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Reticuloendotheliosis Virus Nucleic Acid Sequences in Cellular DNA

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Reticuloendotheliosis virus 60S RNA labeled with ^{125}I, or reticuloendotheliosis virus complementary DNA labeled with ^{3}H, were hybridized to DNAs from infected chicken and pheasant cells. Most of the sequences of the viral RNA were found in the infected cell DNAs. The reticuloendotheliosis viruses, therefore, replicate through a DNA intermediate. The same labeled nucleic acids were hybridized to DNA of uninfected chicken, pheasant, quail, turkey, and duck. About 10% of the sequences of reticuloendotheliosis virus RNA were present in the DNA of uninfected chicken, pheasant, quail, and turkey. None were detected in DNA of duck. The specificity of the hybridization was shown by competition between unlabeled and ^{125}I-labeled viral RNAs and by determination of melting temperatures. In contrast, ^{125}I-labeled RNA of Rous-associated virus-O, an avian leukosis-sarcoma virus, hybridized 55% to DNA of uninfected chicken, 20% to DNA of uninfected pheasant, 15% to DNA of uninfected quail, 10% to DNA of uninfected turkey, and less than 1% to DNA of uninfected duck.

Avian leukosis-sarcoma viruses (ALV), and reticuloendotheliosis viruses (REV) form the two groups of avian ribodeoxyviruses (15). The virions of both ALV and REV have C-type morphology and contain 60S RNA and a DNA polymerase. ALV and REV differ in the antigenicity of their virion proteins, the nucleotide sequences of their 60S RNA, and in their biological effects on young fowl and on avian fibroblasts in cultures. In addition, there are some serological relationships between purified DNA polymerases from ALV and REV virions (8).

ALV replicate through a DNA intermediate, the DNA provirus, and in chicken DNA there are endogenous nucleic acid sequences homologous to much of the genome of a nontransforming ALV (15). In this paper, we report experiments using nucleic acid hybridization to determine if REV replicate through a DNA intermediate and to compare the distribution in DNA of several different fowl of endogenous nucleic acid sequences related to ALV and REV RNAs.

MATERIALS AND METHODS

Cells and viruses. The sources of cells and viruses and a general description of methods for growing cells and propagating viruses have been published (6, 7, 16). Virus production was monitored by measurement of sedimentable exogenous DNA polymerase activity in the cell-free culture media from infected cells (16).

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Trager duck spleen necrosis virus (TDSNV), reticuloendotheliosis virus (strain T) (REV-T), duck infectious anemia virus (DIAV), chick syncytial virus (CSV), and Rous-associated virus-61 (RAV-61) were propagated in C/E, avian leukosis virus (ALV)-negative, chick helper factor-negative, ALV group-specific antigen-negative chicken cells. TDSNV was also grown in pheasant cells.

Rous-associated virus-O (RAV-O) was harvested from line 100 chicken cells in culture (18). (The cells were a kind gift of H. Hanafusa.)

Culture fluids were harvested every 24 h from virus-producing cells and were stored at −10 C. Virus was concentrated and purified as described previously (1, 3).

Cultured chicken REV cells were prepared as follows: REV-T was injected intra-abdominally into a 1-day-old chick. Fourteen days after infection, the moribund chicken was sacrificed, and the spleen was removed. The spleen of the REV-T-infected chicken was enlarged with splenitis and follicular hyperplasia. The spleen cells were cultured in vitro using standard techniques. The cultured spleen cells clumped together, did not form a monolayer like spleen cells from uninfected birds, and grew rapidly in suspension.

Preparation of nucleic acid. RNAs from cells and purified viruses were prepared with the technique previously described (6, 14). DNAs from chicken and pheasant cells in culture, chicken embryos, and livers of an adult pheasant (obtained from the Poynette Game Farm of the Department of Natural Resources, Wis.), an adult turkey (obtained from Kohl's Turkey Farm, Columbus, Wis.), an adult Pekin duck, an adult Muscovy duck (both obtained from W. Thrun, Madison, Wis.), and an adult Japanese quail (ob-

tained from Department of Poultry Science, University of Wisconsin, Madison) were prepared as follows: cultured cells, embryos, or the livers of adult birds were washed three times in phosphate-buffered saline (pH 7.2), resuspended in reticulocyte standard buffer [0.01 M NaCl, 0.0015 M $MgCl_2$, and 0.01 M Tris-hydrochloride (pH 7.5)], chopped with scissors into small pieces, and homogenized in a tight-fitting glass Dounce homogenizer. The homogenates were centrifuged at 8,000 rpm (7,700 × *g*) for 10 min in a Sorvall SS34 rotor, and the nuclei and membrane pellets were resuspended in 10 volumes of 0.01 M Tris-hydrochloride (pH 7.5) containing 0.075 M NaCl and 0.01 M EDTA. Sodium dodecyl sulfate (SDS) (0.5%) and 500 μg of predigested Pronase (Calbiochem) per ml were added, and the mixtures were incubated for 1 h at 37 C with occasional mixing. The DNAs were then extracted once with an equal volume of double distilled phenol saturated with 0.01 M Tris-hydrochloride (pH 7.5) and twice with equal volumes of chloroform-isoamyl alcohol (100:1). The phenol and the chloroform-isoamyl alcohol phases were reextracted with 0.01 M Tris-hydrochloride buffer containing 0.075 M NaCl and 0.01 M EDTA, and the buffers were added to the first aqueous phase. The DNAs in the aqueous phase were precipitated with 3 volumes of 100% ethanol. The precipitated DNAs were spooled out on a pipet and washed twice in 100% ethanol. Approximately 90% of the radioactively labeled DNA from chicken fibroblasts was recovered by this method.

Preparation of DNA for hybridization. The precipitated DNAs were dried by blowing nitrogen gas on them and were resuspended to a final concentration of approximately 5 mg/ml in 0.01 M Tris-hydrochloride (pH 7.5) containing 0.075 M NaCl and 0.01 M EDTA, and then were sonicated in an ice bath at 50 W setting for 90 s by a Sonifier cell disrupter model W185D with microneedle. The DNAs after sonication were approximately 7*S* as determined by sucrose velocity gradient centrifugation. The sonicated DNAs were treated with NaOH at a final concentration of 0.5 N in a 37 C water bath for 1 h. The alkali-treated DNAs were neutralized with HCl to pH 7.5, extracted twice with chloroform-isoamyl alcohol, and precipitated with 3 volumes of 100% ethanol at −20 C. The precipitated DNAs were collected by centrifugation at 10,000 rpm (12,000 × *g*) for 10 min in a Sorvall SS34 rotor, and the DNA pellets were resuspended in 0.1 M Tris-hydrochloride (pH 7.5). The concentration of DNA was measured by optical density at 260 nm. One optical density unit at 260 nm was taken as 50 μg of DNA. The ratio of absorbance at 260 nm and that at 280 nm was approximately 1.85 for all DNA preparations. The recovery of DNA was approximately 90%. The final concentration of DNA was made approximately 20 mg/ml in 0.1 M Tris-hydrochloride (pH 7.5).

