease assays. We have previously reported a procedure whereby the amount of virus-specific RNA present in cellular RNA can be measured (17). This procedure involves determination of the quantity of viral and cellular RNA species required to hybridize a standard amount of single-stranded DNA. The previous data were obtained with the hydroxyapatite assay and indicated that virus-specific RNA constitutes approximately 0.5% of

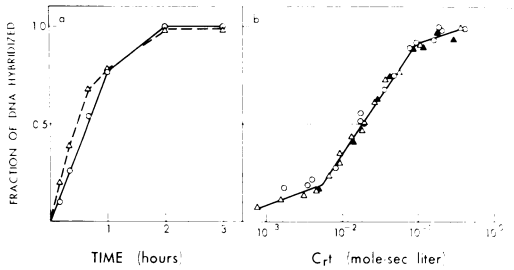

FIG. 3. *Kinetics of hybridization. (a) Kinetics of hybridization at a single concentration of RNA. ^{3}H-labeled single-stranded enzymatic product (0.00025 μg/ml) and either unlabeled 70S viral RNA (○) at 1.0 μg/ml or cytoplasmic RNA (△) at 220 μg/ml were incubated in standard annealing buffer at 68 C. At the indicated times, samples were withdrawn for analysis on hydroxyapatite. Results are expressed as proportion of total input radioactivity hybridized. Maximal hybridization represents the point when elution of single-stranded enzymatic product and enzymatically synthesized hybrid are identical (ca. 85–90% in 0.4 M sodium phosphate; see Table 1). (b) Hybridization of single-stranded enzymatic product to viral RNA. A standard amount (0.0002 μg) of ^{3}H-labeled single-stranded enzymatic product was incubated with 70S viral RNA in annealing buffer at 68 C. Either the concentration of RNA (○) or the time of incubation (△, ▲) was varied, and the extent of hybridization was determined by fractionation on hydroxyapatite. Results are expressed as the porportion of total DNA hybridized at a given value of C_rt (RNA concn × time of incubation), the latter computed as described by Britten and Kohne (8), and corrected to standard conditions of salt concentration (9).*

the total RNA obtained from infected cells (17, 18). We have now extended these observations to include cytoplasmic and nuclear fractions of cellular RNA and have performed parallel experiments with single strand-specific nuclease and hydroxyapatite assays to determine the accuracy and specificity of the latter. The results, expressed in terms of the convention C_rt, are illustrated in Fig. 4. Both assays for hybridization give identical results with 70S viral RNA (Fig. 4a). The results for whole cell (Fig. 4b) and cytoplasmic (Fig. 4c) RNA species are also similar with the two assays. However, there is greater scatter in the data obtained with hydroxyapatite, and the half-C_rt species obtained in this manner are somewhat lower than those measured with the nuclease assay. Both assays indicate that single-stranded enzymatic product can be completely hybridized to cellular RNA species, although hybridization of nuclear RNA has only been tested with the nuclease assay (Fig. 4d).

By comparing the C_rt required to hybridize 50% of test DNA to viral and cellular RNA species, the relative amounts of virus-specific sequences contained in cellular RNA can be computed. These computations and their results are illustrated in Table 8. The results with whole cell RNA (ca. 0.5%) conform to those reported previously (17, 18).

Sedimentation velocity of virus-specific RNA species. The specificity of the annealing reaction and the ease with which hybridization can be detected have facilitated analysis of the size of virus-specific RNA found in infected cells. In the experiments which follow, we have compared the results of extracting cellular RNA at two different temperatures: 25 and 60 C. Phenol extraction at 25 C preserves the secondary structure of 70S RSV RNA, whereas extraction at 60 C disrupts 70S RNA into its constituent subunits (*unpublished observation*) in a manner identical to that of either treatment with dimethylsulfoxide or heating in low concentrations of electrolytes (11, 12). These comparisons provide information concerning both the native state of intracellular viral RNA and the maximal size of unit covalent molecules. On some occasions, the extractions were controlled by the inclusion of differentially labeled 70S RNA in quantities too small to influence the eventual hybridization assays. The extracted RNA species were fractionated by rate-zonal centrifugation, and the individual fractions of the sucrose gradients were assayed for their relative content of virus-specific RNA by hybridization.

