Lack of Sequence Homology Among RNAs of Avian Leukosis-Sarcoma Viruses, Reticuloendotheliosis Viruses, and Chicken Endogenous RNA-Directed DNA Polymerase Activity

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Received for publication 23 July 1973

The relatedness of the RNAs of the three avian systems, including six avian leukosis-sarcoma viruses, four reticuloendotheliosis viruses, and the microsome fraction of normal uninfected chicken embryo cells, containing RNA and a DNA polymerase have been studied by nucleic acid hybridization. All six avian leukosis-sarcoma viruses have closely related nucleotide sequences; and all four reticuloendotheliosis viruses have closely related nucleotide sequences. But, almost no similarities were detected between the RNAs of avian leukosis-sarcoma viruses and reticuloendotheliosis viruses. The RNA template of the endogenous RNA-directed DNA polymerase activity of normal uninfected chicken cells had no detectable relationship to RNAs of avian leukosis-sarcoma and reticuloendotheliosis viruses.

There are three avian systems with RNA and a DNA polymerase: avian leukosis-sarcoma viruses (ALV), reticuloendotheliosis viruses (REV), and a fraction from uninfected chicken cells and embryos.

Virions of both ALV and REV contain 60-70S RNA and a DNA polymerase and have C-type morphology (2, 18, 19, 22, 24, 29). However, there are no serological relationships between the structural proteins or the native DNA polymerases of ALV and REV virions (11, 18, 20). In addition, there is no complementation, phenotypic mixing, or recombination between ALV and REV (11).

A microsome fraction isolated from normal, uninfected chicken cells and embryos contained endogenous RNA-directed DNA polymerase activity (14). This endogenous DNA polymerase activity was both RNase sensitive and partially resistant to actinomycin D. The early DNA product of this activity was isolated as a complex with RNA, and the product DNA hybridized to RNA from the same fraction (14, 15).

The experiments reported in this paper were performed to determine if there were any relationships between the RNAs of these three avian systems. In a preliminary study, we did not find a relationship among the three avian

systems with RNA and a DNA polymerase (14). These experiments have now been repeated with better methods of hybridization, with a larger number of viruses, and with DNA products made in the presence of actinomycin D which contain more of the sequences in the RNAs (9).

Two different methods for determining nucleic acid sequence homologies were used. In one method, virion ³²P-labeled RNA was annealed with an excess of DNA product, and the extent of protection of the RNA from digestion by RNase was determined. Previous reports (8, 9) have shown that excess product DNA from the endogenous DNA polymerase reaction of Rous sarcoma virus (RSV) virions protected from RNase digestion all of the homologous virion ³²P-labeled 60-70S RNA. The presence of actinomycin D in the reaction mixture decreased the ratio of product DNA to viral RNA needed for protection from RNase digestion (9).

In the other method, ³H-labeled product DNA was annealed with excess virion or cell RNA, and the extent of protection of the DNA from digestion by the single-strand-specific nuclease, S1 (from *Aspergillus oryzae*), was determined as a function of RNA concentration and time (13, 16). Association kinetics, developed by

Britten and Kohne (3), measures the genetic complexity of nucleic acids by determining the values for the half- C_0t (DNA concentration $\times$ time required to reanneal half of the DNA) or the half- $C_{\text{r}}t$ (RNA concentration $\times$ time required to anneal half of the DNA). Leong et al. (16) estimated the half- $C_{\text{r}}t$ value for 60–70S RSV RNA and complementary DNA to be approximately 2×10^{-2} mol $\cdot$ s/liter.

MATERIALS AND METHODS

Cells and viruses. Cell cultures were made by standard techniques (1, 20) from embryos of chickens, ring-necked pheasants, and rats and were grown in modified Eagle minimum essential medium with 20% tryptose phosphate broth containing, respectively, 5% calf serum and 3% fetal bovine serum, 6% calf serum and 3% fetal bovine serum, and 3% fetal bovine serum. ALV group-specific antigen-free chicken amnion cells, kindly supplied by R. Dougherty, were grown in modified Eagle minimum essential medium containing 10% tryptose phosphate broth and 3% fetal bovine serum. Fertile chicken eggs were obtained from the Sunnyside Hatchery Co., Oregon, Wis. Those embryos used as a source of cell cultures were C/E, ALV-negative, chf-negative, and ALV group-specific antigen-negative. (Sometimes low levels of ALV group-specific antigen have been found in these cells after culture.) Eggs from wild ring-necked pheasants were obtained from the Poynette Game Farm of the Department of Natural Resources, State of Wisconsin. Pregnant Sprague-Dawley rats were obtained from the Holtzman Co., Madison, Wis.

B77 strain of avian sarcoma virus and Schmidt-Ruppin RSV-D (SR-RSV) were grown on chicken cells as described previously (1). RSV-Rous-associated virus-O (RAV-O) (27) was a kind gift of P. K. Vogt and was grown on pheasant cells. Rous-associated virus-61 (RAV-61) (12) was a kind gift of T. Hanafusa and was grown on chicken cells. Two induced leukemia viruses (I-ILV and ILV-UV) (28) were a kind gift of P. K. Vogt and were grown on pheasant cells. Reticuloendotheliosis virus strain T (REV-T) (2, 22) was a kind gift of A. Levine and was grown on chicken cells as described previously. Trager duck spleen necrosis virus (TDSNV) (26) was obtained from Rockefeller University through the kind agency of R. Nowinski and was grown on chicken cells. Chicken syncytial virus (CSV) (6), cloned on duck cells, was a kind gift of H. G. Purchase. CSV was also obtained from the American Type Culture Collection. CSV was grown on chicken cells. Duck infectious anemia virus (DIAV) (17) was a kind gift of H. G. Purchase and was grown on chicken cells.