Preparation of viral 60S RNA for iodination. TDSNV and RAV-O were purified by equilibrium centrifugation in 15 to 65% sucrose density gradients made in 0.02 M Tris-hydrochloride (pH 7.5) containing 0.1 M NaCl and 0.001 M EDTA (TSE) and were concentrated by centrifugation at 25,000 rpm for 75 min in a Spinco SW27 rotor. The pelleted virus was resuspended in about 0.3 ml of TSE buffer. SDS (0.5%) and 250 μg of predigested Pronase per ml were added to the virus suspension, and the viral proteins were digested at room temperature (about 25 C) for 15 min. Approximately 0.2 ml of Pronase-digested virus sample was layered on 5 ml of a linear 10 to 30% sucrose gradient made in TSE buffer, and the gradient was centrifuged at 50,000 rpm (234,000 × *g*) for 80 min at 15 C in a Spinco SW50.1 rotor. Approximately 35 fractions were collected from each gradient through a hole pierced in the bottom of the tube. The optical density of each fraction was measured at 260 nm. The peak in the 60*S* region of the gradient was pooled, and the RNA was extracted with the method previously described (6). ^{14}C-labeled chicken ribosomal and 4*S* RNAs were used as markers in a parallel gradient.

Radioiodination of viral 60S RNA with ^{125}I. The purified viral 60*S* RNA was iodinated with a modification of the technique of Scherberg and Refetoff (13). Approximately 5 μg of viral 60*S* RNA was labeled with carrier-free ^{125}I as NaI (Amersham-Searle). The iodination reaction contained in 0.1 ml: 5 μg of viral RNA, 2 mCi of ^{125}I, 10 μM KI, and 1 mM $TICl_3$ in 0.1 M ammonium acetate buffer (pH 5.0). The reaction mixture was sealed in a 100-μliter Yankee micropipette (Clay Adams) and incubated at 63 C in a water bath for 1 h. After the incubation, the reaction mixture was chilled in an ice bath, and 0.1 ml of 0.1 M Tris-hydrochloride containing 0.01 M sodium sulfite and adjusted to pH 8.7 was added. The labeled viral RNA was separated from the free ^{125}I by filtration on a Sephadex G25 column (5 by 0.5-cm bed volume) equilibrated and eluted with 0.1 M ammonium acetate buffer (pH 5.0). Approximately 30 fractions of 0.2 ml were collected. Five microliters of each fraction was precipitated with 5 ml of 10% ice-chilled trichloroacetic acid in the presence of 500 μg of bovine serum albumin as carrier.

The trichloroacetic acid-precipitable ^{125}I counts per minute were counted in a Nuclear Chicago γ-counter. The [^{125}I]RNA peak was pooled and dialyzed at 4 C overnight against 1,000 volumes of 0.01M Tris-hydrochloride (pH 7.3) containing 0.1 M NaCl. RNase sensitivity of the dialyzed ^{125}I-labeled RNA was determined using 100 μg of RNase A per ml (Worthington Biochemical Corp.) and 50 IU/ml of RNase T1 (Calbiochem) in 0.02 M Tris-hydrochloride (pH 7.3) containing 0.4 M NaCl. Approximately 4% of the ^{125}I-labeled TDSNV RNA was resistant to RNase digestion, whereas approximately 40% of the RAV-O ^{125}I-labeled RNA was resistant to RNase digestion. The ^{125}I-labeled RAV-O RNA was, therefore, further purified in a Cs_2SO_4 equilibrium density gradient with the method previously described (5). ^{125}I-labeled RAV-O RNA banding in approximately the 1.65 g/cm^3 region of the gradient was pooled and dialyzed at 4 C overnight against 1,000 volumes of 0.01 M Tris-hydrochloride buffer (pH 7.3) containing 0.1 M NaCl. Over 95% of this purified RAV-O [^{125}I]RNA was RNase sensitive. The specific activities of the TDSNV

and RAV-O ^{125}I-labeled RNAs were approximately 1.3 $\times$ 10^7 counts/min per μg (5 $\times$ 10^7 dpm/μg) assuming that all of the input RNA was recovered.

Synthesis and purification of TDSNV product DNA. The standard DNA polymerase reaction of Mizutani et al. (9) was used. Exogenous DNA polymerase reactions were carried out in a complete reaction mixture containing TDSNV RNA and purified DNA polymerase from Rous sarcoma virus-Rous-associated virus-O (RSV-RAV-O) (a kind gift of S. Mizutani) (8).

The reaction mixtures contained 100 nmol of dATP, 75 nmol of ATP, 18.75 μmol of $MgCl_2$, 25 μmol of KCl, 24 μg of phosphoenol pyruvate, 100 μg of pyruvate kinase, 37.5 μCi (1 nmol) of [^{3}H]-dTTP, 37.5 μCi (1.3 nmol) of [^{3}H]dCTP, and 37.5 μCi (2.4 nmol) of [^{3}H]dGTP in 1 ml of 20 mM Tris-hydrochloride (pH 8.0) containing 0.4 mM EDTA, 5 mM dithiothreitol, and 125 μg of actinomycin D. Two hundred fifty microliters of 0.01 M Tris-hydrochloride (pH 7.3) containing 0.1 M NaCl, 25% glycerol, approximately 50 μg of purified TDSNV RNA, and about 25 μg of purified RSV-RAV-O DNA polymerase were added to 1 ml of the reaction mixture, and the 1.25 ml of complete reaction mixture was incubated at 39 C for 3 h. At the end of the DNA polymerase reaction, 0.5% of SDS and 250 μg per ml of predigested Pronase were added to the reaction mixture which was incubated at 37 C for 30 min. Two hundred fifty micrograms of heat-denatured calf thymus DNA (Calbiochem) were then added as carrier, and the DNA product was extracted and purified as described previously (6). Approximately 2 $\times$ 10^6 dpm of TDSNV-DNA product was synthesized from the reaction described above. The specific activity of the TDSNV DNA product was calculated to be approximately 150 dpm/pg. The size of the TDSNV DNA product was approximately 5 to 7*S* as determined by sucrose gradient centrifugation using M13 phage DNA, and chicken 4*S* and ribosomal RNAs as markers. The TDSNV DNA product consisted of approximately 8% double-stranded DNA as determined by S1 nuclease digestion (6).