Virus-specific RNA extracted from whole infected cells at 25 C is extremely heterogeneous with respect to size (Fig. 5a and b) and includes a relatively discrete species which sediments more rapidly than 70S RNA (ca. 90–95S). The condition of the internal marker (70S RNA) shows some evidence of both aggregation and chain scission, but the limited extent of these effects suggests that the extensive heterogeneity of virus-specific RNA is not merely an artifact of either the extraction procedure or subsequent manipulations of the RNA. Extraction of whole cells at 60 C leaves a heterogeneous population of virus-specific RNA, the largest molecules of which sediment at approximately 40–50S (Fig. 5c and d). The loss of the 90–95S species has also been observed after treatment of RNA with dimethylsulfoxide. No virus-specific RNA was detected in the region of the 70S marker in Fig. 5c. This observation confirms our computation that the amount of viral RNA added as internal standard in these studies was insufficient to affect the outcome of the hybridization assay (Fig. 6c).

Much of the native virus-specific RNA in some preparations of cytoplasm from infected cells appears to be 70S RNA (Fig. 6a), although

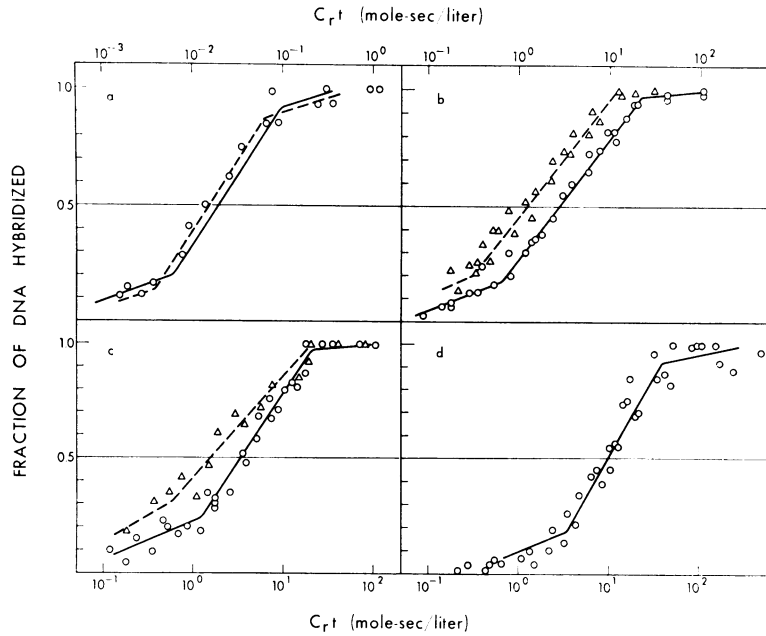

FIG. 4. *Hybridization of viral and cellular RNA species with enzymatic product. ^{3}H-labeled single-stranded DNA was annealed to various RNA species, and the results are expressed as in Fig. 3b. All the data were obtained by varying the concentration of RNA and using a constant period of incubation (4 hr for samples to be analyzed by hydroxyapatite, 4 or 20 hr for those tested with S-1 nuclease; results obtained with the nuclease were independent of incubation time). (a) 70S viral RNA. Solid line, hydroxyapatite (these data are the same as those illustrated in Fig. 3b); ○, S-1 nuclease. (b) Whole cell RNA, extracted at 60 C. △, Hydroxyapatite; ○, S-1 nuclease. Similar results were obtained with RNA extracted at room temperature, although a slightly higher half-C_rt was obtained (see Table 8). (c) Cytoplasmic RNA, extracted at 25 C. △, Hydroxyapatite; ○, S-1 nuclease. (d) Nuclear RNA, extracted at 40 C and treated with an alkylated preparation of deoxyribonuclease. Only the S-1 nuclease assay was used to measure hydrization.*