Chicken or pheasant cells, as indicated, were infected with virus in the fashion previously described for infection of chicken cells by RSV (1, 4). Multiplication of transforming viruses was monitored by morphological transformation. Multiplication of other viruses was monitored by the appearance of cytopathic effects or by the measurement of sedimentable DNA polymerase activity in the supernatant medium of infected cells (H. M. Temin and V. K. Kassner,

submitted for publication). Culture fluids were harvested every 24 h from cells producing virus and were stored at -10°C . Virus was concentrated and purified as described previously (1, 4).

Synthesis and purification of product DNA. The standard DNA polymerase reaction of Temin and Mizutani (25) was used. Endogenous DNA polymerase reactions were carried out in a complete reaction mixture containing 10 nmol of dATP, 7.5 nmol of ATP, 1.875 μmol of MgCl_2 , 2.5 μmol of KCl, 2.4 μg of phosphoenol pyruvate, 10 μg of pyruvate kinase, 3.75 μCi (0.1 nmol) of [^3H]dTTP, 3.75 μCi (0.13 nmol) of [^3H]dCTP, and 3.75 μCi (0.24 nmol) of [^3H]dGTP in 100 μliters of 20 mM Tris-hydrochloride (pH 8.0) containing 0.4 mM EDTA, 5 mM dithiothreitol, and 12.5 μg of actinomycin D. Twenty-five microliters containing approximately 80 μg of protein of Nonidet P-40 (NP40)-disrupted purified virions or 500 μg of protein of chicken microsome fraction (14, 15) was added to 100 μliters of the reaction mixture, and the whole reaction mixture was incubated at 39°C . All of the radioactive biochemicals were purchased from Schwarz/Mann.

For the preparation of unlabeled DNA products, the DNA polymerase reaction was carried out for 1 h at 39°C in the same reaction conditions except for the presence of 10 nmol of each of the four deoxyribonucleoside triphosphates per μliter and the use of about 10 mg of virus protein in 10 ml of the reaction mixture. The amount of cold DNA product was estimated by a parallel reaction by using labeled deoxyribonucleoside triphosphates. Approximately 2.5-fold more DNA was synthesized in a 1-h reaction in the presence of 10 nmol of deoxyribonucleoside triphosphates per 125 μliters of reaction mixture than under the conditions described in the previous paragraph. One microgram of protein of purified virus synthesized approximately 80 μg of DNA during a 1-h reaction in the presence of 10 nmol of deoxyribonucleoside triphosphates/125 μliters of reaction mixture and 100 μg of actinomycin D per ml.

DNA polymerase reactions with purified TDSNV RNA and purified RSV-RAV-O DNA polymerase were carried out by using 40 μg of RNA and 20 μg of DNA polymerase in a 2.5-ml reaction volume using the reaction mixture with the lower deoxyribonucleoside triphosphate concentrations. RSV-RAV-O DNA polymerase was a kind gift of S. Mizutani. It was purified by successive chromatography on DEAE-cellulose and phosphocellulose columns, followed by glycerol gradient centrifugation (20). The purified RSV-RAV-O DNA polymerase had no endogenous DNA polymerase activity.

At the end of a 1-h DNA polymerase reaction, 0.5% sodium dodecyl sulfate (SDS) and 250 μg self-digested Pronase per ml (purchased from Calbiochem and incubated for 2 h at 37°C in a final concentration of 5 mg/ml in 0.01 M Tris-hydrochloride [pH 7.5] containing 0.014 M NaCl) were added to the reaction mixture, and the mixture was incubated at 37°C for 20 min. Heat-denatured calf thymus DNA (250 μg /ml) was then added as a carrier. The sample was extracted twice with an equal volume of double-distilled phenol saturated with 0.05 M Tris-hydrochloride (pH 7.3) and three times with chloroform-isoamyl alcohol

(100:1). The sample was then precipitated at -20°C overnight with three volumes of 100% ethanol containing 2% (wt/vol) potassium acetate. The precipitate was centrifuged at 15,000 rpm for 15 min in a Sorvall SS34 rotor, and the pellet was resuspended in 0.3 ml of double-distilled water. Thirty microliters of 5.5 M NaOH was added to the sample to give a final concentration of 0.5 M NaOH, and the sample was incubated at 40°C for 1 h to hydrolyze the RNA. Tris-hydrochloride buffer (1 M) (pH 7.5) was added to the alkaline hydrolysate to give a final concentration of 0.02 M Tris-hydrochloride. A small amount of phenol red was added, and the pH of the sample was adjusted to approximately 7.5 with 5.5 M HCl solution.