The radioactive deoxyribonucleoside triphosphates were purchased from Schwarz/Mann.

Preparation of [^{3}H]labeled cellular DNA. Five cultures of chicken, pheasant, and Muscovy duck embryo fibroblasts were grown for 3 days in 100-mm petri dishes in 12 ml of modified Eagle minimum essential medium with 20% tryptose phosphate broth containing, respectively, 5% calf serum and 2% fetal bovine serum, 4% fetal bovine serum, and 5% calf serum in the presence of 50 μCi of [^{3}H]TdR (17 Ci/mmol) (Schwarz/Mann) per dish. Cells were harvested by scraping with a rubber policeman. The cells were washed with phosphate-buffered saline (pH 7.2), and the ^{3}H-labeled DNAs were extracted with the techniques described above. The ^{3}H-labeled DNAs were sonicated and alkali-treated before annealing with cold DNA from cells. The specific activities of the ^{3}H-labeled DNAs were 3,100 counts/min per μg, 2,400 counts/min per μg, and 5,500 counts/min per μg, respectively, for chicken, pheasant, and duck DNA.

Nucleic acid hybridization. The hybridization mixtures contained 1 M NaCl, 0.1% SDS, 0.001 M EDTA, and 0.05 M Tris-hydrochloride (pH 7.3). Annealing was carried out at 63 C by using 25 μliters of annealing mixture in sealed 25-μliter Yankee disposable micropipettes. For ^{3}H-labeled viral DNA and infected cell RNA hybridizations, 5 mg of RNA per ml was used. For the [^{125}I]RNA and cellular DNA hybridizations, approximately a 100-fold excess of virus-specific cellular DNA was used in the standard hybridization buffer containing 300 ng of yeast RNA in 25 μliters. For [^{3}H]DNA and unlabeled cellular DNA annealing, 10 mg of unlabeled cellular DNA per ml was used with ^{3}H-labeled virus DNA product or ^{3}H-labeled cellular DNA, and the annealing times were varied. Samples were frozen immediately after different times of incubation and were stored at −60 C until all samples were collected.

The extent of hybridization was analyzed by digestion by RNase A and RNase T1 for the [^{125}I]RNA and by S1 nuclease digestion for the [^{3}H]DNA as described previously (6). For RNase digestion, samples were digested in 0.02 M Tris-hydrochloride (pH 7.3) containing 0.4 M NaCl at 37 C for 45 min with 100 μg of heat-treated RNase A per ml and 50 IU of heat-treated RNase T1 per ml. The amount of trichloroacetic acid-precipitable ^{125}I counts was determined. The conditions for S1 nuclease digestion using excess single-stranded specific S1 nuclease have been described (6). The C_0t (concentration of deoxyribonucleotides in moles/liter $\times$ time of annealing in seconds) values were corrected to phosphate concentration of 0.12 M according to Britten and Smith (2).

Competition hybridization with viral RNA. The standard hybridization mixtures contained 10 mg of TDSNV-infected chicken cell DNA per ml, approximately 0.1 ng of TDSNV ^{125}I-RNA, and varying amounts of competing cold RNAs from TDSNV, REV-T, CSV, DIAV, and RAV-61. The reaction mixtures were incubated at 63 C for 120 h to achieve C_0t values of approximately 8 $\times$ 10^4 mol/liter $\times$ s. The extent of [^{125}I]RNA hybridization was determined by RNase digestion in 0.4 M NaCl as previously described (6).

Determination of melting temperature of hybrids. RNA-DNA hybrids and double-stranded DNAs were made by hybridization of virus-specific [^{3}H]DNA product or [^{125}I]RNA and cellular DNAs at C_0t values of greater than 5 $\times$ 10^4 mol/liter $\times$ s. The melting temperatures were then determined by incubating samples at the designated temperatures for 20 min, followed by RNase digestion in 0.4 M NaCl or S1 nuclease digestion as previously described (6).

RESULTS

Extent of copying of TDSNV RNA into DNA. The approximately 20-fold increase in the specific activity of ^{125}I-labeled viral RNA over ^{32}P-labeled RNA allowed us to study the complexity of the TDSNV ^{3}H-labeled DNA product (6). Approximately 50 ng of TDSNV

^{3}H-labeled DNA product was prepared from a 3-h DNA polymerase reaction in 5 ml of reaction mixture containing about 200 μg of TDSNV RNA, 100 μg of purified RSV-RAV-O DNA polymerase, and 100 μg of actinomycin D per ml. A constant amount of TDSNV ^{125}I-labeled RNA was hybridized with different amounts of the TDSNV DNA product for 40 h, and the RNase resistance of the TDSNV ^{125}I-labeled RNA was determined (Fig. 1).

After annealing 0.5 ng of TDSNV[^{3}H]DNA product to 540 counts/min of TDSNV ^{125}I-labeled RNA (a DNA-RNA ratio of approximately 10:1) at a C_0t value of 2×10^{-2} mol/liter × s, approximately 45% of the TDSNV RNA became resistant to RNase digestion. Six nanograms of TDSNV[^{3}H]DNA product protected over 75% of the TDSNV[^{125}I]RNA (a DNA-RNA ratio of approximately 120:1) at a C_0t value of approximately 2.4×10^{-1} mol/liter × s. Thus, the TDSNV[^{3}H]DNA product prepared from an exogenous DNA polymerase reaction contained most of the sequences of TDSNV RNA.

However, the exact distribution of the relative abundances of the sequences in the DNA product could not be determined. An increasing portion of [^{125}I]RNA was protected when the C_0t values were increased by addition of more [^{3}H]DNA product to the annealing mixture (Fig. 1). The increase in the percent of [^{125}I]-RNA protected with the increase in the amount of DNA product used in the annealing mixture indicates that the TDSNV DNA product may contain the entire sequence of TDSNV RNA.

TDSNV-specific DNA sequences in cells chronically infected and producing TDSNV. To determine whether REV replicate through a DNA intermediate like ALV, radioactively labeled virus-specific DNA or RNA were hybridized to DNAs from virus-infected cells.