TABLE 8. *Virus-specific RNA in infected cells*[a]

RNA	Half-C_rt (mole-sec/liter)	% Viral RNA
70S viral	10^{-2} to 2×10^{-2}	100
Whole cell (60 C)	2.8	0.3–0.7
Whole cell (25 C)	4.6	0.18–0.43
Cytoplasm	3.4	0.25–0.6
Nuclei	10	0.1–0.2

[a] The half-C_rt of various RNA species annealed to single-stranded enzymatic product were determined as illustrated in Fig. 4, with S-1 nuclease in the assay for hybridization. The proportion of cellular RNA representing virus-specific sequences is computed by direct comparison of the half-C_rt of cellular and purified viral RNA. Thus, the half-C_rt for whole cell RNA extracted at 60 C is ca. 2.8, indicating that the concentration of virus-specific sequences is approximately 0.3 to 0.7% that in purified viral RNA (half-C_rt 10^{-2} to 10^{-2}).

smaller molecules are also evident (Fig. 6a and b). Extraction at 60 C denatures the 70S cytoplasmic RNA (Fig. 6c), leaving a prominent 35S component (presumably subunits derived from 70S RNA) and a heterogeneous population of virus-specific RNA in the range of 4 to 28S (Fig. 6d). On occasion, preparations of cytoplasmic RNA contain far less 70S RNA, the principal virus-specific species being in the range of 4 to 50S (Fig. 7). We attribute these inconsistencies to variations in the amount of budding virions still associated with cell surfaces following trypsinization.

All of the analyses illustrated in Fig. 5 and 6 were performed with the hydroxyapatite assay for hybridization. We have also compared hydroxyapatite with S-1 nuclease, using cytoplasmic RNA centrifuged as in Fig. 7a. The results with the two procedures are virtually identical (*unpublished observation*).

DISCUSSION

Assays for hybridization. The single-stranded DNA synthesized by the RNA-directed DNA polymerase of RSV is entirely complementary to the 70S RNA of the virus (16, 17). This fact provides a high order of specificity in hybridization procedures and eliminates DNA-DNA interactions from consideration. Hybridization of