Extraction of RNA. The chicken microsome fraction (14) or purified virus was lysed with 0.5% SDS and was treated with 250 μg of predigested Pronase per ml at 37°C for 30 min. The sample was then extracted with an equal volume of phenol saturated with 0.1 M sodium acetate buffer (pH 5.0) containing 10% meta-cresol, followed by three extractions with chloroform-isoamyl alcohol. The aqueous phase from the final extraction was precipitated overnight at -20°C with 100% ethanol and 2% (wt/vol) potassium acetate. The precipitate was collected by centrifugation, and the pellet was resuspended in 0.01 M Tris-hydrochloride (pH 7.5) containing 0.1 M NaCl, 0.01 M MgCl_2 , and 0.01 M CaCl_2 . Twenty-five micrograms of RNase-free DNase per milliliter (Worthington Biochemical Corp.) was added, and the sample was digested at room temperature for 30 min. After DNase digestion, the RNA was extracted twice with chloroform-isoamyl alcohol and was precipitated with 100% ethanol in the presence of 2% potassium acetate. The RNA precipitate was collected by centrifugation, and the pellet was dissolved in 0.02 M Tris-hydrochloride buffer (pH 7.3) containing 0.1 M NaCl and 0.001 M EDTA (TSE). The concentration of RNA was measured by determining the optical density at 260 nm; 1 OD unit at 260 nm was taken as 50 μg of RNA. The 260 nm/280 nm ratio was greater than 2 for all RNA preparations.

Preparation of ^{32}P -labeled viral 60-70S RNA and cellular RNA. Cells producing virus were washed with phosphate-free Eagle medium (7) and starved for phosphate for 20 h in phosphate-free Eagle medium. The culture fluid was then changed to fresh phosphate-free Eagle medium, and 200 μCi of ^{32}P per ml was added. After 24 h, culture fluids containing ^{32}P -labeled virus were collected and centrifuged at 10,000 rpm for 10 min in a Sorvall SS34 rotor. The supernatant fluid was then centrifuged at 26,000 rpm for 1 h in a Spinco SW27 rotor. The pelleted virus was resuspended in approximately 0.2 ml of TSE buffer. The virus was lysed with 1% SDS at room temperature and layered on a 5 to 20% linear sucrose gradient made in TSE buffer containing 0.1% SDS. For sedimentation markers, 28S and 18S chicken rRNAs and 4S chicken RNA were used. The gradient was centrifuged at 50,000 rpm for 1 h at 15°C in a Spinco SW50.1 rotor and fractionated from the top with the aid of a Buchler Auto-Densi-Flow. The fractions containing ^{32}P which sedimented in the 60-70S region

of the gradient were pooled, and these fractions were extracted three times with chloroform-isoamyl alcohol in the presence of 200 μg of purified yeast RNA (Worthington Biochemical Corp.). The samples were precipitated at -20°C overnight with three volumes of 100% ethanol in the presence of 2% potassium acetate. The precipitate was collected by centrifugation at 15,000 rpm for 15 min, resuspended in TSE buffer, and sedimented in a 10 to 30% sucrose gradient made in TSE buffer. RNA from the 60S region of the gradients shown in Fig. 1, fractions 11 to 14, was used as 60-70S viral RNA. RNAs from RSV-RAV-O, RAV-61, and REV-T had sedimentation patterns similar to those of RNAs from B77V and TDSNV.

The specific activity of the ^{32}P -RNA was determined after labeling pheasant cells with ^{32}P by the same procedures described for labeling virus RNA. ^{32}P -labeled cells were harvested, washed twice with phosphate buffer (pH 7.0), and homogenized with a glass Dounce homogenizer in Tris-hydrochloride buffer (pH 7.5) containing 0.25 M sucrose and 0.001 M EDTA. The cytoplasmic membrane fractions and nuclei were removed by centrifugation at 8,000 rpm for 10 min. The RNA was extracted from the supernatant fluid as described above. The RNA was resuspended in TSE buffer, and the optical density was determined at 260 and 280 nm. The ratio of $\text{OD}_{260}/\text{OD}_{280}$

FIG. 1. Velocity sedimentation of ^{32}P -labeled RNAs of B77 virus and TDSNV. Partially purified viral ^{32}P -labeled RNAs prepared as described in Materials and Methods were layered on 10 to 30% linear sucrose gradients made in TSE buffer and centrifuged at 50,000 rpm for 80 min at 7°C in a Spinco SW50.1 rotor. Thirty fractions were collected from each gradient through a hole pierced in the bottom of the tube. The amount of radioactivity in a 5- μliter portion of each fraction was determined. The arrow markers represent the position of ^{14}C -labeled chicken rRNA and tRNA in a parallel gradient. Sedimentation was from right to left. ^{32}P -labeled RSV-RAV-O RNA, RAV-61 RNA, and REV-T RNA were analyzed on similar linear sucrose gradients. The sedimentation patterns of ^{32}P -labeled RSV-RAV-O RNA, RAV-61 RNA, and REV-T RNA were similar to those of B77V and TDSNV RNAs.

was over 2.1. This ^{32}P -RNA was analyzed on a 10 to 30% sucrose gradient, and a typical pattern of rRNAs and tRNAs was found. The specific activity of this ^{32}P -RNA was approximately 2,000 counts per min per ng, taking 1 OD unit at 260 nm as 50 μg of RNA. Similar specific activities of chicken cell RNAs labeled in this fashion have been found by others (5, 9, 21).