^{3}H-labeled TDSNV DNA product was annealed to DNAs extracted from TDSNV-infected chicken and pheasant fibroblasts and to DNA extracted from reticuloendotheliosis cells originally isolated from the spleen of a REV-T-infected chicken (Fig. 2). The TDSNV-infected, virus-producing chicken and pheasant cells, and the REV-T-infected chicken spleen cells contained virus-specific DNA sequences. Almost all of the TDSNV[^{3}H]DNA product hybridized to the DNA extracted from the TDSNV-infected chicken cells. The half C_0t value for this hybridization indicates that the TDSNV-infected chicken cells contained approximately five copies of virus-specific DNA sequences per haploid chicken cell genome. REV-T-infected chicken spleen cells contained

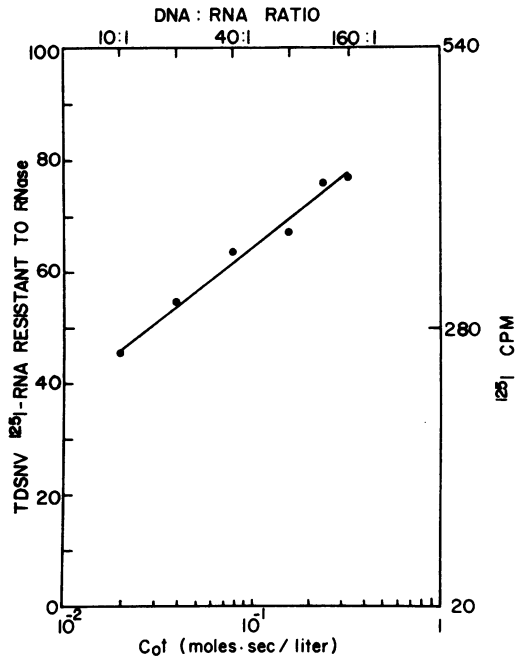

FIG. 1. *Protection of TDSNV [^{125}I]RNA against RNase digestion after hybridization with TDSNV [^{3}H]DNA product. ^{3}H-labeled TDSNV DNA product was made from an exogenous DNA polymerase reaction using purified TDSNV RNA and purified RSV-RAV-O DNA polymerase as described. The specific activity of the DNA product was approximately 150 dpm/pg. The TDSNV 60S RNA was iodinated with ^{125}I as described. The specific activity of the [^{125}I]-RNA was estimated as about 1.3×10^7 counts/min per μg (5×10^7 dpm/μg). TDSNV [^{125}I]RNA (540 counts/min) was hybridized with 0.5, 1, 2, 4, 6, and 8 ng of TDSNV [^{3}H]DNA, giving approximate DNA-RNA ratios of from 10:1 to 160:1. The hybridizations were carried out in 1 M salt for 40 h under the conditions described. The C_0t values for Fig. 1 have not been corrected for salt concentration since there are no available standards for correcting values for hybridization between single-stranded DNA and single-stranded RNA. At the end of the 40-h incubation, samples were digested with RNase, and trichloroacetic acid-precipitable [^{125}I]counts were determined. One hundred percent of the [^{125}I]counts was 540 counts/min, and the RNase-resistant background counts were 20 counts/min. The background was subtracted before the calculation of the percent RNase-resistant RNA.*

a similar number of copies of virus DNA sequences, whereas the TDSNV-infected pheasant cells apparently had approximately 10 copies per haploid cell genome. However, we were not able to achieve 100% hybridization of the TDSNV[^{3}H]DNA product with DNAs extracted from the pheasant and chicken spleen cells. This failure to achieve 100% hybridization

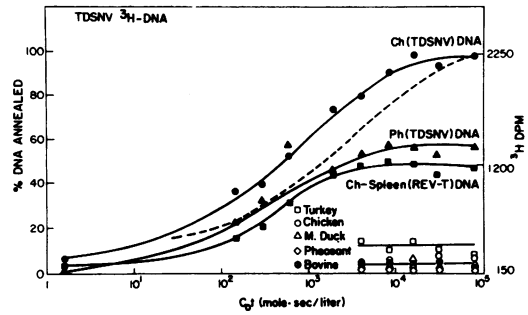

FIG. 2. *Kinetics of hybridization of TDSNV DNA product to DNAs of infected and uninfected cells. ³H-labeled TDSNV DNA was annealed with 10 mg of infected and uninfected cell DNAs per ml. DNAs from infected chicken and pheasant fibroblasts were prepared from cells grown and infected in culture. The chicken spleen (REV-T) DNA was prepared from cultured chicken cells taken from the spleen of a new-born chicken infected with REV-T. The cells were harvested by centrifugation, and DNA was extracted with the standard techniques described. DNAs from uninfected pheasant, turkey, and Muscovy duck were extracted from the livers of adult birds. Uninfected chicken DNA was extracted from pooled livers of 14-day chicken embryos which were ALV group-specific antigen-negative. The calf thymus (bovine) DNA was purchased from Calbiochem. After different times of hybridization, samples were withdrawn and were frozen at −60 C. At the end of the final incubation, the extent of hybridization was determined by S1 nuclease digestion. The results are expressed as the proportion of the total [³H]DNA hybridized at a given C_0t value corrected according to Britten and Smith (2). Approximately 7% (150 dpm) of S1 nuclease-resistant [³H]DNA was found in controls incubated without DNA. The background was subtracted before the calculation of % DNA annealed. The dotted line represents the kinetics of reassociation of unique sequences of chicken cell DNA (data from Fig. 4).*

may relate to a different state, that is, not integrated, of the viral DNA in the pheasant and chicken spleen cells.

The uninfected turkey liver DNA hybridized about 10% of the virus-specific DNA, whereas the uninfected chicken cell DNA hybridized approximately 5% of the virus DNA. Uninfected pheasant and Muscovy duck DNA hybridized no significant amounts of virus-specific DNA.

Although the ³H-labeled TDSNV DNA product represented over 75% of the TDSNV genome, we do not know the molar ratio of the DNA products or the minimal DNA-RNA ratio required to protect 100% of virus RNA. Therefore, to determine the fraction of virus nucleic acid sequences present in these cells we used ¹²⁵I-labeled 60S RNA from TDSNV, which represents the entire viral genome, for hybridization. Figure 3 shows that over 70% of TDSNV ¹²⁵I-labeled RNA hybridized to DNA extracted from TDSNV-infected chicken fibroblasts. The failure to achieve 100% hybridization resulted from the more rapid rate of DNA-DNA reassociation than RNA-DNA hybridization (see ref. 12, and compare Fig. 2 and 3). The uninfected chicken cells contained about 10% of the TDSNV-specific nucleic acid sequences.