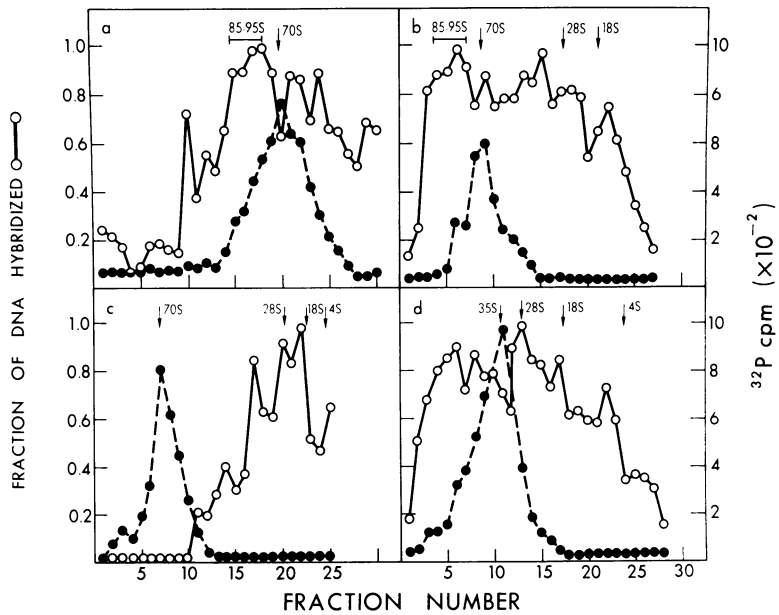

FIG. 5. *Analysis of virus-specific RNA from infected cells. RNA was extracted with SDS-phenol from infected-transformed cells as described in Materials and Methods. Samples (1–2 mg) were centrifuged in gradients of 15 to 30% sucrose (SW 25.3 rotor, 4 C). A portion of each gradient fraction was annealed with ^{3}H-labeled virus-specific DNA (ca. 0.0002 μg) and then analyzed on hydroxyapatite. The results are expressed as the amount of DNA hybridized relative to a standard 70S DNA-RNA hybrid (see Materials and Methods, Table 1, and reference 17) without further normalization. Arrows indicate the positions of cellular (optical density) and viral RNA markers, the latter centrifuged simultaneously in a separate bucket.* ○, *Relative amount of DNA hybridized;* ●, ^{32}*P counts/min. (a) Whole cells extracted at 25 C. A small amount of ^{32}P-70S RNA was added at the outset of the extraction to serve as an internal standard. Centrifugation was at 17,500 rev/min for 15 hr. (b) Whole cells extracted at 25 C. ^{32}P-70S RNA was added as in (a). Centrifugation was at 22,500 rev/min for 17 hr. (c) Whole cells extracted at 60 C. ^{32}P-70S RNA was added immediately prior to centrifugation to serve as an internal sedimentation reference. Centrifugation was at 24,000 rev/min for 17 hr. (d) Whole cells extracted at 60 C. ^{32}P-35S poliovirus RNA was added immediately prior to centrifugation, which was at 24,000 rev/min for 21 hr.*

radio-labeled DNA to RNA has generally been detected by either fractionation on hydroxyapatite (17, 18, 20) or centrifugation in Cs_2SO_4 (16, 25, 26). However, neither of these procedures can discriminate between partial and complete base-pairing of DNA with RNA. We have therefore turned to the use of single strand-specific nucleases to obtain a more stringent criterion for hybridization. At least two of the currently available nucleases (those of *N. crassa* and *A. oryzae*) have been used successfully, although the *Aspergillus* enzyme (S-1 nuclease) is preferable by virtue of its activity over a wide range of NaCl concentrations and the ease with which it can be prepared. The use of nuclease assays such as those developed in this and other (31) laboratories should be of special value in the precise estimation of nucleic acid homologies using DNA-RNA interactions. Similar considerations have led to the use of these enzymes in following the reassociation of denatured DNA (27).

Interaction of virus-specific DNA and viral RNA. The hybrids formed between viral RNA and enzymatic product have a T_m very similar to that of either enzymatically synthesized DNA-RNA hybrids or the homologous double-stranded DNA (Fig. 2). This fact indicates a high degree of fidelity in the base-pairing of nucleotide sequences. Nevertheless, a limited amount of mismatching between complementary sequences can be detected by examining the thermal stability of the hybrids in detail (Fig. 2a). This mispairing may be a consequence of the conditions used for forming the hybrids (0.3 M NaCl, 68 C). It is of no major concern in the work reported here but will have to be considered in any future studies of nucleic acid homologies. Similar results have been obtained with hybrids between enzymatic product and cellular RNA.

The enzymatically synthesized hybrids melt-out over a somewhat broader range of temperatures than do the other double-helical molecules examined here (Fig. 2a). This could reflect the presence in the hybrids of partially double-