Preparation of S1 nuclease. Single-stranded-specific nuclease, S1, was purified by DEAE-cellulose column chromatography, as described by Sutton (23). The starting material was Takadiastase powder from *A. oryzae* purchased from the Enzyme Development Corporation. The partially purified enzyme was stored at -60°C in the presence of 25% glycerol. The enzyme preparation digested more than 96% of single-stranded M13 bacteriophage DNA and less than 0.1% of double-stranded rat cell DNA under the assay conditions used in the hybridization analysis. The M13 bacteriophage was a kind gift of J. Salstrom and was grown in K38 strain of *E. coli* in Hershey broth in the presence of 0.5 μCi of ^3H -TdR per ml. The radioactive DNA was extracted by standard methods.

Nucleic acid hybridization. The hybridization mixture contained 0.3 M NaCl, 0.1% SDS, 0.001 M EDTA, 2% phenol, and 0.05 M Tris-hydrochloride (pH 7.3). Annealing was carried out at 63°C by using either 25 or 50 μl of annealing mixture in sealed 25- or 50- μl Yankee disposable micropipettes. For the ^{32}P -RNA protection experiments, a series of annealing reactions was performed by using approximately 2,000 to 6,000 counts per min of ^{32}P -RNA (specific activity 2,000 counts per min per ng) and a 25- to 35-fold excess of unlabeled DNA product. For kinetic analysis of hybridization of ^3H -DNA with viral RNA, a constant amount of viral RNA and ^3H -labeled viral DNA product was used, and the annealing times were varied. Samples were frozen immediately after the appropriate time of incubation and were stored at -60°C until all samples were collected.

The extent of hybridization was analyzed either by digestion by heat-treated RNase A (Worthington Biochemical Corp.) and T1 (Calbiochem) for the ^{32}P -RNA protection experiments or by S1 nuclease digestion for the labeled DNA product annealed with RNA. For RNase digestion, samples were digested in the standard hybridization buffer at 37°C for 30 min with 50 μg of heat-treated RNase A per ml and 50 IU of heat-treated RNase T1, per ml. The amount of trichloroacetic acid-precipitable ^{32}P counts was determined. For S1 nuclease digestion, annealing mixtures were diluted into 0.2 ml of buffer solution containing 0.03 M sodium acetate buffer (pH 4.5), 0.0018 M ZnCl_2 , 0.3 M NaCl, 50 μg of heat-denatured calf thymus DNA, and an excess of S1 nuclease (16). The samples were incubated at 38°C for 60 min, and each sample was precipitated with 5 ml of ice-cold 10% trichloroacetic acid in the presence of 500 μg of bovine serum albumin. The precipitate was collected by centrifugation at 1,500 rpm for 10 min at 4°C in an International centrifuge, and the pellet was washed once with 5 ml of 10% trichloroacetic acid. The final pellet was dissolved in 0.1 ml of 2 N NaOH. The NaOH concentration was adjusted to 0.2 N by addi-

tion of 0.9 ml of water, and the amount of radioactivity was determined in a Packard scintillation counter by using Scintisol scintillation fluid. Two to 15% of the virus product DNAs were resistant to S1 nuclease after self-annealing to C_{0t} values of 5×10^{-4} mol·s/liter in 0.3 M salt. Approximately 50% of the product DNAs from the endogenous DNA polymerase activity of the chicken microsome fraction were resistant to S1 nuclease after self-annealing to C_{0t} values of 5×10^{-4} mol·s/liter in 0.3 M salt.

Determination of melting temperatures of hybrids. RNA and DNA were annealed to C_{0t} values of greater than 2.5 mol·s/liter in 0.3 M salt in 250 μl of water. After annealing, the samples were divided into nine portions containing 25 μl each in 25- μl pipettes. The pipettes were sealed. Separate samples were then incubated for 20 min at the indicated temperatures by using a Haake circulating water bath and were rapidly chilled in an ice bath. The fraction of DNA remaining in hybrid molecules was then determined by S1 nuclease digestion.

RESULTS

Extent of transcription of ALV RNA. The fraction of the nucleotide sequences in the viral RNA represented in the DNA product of an endogenous DNA polymerase reaction was determined by a ^{32}P -RNA protection experiment (8). Large quantities of nonradioactive product DNA were prepared after 1 h of incubation of NP40-disrupted B77V and RSV-RAV-O virions in the presence of polymerase reaction mixtures containing 100 μg of actinomycin D per ml. The viral DNA product was then annealed to homologous ^{32}P -labeled 60–70S viral RNA for different periods of time, and the RNase resistance of the ^{32}P -labeled viral RNA was determined.

After annealing approximately 55 ng of B77V DNA product to about 2 ng of ^{32}P -labeled B77V RNA for 30 h, 100% of the B77 virus RNA became resistant to RNase digestion (Fig. 2, top). In a similar experiment, approximately 90% of ^{32}P -labeled RSV-RAV-O RNA became resistant to RNase digestion after annealing for 24 h to approximately a 35-fold excess of RSV-RAV-O DNA product (Fig. 2, bottom). Therefore, the DNA products synthesized in a 1-h endogenous DNA polymerase reaction in the presence of 100 μg of actinomycin D per ml contained almost all of the sequences of the viral RNA.