We conclude from these data that the REV replicate through a DNA provirus intermediate like the avian leukosis-sarcoma viruses.

Endogenous virus-specific sequences in different avian species. The results of the experiments described in Fig. 2 and 3 indicated that uninfected avian cells contained a small portion of TDSNV-specific DNA sequences.

Before studying further the distribution of endogenous virus nucleic acid sequences, we determined the extent of cross-hybridization between DNAs of several species of fowl. We prepared ³H-labeled DNA of chicken, pheasant,

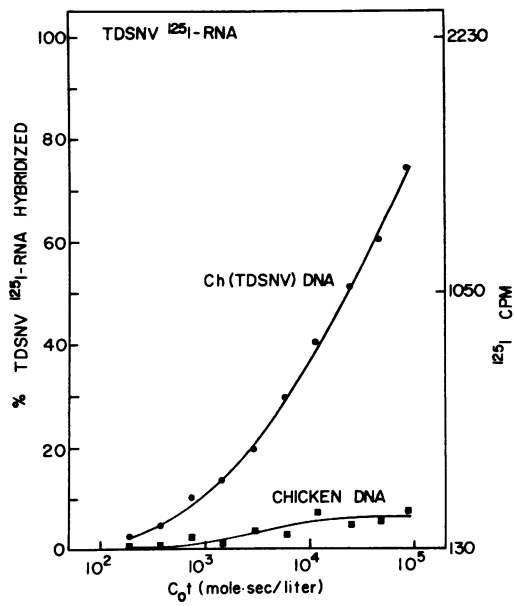

FIG. 3. *Kinetics of hybridization of TDSNV ¹²⁵I-labeled RNA to DNAs from TDSNV-infected and uninfected chicken cells. TDSNV ¹²⁵I-labeled RNA (2,300 counts/min) was hybridized for different times with 250 μg of DNAs from TDSNV-infected and uninfected chicken cells in 25 μliters of standard hybridization buffer. The conditions of hybridization and the presentation of the results are the same as in Fig. 2 with the exception that the extent of hybridization was determined by RNase A and RNase T1 digestion (Fig. 1) instead of S1 nuclease digestion. The RNase-resistant background counts (130 counts/min) were subtracted before the calculation of percentage hybridized.*

and duck. The extent of reassociation was determined by S1 nuclease digestion (Fig. 4). Using chicken [^{3}H]DNA, we found that there was more homology between chicken DNA and turkey DNA than between chicken DNA and Pekin or Muscovy duck DNAs. Similarly, using a pheasant [^{3}H]DNA probe, we found more homology between pheasant DNA and chicken and turkey DNAs than between pheasant DNA and Muscovy duck DNA. When a Muscovy duck [^{3}H]DNA probe was used, we found more homology between Muscovy and Pekin duck DNAs than between Muscovy duck DNA and chicken and turkey DNAs. These studies confirmed the previous classification of these fowl. Pheasants and turkeys are in the same order as chickens, whereas ducks are in a different order, but still in the same class as chickens.

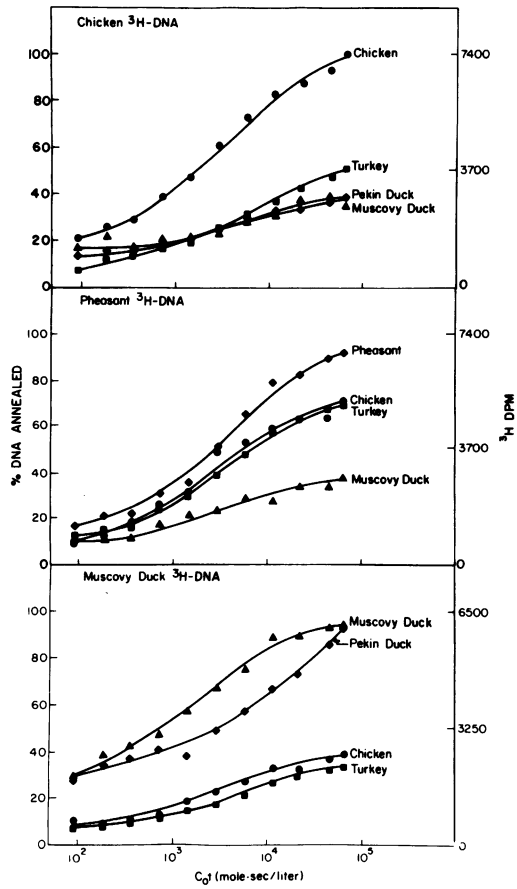

FIG. 4. *Kinetics of cellular DNA reassociation. ^{3}H-labeled DNAs were prepared from chicken, pheasant, and Muscovy duck fibroblasts in culture. Unlabeled DNAs from pheasant, turkey, Pekin duck, and Muscovy duck were prepared from livers of adult birds, and unlabeled chicken DNA was prepared from ALV group-specific antigen-negative chicken embryo livers as described in the legend to Fig. 2. The extent of hybridization was determined by S1 nuclease digestion. The S1 nuclease-resistant [^{3}H]DNAs of chicken, pheasant, and Muscovy duck in background controls incubated without unlabeled DNA were between 10 and 15%. The high background is probably due to the reassociation of repetitive sequences of ^{3}H-labeled DNAs. The background counts were subtracted.*

To look for endogenous nucleic acid sequences related to ALV and REV in normal avian cells, nucleic acid hybridization experiments were performed using virus ^{125}I-labeled RNAs and normal cell DNAs.

Table 1 shows the amount of endogenous ALV nucleic acid sequences in five different avian DNAs. We hybridized RAV-O ^{125}I-labeled RNA with DNAs of chicken, pheasant, quail, turkey, and Pekin duck. We found approximately 55% of the RAV-O [^{125}I]RNA hybridized with DNA extracted from normal ALV group-specific antigen-negative chicken cells, whereas about 20% of the RAV-O sequences were present in normal pheasant DNA, 15% in normal quail DNA, 10% in normal turkey DNA, and none were detected in Pekin duck DNA. There was a good correlation between the amount of endogenous RAV-O nucleic acid sequences in avian cell DNA and the closeness of relationship of the avian DNA to chicken DNA (Fig. 4).