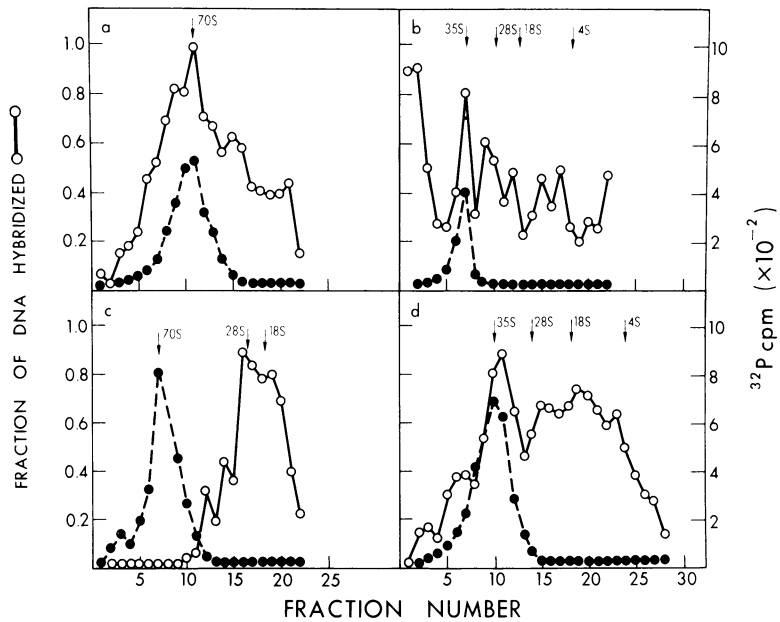

FIG. 6. *Analysis of virus-specific RNA from cytoplasm of infected cells. Cytoplasmic extracts were prepared from infected cells, then extracted with SDS-phenol. Samples of the RNA (1–2 mg) were centrifuged (SW 25.3 rotor) and the gradients analyzed as in Fig. 5. Arrows indicate the position of cellular RNA species (optical density) and viral RNA markers, the latter centrifuged simultaneously in a separate bucket.* ○, *Relative amount of DNA hybridized;* ●, ^{32}*P counts/min. (a) Cytoplasm extracted at 25 C. A small amount of* ^{32}*P-70S RNA was added to the cytoplasm prior to removal of nuclei. Centrifugation was at 23,000 rev/min for 12 hr. (b) Cytoplasm extracted at 25 C.* ^{32}*P-35S poliovirus RNA was added immediately prior to centrifugation at 24,000 rev/min for 21 hr. (c) Cytoplasm extracted at 60 C.* ^{32}*P-70S RNA was added immediately prior to centrifugation at 24,000 rev/min for 17 hr. (d) Cytoplasm extracted at 60 C.* ^{32}*P-35S poliovirus RNA was added immediately prior to centrifugation at 24,000 rev/min for 21 hr.*

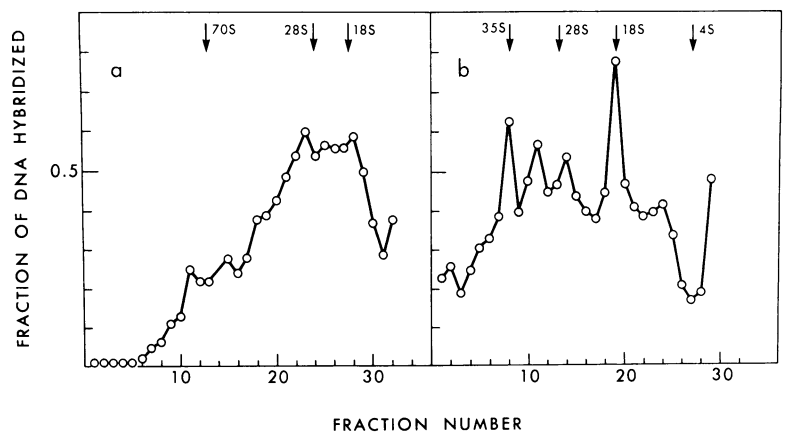

FIG. 7. *Virus-specific RNA in cytoplasmic fractions from infected cells. Cytoplasmic fractions were extracted at 25 C. The RNA was analyzed by centrifugation and subsequent hybridization as in Fig. 5, except that both S-1 nuclease and hydroxyapatite were used with essentially identical results. The illustrated data were obtained with the nuclease. Centrifugation was performed with an SW 41 rotor at 4 C.* ○, *Relative amount of DNA hybridized. (a) Centrifugation for 3.5 hr at 40,000 rev/min, with 50 μg of cytoplasmic RNA. The use of S-1 nuclease permits much longer incubations for hybridization and consequent reductions in the amount of RNA analyzed.* ^{32}*P-labeled 70S RNA was centrifuged in a separate bucket. (b) Centrifugation for 18 hr at 28,000 rev/min, with 50 μg of cytoplasmic RNA.* ^{32}*P-labeled poliovirus RNA (35S) was centrifuged in a separate bucket.*

stranded DNA (ca. 20% of total DNA, see references 14 and 21) which has a T_m slightly higher than that of the hybrids beteween single-stranded DNA and RNA.