However, annealing product DNA to ^{32}P -RNA at lower DNA to RNA ratios gave only partial protection of the RNA. For example, at a ratio of B77V DNA to B77V RNA of 6 to 1, only 50% of the B77V RNA was protected from RNase digestion after annealing for 40 h.

The DNA product of B77 virus also contained most of the sequences of RAV-61 (Fig. 2, top),

FIG. 2. Protection of viral- ^{32}P RNAs against RNase digestion after annealing with viral DNA products. Unlabeled DNA products were made from an endogenous DNA polymerase reaction of NP40-disrupted virions of B77V or RSV-RAV-O. The amount of unlabeled viral DNA product was estimated from the rate of a parallel reaction with labeled substrates. The specific activity of the ^{32}P -labeled viral RNA was estimated as 2,000 counts per min per ng. For each time point, approximately 55 ng of cold DNA product was annealed with 2,500 to 5,000 counts/min (about 1 to 2.5 ng) of ^{32}P -labeled 60–70S viral RNA. At the end of the last incubation, samples were digested with RNase, and trichloroacetic acid-precipitable ^{32}P -counts were determined as has been described in the Materials and Methods. One hundred percent of ^{32}P counts of B77V, RAV-61, RSV-RAV-O, REV-T, and TDSNV were approximately 4,500, 3,600, 2,500, 2,600, and 5,000 counts/min, respectively. The RNase-resistant background counts in all ^{32}P -RNA preparations were less than 3%. The background was subtracted.

an avian leukosis virus produced from pheasant cells after infection with helper-dependent RSV (12).

Relationship between RNAs of ALV and REV determined by a ^{32}P protection experiment. The relationship between the RNAs of ALV and REV was studied by preparing 60–70S ^{32}P -labeled RNA of REV-T and TDSNV and performing ^{32}P -RNA protection experiments by using the DNA products of B77V and RSV-RAV-O. As can be seen in Fig. 2, we found no protection of REV-T or TDSNV RNAs after over 40 h of annealing in the presence of an approximately 20-fold excess of ALV DNAs.

To determine whether REV RNAs had some

nucleotide sequences complementary to those in ALV RNA, ^{32}P -labeled TDSNV RNA was annealed with cold B77V RNA to a C_{t} value of 2 mol·s/liter in 0.3 M salt, and the RNase resistance of the TDSNV ^{32}P -RNA was measured. No RNase resistance was found. Therefore, TDSNV RNA does not contain nucleotide sequences either the same as or complementary to those in ALV RNA.

Relationship between ALV and REV RNAs as studied by kinetics of hybridization. The kinetics of hybridization between ALV and REV DNA products and RNA extracted and purified from ALV or REV was studied. We first prepared ^3H -DNA products from a 1-h endogenous DNA polymerase reaction of purified RSV-RAV-O, B77V, and TDSNV virions in the presence of 100 μg of actinomycin D per ml (Fig. 3A). Under these conditions, no endogenous actinomycin D resistant DNA polymerase activity of TDSNV was found (Fig. 3A). (The virions do contain a DNA polymerase, and some en-

FIG. 3. Kinetics of endogenous and exogenous DNA synthesis by ALV and REV virions. A, Purified virions of B77V, RSV-RAV-O, and TDSNV were disrupted with NP40, and endogenous DNA polymerase reactions were carried out at 39 C in 250 μl of reaction mixture containing 100 μg of actinomycin D per ml. At each time point 25 μl of reaction mixture was withdrawn and the trichloroacetic acid-precipitable radioactivity was determined. The amount of ^3H -dCMP, ^3H -dGMP, and ^3H -dTTP incorporated per 10 μg of viral protein is plotted against time of incubation. B, Approximately 3 μg of purified RNA of either B77V or TDSNV was incubated with purified RSV-RAV-O DNA polymerase in 125 μl of a standard DNA polymerase reaction mixture containing 100 μg of actinomycin D per ml. At the times indicated, 25 μl of the sample was withdrawn, and the acid-precipitable radioactivity was determined.

FIG. 4. Denaturation of RNA-DNA hybrids. ^3H -labeled DNA products were annealed to the homologous unlabeled viral RNA to a C_t value of approximately 3 mol·s/liter in 0.3 M salt. As shown in Fig. 5 to 8, essentially 100% of the ^3H -DNA hybridized at this C_t value. Samples of hybrid (approximately 3,500 counts per min per sample of B77V and RSV-RAV-O, approximately 7,500 counts per min per sample of I-ILV, and approximately 700 counts per min per sample of TDSNV) were heated for 20 min at the indicated temperatures in a standard hybridization buffer, rapidly chilled in ice, and assayed for extent of denaturation with S1 nuclease digestion. A, RSV-RAV-O ^3H -DNA with RSV-RAV-O RNA, and B77V ^3H -DNA with B77 RNA. B, I-ILV ^3H -DNA with I-ILV RNA, I-ILV ^3H -DNA with RAV-61 RNA, and I-ILV ^3H -DNA with SR-RSV-D. C, TDSNV ^3H -DNA with TDSNV RNA.

ogenous DNA-directed RNase-resistant DNA polymerase activity was present.) Therefore, we synthesized a ^3H -DNA product by using TDSNV RNA as template-primer with purified RSV-RAV-O DNA polymerase. Figure 3B shows the kinetics of ^3H -dCMP, ^3H -dGMP, and ^3H -dTTP incorporation into product DNA in this reaction. It is interesting that, although there was no endogenous actinomycin D-resistant reaction of TDSNV virions, TDSNV RNA was almost as good a template-primer for the RSV-RAV-O polymerase as was B77V RNA.