TDSNV ^{125}I-labeled RNA was hybridized

TABLE 1. *Endogenous virus-specific sequences in DNAs of different fowl*[a]

[^{125}I]RNA	Sources of DNA					
	Chicken[b]	Pheasant	Quail	Turkey	Duck	Calf thymus
TDSNV	10[c]	10	10	10	<2	<2
RAV-O	55	20	15	10	<1	<1

[a] The sources of cell DNAs were the same as in Fig. 2 and 4, and the conditions for hybridization were identical to those of Fig. 3. Twenty-two hundred counts/min of TDSNV [^{125}I]RNA or three thousand counts/min of RAV-O [^{125}I]RNA were used. TDSNV-infected chicken cell DNA protected about 60% of the TDSNV [^{125}I]RNA, and chicken line 100 DNA protected approximately 50% of RAV-O [^{125}I]RNA at C_0t values of 8×10^4 mol/liter $\times$ s. The RNase-resistant background counts of TDSNV and RAV-O [^{125}I]RNAs were 130 and 140 counts per min, respectively. The background counts were subtracted before calculation of percentage of hybridization.

[b] DNA extracted from livers of 14-day-old embryos.

[c] Percentage of [^{125}I]RNA hybridized at saturation of experiments like that of Fig. 3. Average of two separate experiments.

with 10 mg of DNAs per ml from chicken, pheasant, quail, turkey, and duck cells to C_0t values of 8×10^4 mol/liter $\times$ s. Approximately 10% of TDSNV ^{125}I-labeled RNA hybridized to DNA of chicken, pheasant, quail, and turkey (Table 1). In contrast, no detectable amount of TDSNV ^{125}I-labeled RNA hybridized to Pekin duck DNA.

To test the specificity of these low levels of TDSNV hybridization, we carried out a competition hybridization experiment using an excess of unlabeled TDSNV RNA. TDSNV ^{125}I-labeled RNA was hybridized at C_0t values of about 8×10^4 mol/liter $\times$ s to DNAs from uninfected chicken and turkey in the presence of two different concentrations of unlabeled TDSNV RNA. The extent of hybridization was determined by RNase digestion. The unlabeled TDSNV RNA competed with all of the hybridizable ^{125}I-RNA (Table 2). Accordingly, we conclude that uninfected chicken and turkey, and presumably pheasant and quail, cells contain a fraction of REV-specific endogenous DNA sequences.

The fidelity of the hybridization of the TDSNV[^{3}H]DNA product to the DNAs from virus-infected cells and of the TDSNV ^{125}I-labeled RNA to the DNAs from the virus-infected and uninfected cells were determined by measuring the melting temperatures of the hybrids. Hybrids of TDSNV ^{3}H-labeled DNA and DNA of TDSNV-infected chicken cells, TDSNV[^{125}I]RNA and DNA of TDSNV-infected chicken cells, TDSNV[^{125}I]RNA and DNA of uninfected chicken, and TDSNV[^{125}I]-RNA and DNA of uninfected turkey were prepared by standard hybridization reactions to C_0t values of 8×10^4 mol/liter $\times$ s. Portions of the double-stranded DNAs and of the hybrids were incubated at different temperatures for 20 min. The fractions of the double-stranded DNA and of the hybrids which were denatured were determined by either S1 nuclease or RNase digestion. The melting curves presented in Fig. 5 demonstrate that extensive mismatching did not occur. The melting temperatures measured in 0.05 M Tris-hydrochloride (pH 7.3) containing 1 M NaCl, 0.1% SDS, and 0.001 M EDTA, were 83 C for double-stranded DNA of TDSNV[^{3}H]DNA and DNA from TDSNV-infected chicken cells, 87 C for hybrids between TDSNV[^{125}H]RNA and DNA from TDSNV-infected chicken, 85 C for hybrids between TDSNV[^{125}I]RNA and DNA from uninfected chicken, and 88 C for hybrids between TDSNV[^{125}I]RNA and DNA from uninfected turkey.

TABLE 2. *Competition between unlabeled TDSNV RNA and TDSNV [^{125}I]RNA for hybridization with uninfected cell DNA*[a]

DNA	Unlabeled TDSNV RNA (μg)		
	0	1.8	3.6
Chicken	300[b]	30	0
Turkey	310	0	0

[a] One hundred microliters of standard hybridization mixture contained 1 mg of cell DNA, 4,100 counts/min (300 pg) of TDSNV [^{125}I]RNA, and the indicated amounts of competing cold TDSNV RNA. Hybridization was carried out at 63 C for 120 h to achieve a C_0t value of 8×10^4 mol/liter $\times$ s. The extent of hybridization was determined by RNase digestion as described. Approximately 6% (230 counts/min) of RNase-resistant [^{125}I]RNA was found in controls incubated without RNA. The background was subtracted.

[b] ^{125}I-counts/min hybridized from 4,100 counts/min of input TDSNV [^{125}I]RNA. Average of duplicate samples.

Relationships between REV RNAs as studied by competition hybridization. The extent of nucleic acid sequence homology between the four members of the REV group was studied by competition hybridization (Fig. 6). The extent of hybridization obtained at a C_0t value of 8×10^4 mol/liter $\times$ *s* in the absence of competing unlabeled RNA was normalized to 100% to facilitate comparisons. The addition of unlabeled TDSNV RNA in the hybridization reaction between TDSNV[^{125}I]RNA and DNA from TDSNV-infected chicken cells reduced by 97% the fraction of [^{125}I]RNA entering hybrids. When the unlabeled RNAs from REV-T, CSV, and DIAV were used at the same concentrations, the same degrees of competition were observed. In contrast, RAV-61 RNA did not compete with REV RNA. These results are consistent with our previous results (6) that the sequences of the RNAs of all four members of the REV group were closely related, whereas there was no detectable sequence homology between REV and ALV RNAs.

Transcription of REV-specific DNA sequences in infected and uninfected cells. To measure the amount of TDSNV-specific RNAs present in uninfected and TDSNV-infected avian cells, TDSNV ^{3}H-labeled DNA product was annealed with RNAs isolated from uninfected chicken and turkey cells and with RNAs from TDSNV-infected chicken cells. The RNA from uninfected cells hybridized 4 to 20% of the TDSNV[^{3}H]DNA (Table 3) (6). Thus, the unin-