The rate at which complementary nucleic acids reassociate, expressed in terms of the convention C_ot (or C_rt), can be used to define the complexity of the nucleotide sequences participating in the reaction (8). This convention has been applied extensively to the characterization of DNA sequences and is also valid for the study of RNA (4). We have obtained a reasonably precise half-C_rt for the interaction between the 70*S* RNA of RSV and complementary DNA (Fig. 3b and 4a). However, estimation of the complexity of the viral RNA on this basis would be premature because RNA species of known complexity have yet to be analyzed under identical circumstances.

Virus-specific RNA in cells infected with RSV. The data presented above provide a preliminary survey of the nature of virus-specific RNA present in cells transformed by and producing RSV (Fig. 5–7). The preponderance of 70*S* RNA in some cytoplasmic fractions is most likely due to the presence of incomplete virions still associated with cell surfaces following trypsinization. Preliminary results in this laboratory indicate that all virus-specific RNA isolated from polyribosomes is smaller than 70*S* and quite heterogeneous in size (*unpublished observations*). The most striking feature of virus-specific RNA obtained from unfractionated cells is the presence of molecules which sediment more rapidly than 70*S* RNA and which are presumably derived from nuclei. This latter conclusion requires confirmation by analysis of RNA extracted from nuclei under conditions which do not disrupt secondary structure. The sedimentation coefficient of 90 to 95*S* is apparently not an artifact of the extraction procedure per se, but the RNA in question does undergo a major transition as a consequence of thermal or solvent denaturation. Experiments to define the precise products of this transition and to further characterize the native 90 to 95*S* RNA are now in progress. It is conceivable that this material represents covalently linked sequences of cellular and viral RNA analogous to those found in cells infected with DNA tumor viruses (19, 33).

The nuclear RNA used in the present studies was extracted at 40 C in an effort to preserve all secondary structure of viral RNA. For logistical reasons, we have yet to analyze nuclear RNA by the procedures illustrated in Fig. 5 to 7. However, electrophoresis in gels of 2.25% polyacrylamide has confirmed the purity of the nuclear preparations (judged by the absence of 18*S* RNA), and the RNA is otherwise similar to that extracted from nuclei of HeLa cells at either 40 or 60 C (*unpublished observations*). We presently have no data concerning the relative yields of RNA at these two temperatures, but the amount of virus-specific RNA detected in nuclear preparations (Fig. 4d) is considerably lower than that reported for a murine system by Green et al. (18). It remains to be determined whether this discrepancy is simply a function of differences in extraction procedures.

We conclude that virus-specific RNA is present in both nuclei and cytoplasm of cells producing RSV and that this RNA is remarkably heterogeneous in size. The present data pertain only to single-stranded RNA with nucleotide sequences identical to those of the viral genome. To date, we have been unable to detect RNA complementary to 70*S* RNA, using the appropriate DNA sequences prepared from denatured double-stranded molecules synthesized by the RNA-directed polymerase of RSV (*unpublished observations*).

Virus-specific RNA in normal avian cells. Normal avian embryos frequently contain a group-specific antigen of avian leukosis-sarcoma viruses (22) and therefore might also contain detectable amounts of virus-specific RNA. Our preliminary results indicate that this is the case (*work in progress*). By contrast, we have yet to detect viral RNA in avian cells which are devoid of viral antigen (Table 7) when tested by complement fixation by the method of Vogt and Friis (32). These results may change when the assays for virus-specific RNA are repeated at a higher level of sensitivity.

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