Before carrying out cross-hybridization experiments between ALV and REV, we measured the melting temperatures (T_m) of homologous RNA-DNA hybrids to determine the fidelity of hybridization of the DNA products to the homologous RNA templates. Homologous hybrids of B77V, RSV-RAV-O, I-ILV, and TDSNV and hybrids of I-ILV DNA with RNAs of SR-RSV-D and RAV-61 were prepared in standard hybridization reactions to C_t values of 3 mol·s/liter in 0.3 M salt. Portions of the hybrids were incubated at the indicated temperatures for 20 min. The fraction of the hybrid which was denatured was determined by S1 nuclease digestion. The steep melting curves presented in Fig. 4 demonstrate that extensive mismatching did not occur

in the homologous systems or between different ALV. The half T_m , measured in 0.05 M Tris-hydrochloride (pH 7.3) containing 0.3 M NaCl, 0.1% SDS, 2% phenol, and 0.001 M EDTA, were 83, 79, 84, and 83°C for homologous hybrids of B77V, RSV-RAV-O, I-ILV, and TDSNV, respectively. The half T_m of hybrids of I-ILV DNA with SR-RSV-D RNA and I-ILV DNA with RAV-61 RNA were 81 and 83°C, respectively.

The hybrid molecules were then characterized further. Homologous hybrids of RSV-RAV-O DNA and RNA were prepared by a standard hybridization reaction to a C_t value of 3 mol·s/liter in 0.3 M salt. The hybrids were treated with S1 nuclease, and their size and density were determined. The hybrids sedimented slightly faster than tRNA in a neutral sucrose density gradient, giving an S value of about 5–6S. The hybrid had a density of approximately 1.54 g/cm³ in equilibrium Cs_2SO_4 density gradient centrifugation. The ^3H -DNA product before hybridization with RNA banded at a density of 1.48 g/cm³, and control chicken cell ^3H -RNA banded at a density of 1.65 g/cm³ in such a gradient (unpublished data).

To determine further if the nucleotide sequences of RNAs of different ALV were similar, kinetics of association were studied for anneal-

FIG. 5. Kinetics of hybridization of ALV DNA products to different ALV RNAs. ^3H -labeled RSV-RAV-O DNA (3,600 counts per min per sample), I-ILV DNA (5,500 counts per min per sample), and RAV-61 DNA (5,900 counts per min per sample) were incubated at 63 C in 25 μl of standard annealing buffer sealed in 25- μl micropipettes with 0.3 μg of RNA of RSV-RAV-O, SR-RSV-D, I-ILV, ILV-UV, and RAV-61 as indicated in the figure. At different times, samples were withdrawn and were frozen at -60 C. At the end of all incubations, the extent of hybridization was determined by S1 nuclease digestion. The results are expressed as the proportion of total ^3H -DNA hybridized at a given C, t value in 0.3 M salt (RNA concentration in moles/liter $\times$ time of incubation in seconds). Approximately 3% (100 counts/min) of S1 nuclease-resistant ^3H -DNA was found in controls incubated without RNA. It was subtracted from the values plotted.

ing of ^3H -DNA products of RSV-RAV-O, I-ILV, and RAV-61 with RNAs of different ALV (Fig. 5). The half- C, t values were 2×10^{-2} to 5×10^{-2} mol-s/liter, in good agreement with previous results obtained for RSV (13, 16). In almost all cases, 100% of the DNA hybridized. (We do not know the significance of the slight shift in the curves for SR-RSV-D RNA.) Therefore, any ALV DNA product prepared in this way seems to represent sequences present in all ALV.

FIG. 6. Kinetics of hybridization of RSV-RAV-O DNA product to RSV-RAV-O, REV-T, and TDSNV RNAs. ^3H -labeled RSV-RAV-O DNA product (3,500 counts per min per sample) and 0.625 μg of purified RNA from RSV-RAV-O (Δ), REV-T ($\circ$), and TDSNV ($\square$) were incubated at 63 C in 25 μl of standard annealing buffer sealed in 25- μl micropipettes. Samples were withdrawn at different times and were frozen immediately at -60 C. At the end of the incubation, the extent of hybridization was determined by S1 nuclease digestion. The results are expressed as the proportion of total ^3H -DNA hybridized at a given value of C, t in 0.3 M salt (RNA concentration in moles/liter $\times$ time of incubation in seconds). Approximately 3% (100 counts/min) of S1 nuclease-resistant ^3H -DNA was found in a background control incubated without RNA. It was subtracted from the values plotted. The results from two separate experiments are shown. One experiment was also plotted at the top of Fig. 5.