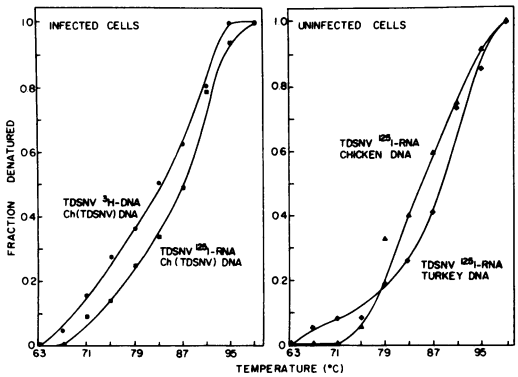

FIG. 5. *Denaturation of RNA-DNA hybrids and double-stranded DNA. TDSNV ³H-labeled DNA product was annealed with TDSNV-infected chicken cell DNA to a C_0t value of 8×10^4 mol/liter × s. As was also found in Fig. 2, almost 100% of the [³H]DNA hybridized at this C_0t value. ¹²⁵I-labeled TDSNV RNA was hybridized with TDSNV-infected chicken cell DNA and with uninfected turkey DNA to C_0t values of 8×10^4 mol/liter × s. Hybridization was performed in 25 μliters for infected cell DNA and in 100 μliters for uninfected cell DNA. Approximately 60% of the ¹²⁵I-labeled TDSNV RNA was hybridized with DNAs from TDSNV-infected chicken cells and about 10% with DNAs from both the uninfected chicken and turkey cells (Fig. 3). The labeled double-stranded DNA and hybrids were obtained as described. Samples of double-stranded DNA [1,700 dpm per point of TDSNV [³H]DNA with Ch(TDSNV) DNA], and samples of RNA-DNA hybrids [1,080 counts/min per point of TDSNV [¹²⁵I]RNA with Ch(TDSNV) DNA, 430 counts/min per point of TDSNV [¹²⁵I]RNA with uninfected turkey DNA, and 450 counts/min per point of TDSNV [¹²⁵I]RNA with uninfected chicken DNA] were heated for 20 min at the indicated temperatures in the standard hybridization buffer, rapidly chilled in an ice bath, and assayed for extent of denaturation with either S1 nuclease or RNase digestion.*

fected chicken and turkey cells transcribed some of the REV-related DNA sequences into RNA. We noted that cells grown in tissue culture apparently contained less TDSNV-specific RNA than cells from adult liver or total embryos.

The amount of TDSNV-specific RNA in TDSNV-infected chicken cells (Fig. 7) was similar to that of ALV-specific RNA we have found in B77 virus-infected chicken cells (E. Humphries and H. M. Temin, J. Virol. 14:531–546, 1974; data not shown). The half C_rt value for TDSNV RNA in infected chicken cells was approximately 10 mol/liter × s when the TDSNV[³H]DNA product was hybridized to an excess of RNA from TDSNV-infected chicken cells. Therefore, approximately 0.5% of the RNA in TDSNV-infected chicken cells was virus-specific RNA.

DISCUSSION

The experiments described in this paper were performed to determine the amount of REV nucleic acid sequences in cell DNAs. The technique used was primarily hybridization in liquid with excess cellular DNA. Two labeled viral nucleic acids were used: [³H]DNA transcribed from viral 60S RNA by an ALV DNA polymerase and ¹²⁵I-labeled 60S RNA isolated from TDSNV virions. The [³H]DNA had been partially characterized previously (6). It and the [¹²⁵I]RNA were further characterized by hybridization with each other (Fig. 1), and the [¹²⁵I]RNA was characterized by competition by unlabeled virion RNA (Fig. 6), and by measurement of melting temperatures of the hy-

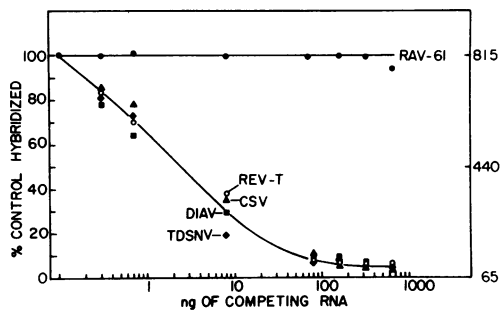

FIG. 6. *Competition between unlabeled REV RNAs and TDSNV [¹²⁵I]RNA for infected cell DNA. For each point, 25 μliters of reaction mixture contained 1,200 counts/min of TDSNV [¹²⁵I]RNA, 250 μg of TDSNV-infected chicken cell DNA, and different amounts of unlabeled total viral RNAs from purified virions of TDSNV, REV-T, CSV, DIAV, and RAV-61. Hybridizations were carried out in the standard hybridization buffer to a C_0t value of 8×10^4 mol/liter × s. Hybridization in the absence of unlabeled competing viral RNA was approximately 60%, which was normalized to 100% in the ordinate. The virus-specific DNA to viral RNA ratio in the reaction containing no competing RNA was approximately 400:1 assuming that the TDSNV-infected chicken cells contained five copies of provirus DNA per haploid cell genome (Fig. 2), that the provirus had a mol wt of 2×10^7 (G. M. Cooper and H. M. Temin, this issue), and that the DNA per diploid chicken cell had a mol wt of 12×10^{11} (4). The extent of [¹²⁵I]-RNA hybridization was determined by RNase A and RNase T1 digestion. The ¹²⁵I counts in the control sample without RNase digestion were 1,250 counts/min, and the RNase-resistant background count was 65 counts/min. The background was subtracted before calculation of % hybridization.*

TABLE 3. *TDSNV-specific RNA in uninfected cells*[a]

Source of RNA	TDSNV [^{3}H]DNA hybridized (%)
Livers from 14-day chicken embryos	4–20[b]
Chicken embryo fibroblasts in culture	5–10
Adult turkey liver	6–20

[a] The conditions for hybridization were the same as in Fig. 7. Eight milligrams of uninfected cell RNAs per ml were hybridized in 1 M NaCl with 2,100 counts per min of TDSNV [^{3}H]DNA product to C_rt values of 10^4 mol/liter × s. The extent of hybridization was determined by S1 nuclease digestion. Approximately 6% (120 counts/min) of S1 nuclease-resistant [^{3}H]-DNA was found in controls incubated without RNA. The background was subtracted before calculation of percent of hybridization.

[b] Range of data from 2 to 4 separate experiments.

brids (Fig. 5). The ^{125}I-labeling had no apparent effect on the specificity of the hybridization (12). In addition, qualitatively similar results were found hybridizing [^{3}H]DNA and [^{125}I]RNA to cell DNAs (Fig. 2 and 3; Table 1).

Hybridization to infected cell DNA. TDSNV-infected chicken and pheasant cells contained DNA sequences homologous to those in TDSNV RNA. These DNA sequences represented at least 75% of the viral RNA sequences (Fig. 3). Other studies, using infectious DNA show that all of the viral RNA sequences are present as DNA in TDSNV-infected chicken cells (G. M. Cooper and H. M. Temin, J. Virol., this issue). Therefore, REV replicate through a DNA intermediate. We do not know whether or not this DNA intermediate is integrated with cell DNA.

The number of copies of DNA per cell was estimated at about 5 to 10. This number is similar to that reported by others for RSV (10).

Hybridization to uninfected cell DNA. DNAs from uninfected avian cells hybridized to each other as expected from the classification of fowl. RAV-O RNA hybridized to different uninfected cell DNAs to different extents depending on the closeness of their relationship to chickens. Similar results were reported by Neiman (10, 11), and Varmus et al. (17) found no sequences in duck DNA related to a partial DNA copy of RSV RNA. This distribution of endogenous RAV-O nucleic acid sequences is consistent with the protovirus hypothesis (H. M. Temin, Annu. Rev. Genet., in press; H. M. Temin, Harvey Lect., in press).

No REV nucleic acid sequences were found in duck DNA, but 10% were found in all the DNAs from gallinaceous birds. These sequences appeared to be REV-specific as shown by the results of melting temperature and viral RNA competition experiments. The failure to find hybridization with duck DNA indicates that the hybridization seen with the DNAs from gallinaceous birds was not the result of possible 60*S*-associated RNAs. The species of origin of REV is, therefore, not clear.

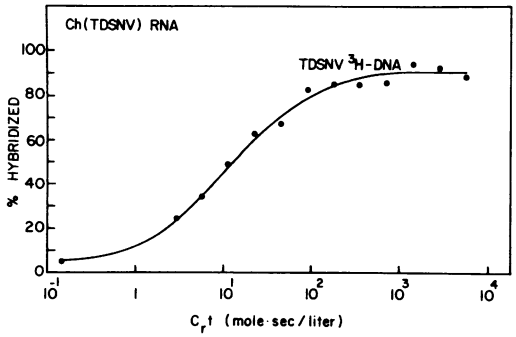

FIG. 7. *Kinetics of hybridization of Ch(TDSNV) RNA to TDSNV [^{3}H]DNA product. Total cytoplasmic RNAs from TDSNV-infected chicken cells [Ch(TDSNV)] were extracted by the SDS-hot phenol extraction procedure of Scherrer (14) from cytoplasmic fraction prepared as described previously (E. Humphries and H. M. Temin, J. Virol. 14:531–546, 1974). One hundred twenty-five micrograms of Ch-(TDSNV) RNA was hybridized in a 25-μliter reaction volume with 3,000 dpm of TDSNV [^{3}H]DNA per point. The conditions of hybridization and the presentation of results are the same as in Fig. 2, with the exception that this reaction was hybridization with excess RNA. The C_rt values were calculated for 1 M salt and were not corrected since no standard correction factors are available. Approximately 10% (300 dpm) of S1 nuclease-resistant TDSNV [^{3}H]-DNA was found in a background control incubated without RNA. The background counts were subtracted before calculation of percent hybridization.*

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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 viruses. Virology 40:118–134.
2. Britten, R. J., and J. Smith. 1970. A bovine genome. Carnegie Inst. Washington Yearb. 68:378–386.

3. Coffin, J. M., and H. M. Temin. 1971. Comparison of Rous sarcoma virus-specific deoxyribonucleic acid polymerase in virions of Rous sarcoma viruses and in Rous sarcoma virus-infected chicken cells. J. Virol. **7**:625–634.
4. Davidson, J. N., and W. M. McIndoe. 1949. Phosphorus compounds in the cell nucleus. Biochem. J. **45**:XVI (abstract).
5. Kang, C.-Y., and H. M. Temin. 1973. Early DNA-RNA complex from the endogenous RNA-directed DNA polymerase activity of uninfected chicken embryos. Nature N. Biol. **242**:206–208.
6. Kang, C.-Y., and H. M. Temin. 1973. Lack of sequence homology among RNAs of avian leukosis-sarcoma viruses, reticuloendotheliosis viruses, and chicken RNA-directed DNA polymerase activity. J. Virol. **12**:1314–1324.
7. Mizutani, S., and H. M. Temin. 1973. Lack of serological relationship among DNA polymerases of avian leukosis-sarcoma viruses, reticuloendotheliosis viruses, and chicken cells. J. Virol. **12**:440–448.
8. Mizutani, S., and H. M. Temin. 1974. Specific serological relationships among partially purified DNA polymerases of avian leukosis-sarcoma viruses, reticuloendotheliosis viruses, and avian cells. J. Virol. **13**:1020–1029.
9. Mizutani, S., C.-Y. Kang, and H. M. Temin. 1974. Endogenous RNA-directed DNA polymerase activity in virions of RNA tumor viruses and in a fraction from normal chicken cells. p. 119–124. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. XXIX. Academic Press Inc., New York.
10. Neiman, P. E. 1972. Rous sarcoma virus nucleotide sequences in cellular DNA: measurement of RNA-DNA hybridization. Science **178**:750–752.
11. Neiman, P. E. 1973. Measurement of endogenous leukosis virus nucleotide sequences in the DNA of normal avian embryos by RNA-DNA hybridization. Virology **53**:196-204.
12. Neiman, P. E., S. E. Wright, C. McMillin, and D. MacDonnell. 1974. Nucleotide sequence relationships of avian RNA tumor viruses: measurement of the deletion in a transformation defective mutant of Rous sarcoma virus. J. Virol. **13**:837–846.
13. Scherberg, N. H., and S. Refetoff. 1973. Hybridization of RNA labelled with ^{125}I to high specific activity. Nature N. Biol. **242**:142–145.
14. Scherrer, K. 1969. Isolation and sucrose gradient analysis of RNA. p. 413–432. *In* K. Habel and N. P. Salzman, (ed.), Fundamental techniques in virology. Academic Press Inc., New York.
15. Temin, H. M. 1974. The cellular and molecular biology of RNA tumor viruses, especially avian leukosis-sarcoma viruses, and their relatives. Advan. Cancer Res. **19**:47–104.
16. Temin, H. M., and V. K. Kassner. 1974. Replication of reticuloendotheliosis viruses in cell culture: acute infection. J. Virol. **13**:291–297.
17. Varmus, H. E., P. K. Vogt, and J. M. Bishop. 1973. Integration of deoxyribonucleic acid specific for Rous sarcoma virus after infection of permissive and nonpermissive hosts. Proc. Nat. Acad. Sci. U. S. A. **70**:3067–3071.
18. Vogt, P. K., and R. R. Friis. 1971. An avian leukosis virus related to RSV(O): properties and evidence for helper activity. Virology **43**:223–234.