TABLE 1. Hybridization of I-ILV ^3H -DNA with polyribonucleotides from different sources^a

RNA	C, t (mol · s/liter)	% I-ILV ^3H -DNA hybridized
I-ILV	4.6	96
B77V	4.6	99
TDSNV	4.6	1
REV-T	4.6	2
CSV	4.6	3
Microsomal fraction of chicken amnion cells	188	3
Microsomal fraction of 4-day chicken embryos	745	15
Rat cell	46	3
Poly(rA)	4.6	0
Poly(rI)	4.6	0

^a ^3H -DNA product of I-ILV was incubated with 1.25, 12.5, 50, or 200 μg of RNAs from different sources in 50 μl of standard hybridization buffer at 63 C for 18 h. The extent of hybridization was determined by S1 nuclease digestion as described in Materials and Methods. One hundred percent of the ^3H -DNA was 9,380 counts/min. All synthetic template-primers were purchased from Pabst Laboratories, Milwaukee, Wis. The S1 nuclease-resistant fraction in the DNA probe after 18 h of incubation without RNA was 150 counts/min. The background counts were subtracted. C, t 's were calculated for 0.3 M salt.

FIG. 7. Kinetics of hybridization of TDSNV DNA product to RNAs of TDSNV, REV-T, CSV, and DIAV. ^3H -labeled TDSNV DNA (3,300 counts per min per sample) was annealed with 0.625 μg of RNAs of TDSNV ($\square$), REV-T (∇), CSV ($\bullet$), and DIAV ($\blacktriangle$). The conditions of hybridization and the presentation of results are the same as in Fig. 5. Approximately 15% (590 counts/min) of S1 nuclease-resistant ^3H -DNA was found in a background control incubated without RNA. The background was subtracted from the values plotted.

TABLE 2. Hybridization of TDSNV ^3H -DNA with polyribonucleotides from different sources^a

RNA	C_t (mol. s/liter)	% TDSNV ^3H -DNA hybridized
TDSNV	9.2	100
REV-T	9.2	100
CSV	9.2	86
DIAV	9.2	100
I-ILV	9.2	8
B77V	9.2	8
Microsomal fraction of chicken amnion cells	376	19
Microsomal fraction of 4-day chicken embryos	1,490	24
Rat cell	92	5
Poly(rA)	9.2	3
Poly(rI)	9.2	4

^a ^3H -DNA product of TDSNV was hybridized to 1.25, 12.5, 50, or 200 μg of RNAs from different sources in 50 μl of standard hybridization buffer at 63 C for 36 h. The extent of hybridization was determined by S1 nuclease digestion. One hundred percent of the ^3H -DNA was 3,430 counts/min. The S1 nuclease-resistant fraction in a background control incubated without RNA was 390 counts/min. The background counts were subtracted. C_t 's were calculated for 0.3 M salt.

for annealing of RSV-RAV-O ^3H -DNA product with approximately 25 μg of RNA per ml of RSV-RAV-O, REV-T, and TSDNV (Fig. 6). We did not observe any significant annealing between RSV-RAV-O DNA product and RNA isolated from either REV-T or TDSNV.

We also performed hybridization experiments with ^3H -labeled I-ILV DNA product and differ-

ent REV RNAs (Table 1). I-ILV ^3H -DNA hybridized over 95% to I-ILV and B77V RNAs and 15% to RNA from pooled chicken embryos. The former result was expected from the results of Fig. 5. The latter result is explained by the presence of ALV RNA in some (or most) uninfected chicken embryos (13). There was 0 to 3% hybridization to three REV RNAs—TDSNV, REV-T, and CSV—rat cell RNA, ALV group-specific antigen-negative chicken amnion cell RNA, poly(rA), and poly(rI).

Similar experiments were carried out with ^3H -labeled TDSNV DNA product. First, the ^3H -DNA product of TDSNV was annealed to homologous TDSNV RNA and to RNA of the other three members of the REV group—CSV, DIAV, and REV-T (Fig. 7; Table 2). All of the RNAs of the REV group annealed to 85 to 100% of the TDSNV ^3H -DNA with similar C_t values for 50% hybridization, about 2×10^{-2} to 5×10^{-2} mol. s/liter in 0.3 M salt. These results show that the complexity of REV RNAs is similar to that of ALV RNAs and that all of the REV share similar nucleotide sequences. Unfortunately, we did not determine the fraction of the sequences of TDSNV RNA in the TDSNV DNA because of the limited quantity of TDSNV ^3H -DNA prepared from the purified system of TDSNV RNA and RSV-RAV-O DNA polymerase. Second, the ^3H -DNA product of TDSNV was annealed to RNAs of B77 and I-ILV (Fig. 8; Table 2). Cellular RNAs, poly(rA), and poly(rI) were used as controls. Hybridization between ^3H -DNA of TDSNV and ALV RNAs at C_t val-

FIG. 8. Kinetics of hybridization of TDSNV DNA product to TDSNV RNA and to B77V RNA. ^3H -labeled TDSNV DNA (700 counts per min per sample), from the reaction with TDSNV RNA and the RSV-RAV-O DNA polymerase described in Materials and Methods, was annealed with 0.625 μg of either TDSNV RNA ($\square$) or B77V RNA ($\circ$). The conditions of hybridization and the presentation of the results are the same as in Fig. 5. Approximately 7% (50 counts/min) of S1 nuclease-resistant ^3H -DNA was found in a background control incubated without RNA. It was subtracted from the values plotted.

TABLE 3. Hybridization of chicken microsome fraction ³H-DNA with polyribonucleotides from different sources^a

RNA	Microsome fraction isolated from chicken amnion cells		Microsome fraction isolated from 4-day chicken embryos	
	C _t t (mol·s/liter)	% of 270 counts/min DNA hybridized	C _t t (mol·s/liter)	% of 680 counts/min DNA hybridized
I-ILV	4.6	0	9.2	0
B77V		NT ^b	9.2	3
TDSNV	4.6	0	9.2	0
REV-T	4.6	0	9.2	10
Microsomal fraction of chicken amnion cells	188	100	376	40
Microsomal fraction of 4-day chicken embryos	745	40	1,490	50
Rat cell	46	0	92	10
Poly (rA)	4.6	0	9.2	0
Poly (rI)	4.6	0	9.2	15

^a Microsome fractions were prepared from both ALV group-specific antigen-free chicken amnion cells and 20 dozen 4-day chicken embryos. ³H-DNA products were prepared and hybridized with 1.25, 12.5, 50, or 200 µg of RNAs from different sources in 50 µliters of standard hybridization buffer at 63°C for 18 or 36 h. The extent of hybridization was determined as described in the legends to Tables 1 and 2. One hundred percent of the chicken amnion cell fraction ³H-DNA was 650 counts/min, and the S1 nuclease-resistant fraction in a control incubated without RNA was 380 counts/min. One hundred percent of the 4-day chicken embryo fraction ³H-DNA was 1,570 counts/min, and the S1 nuclease-resistant fraction in a background control incubated without RNA was 890 counts/min. The background counts were subtracted. C_tt's were calculated for 0.3 M salt.

^b NT, Not tested.

ues of over 5 mol·s/liter in 0.3 M salt was less than twice background. The ³H-DNA product of TDSNV hybridized five to six times background to RNAs from ALV group-specific antigen-negative chicken amnion cells and from pooled 4-day chicken embryos at C_tt values of 3.8×10^2 and 1.5×10^3 mol·s/liter in 0.3 M salt, respectively. These results indicate that normal chicken cells contain endogenous REV-related genes. Further work is being carried out to test this possibility.

TDSNV ³H-DNA did not hybridize to any great extent to B77V RNA or I-ILV RNA, nor did RSV-RAV-O or I-ILV ³H-DNAs hybridize to REV-T RNA, CSV RNA, or TDSNV RNA. These results demonstrate no large amount of nucleotide sequence homology between ALV RNAs and REV RNAs.

Relationship of RNA of chicken endogenous RNA-directed DNA polymerase activity to RNAs of ALV and REV. Microsome fractions were prepared from ALV group-specific antigen-negative amnion cells and 20 dozen 4-day-old chicken embryos as described previously (14). ³H-DNA products were synthesized by the endogenous RNA-directed DNA polymerase activity in the presence of 100 µg of actinomycin D per ml and were hybridized to different RNAs (Table 3). The ³H-DNA of the chicken amnion cell fraction hybridized completely to RNA from the same fraction at a C_tt value of 1.9×10^2 mol·s/liter in 0.3 M salt and

about 40% to the RNA from the microsome fraction from 4-day chicken embryos at a C_tt value of 7.5×10^2 mol·s/liter in 0.3 M salt. In contrast, no hybridization was detected to I-ILV RNA, TDSNV RNA, REV-T RNA, rat cell RNA, or two synthetic polyribonucleotides.

The ³H-DNA of the microsome fraction from 4-day chicken embryos annealed 50% to RNA from the same fraction and 40% to the RNA from the microsome fraction of chicken amnion cells at C_tt values of 1.5×10^3 and 3.8×10^2 mol·s/liter in 0.3 M salt, respectively. This ³H-DNA also hybridized from 3 to 14% to RNAs from B77V, REV-T, rat cells, and poly(rI). We found some nucleotide terminal transferase activity in the microsome fraction of 4-day chicken embryos which synthesizes acid-precipitable poly(dC) in the absence of the three other deoxyribonucleoside triphosphates (unpublished data). The 14% hybridization of chicken microsome fraction ³H-DNA to poly(rI) could have resulted from the product of this terminal transferase activity.

There was a high background, approximately 60%, of double-stranded DNA in products prepared from the endogenous RNA-directed DNA polymerase activity of chicken microsome fractions. Since this double-stranded DNA was also present in unannealed samples, it may represent intramolecular hair-pin structures.

The failure to find 100% hybridization be-

tween RNA and ³H-DNA from pooled embryos is explained either by the presence of positive-strand DNA in the labeled product or of a very low concentration of template RNA.

It is interesting that the DNA products of the microsome fractions from amnion cells and from pooled embryos hybridized more to RNA from the homologous fraction than to RNA from the heterologous one.

The results in Table 3 indicate that the RNA template of the endogenous RNA-directed DNA polymerase activity of the chicken microsome fraction has no close relationship to RNAs of ALV or REV.

DISCUSSION

The relationships between the RNAs of the three avian systems with RNA and a DNA polymerase—ALV, REV, and a microsome fraction from chicken cells and embryos—was studied by nucleic acid hybridization. DNA products were synthesized by RNA-directed DNA polymerase activity. The DNA synthesized by the endogenous reactions of B77V and of RSV-RAV-O at a high concentration of deoxyribonucleoside triphosphates was shown to be complementary to all of the sequences of the B77V RNA and of the RSV-RAV-O RNA. We assume that the difference in deoxyribonucleoside triphosphate concentrations used to prepare different DNA products did not affect the complexity of the product and that the other DNA products were similarly representative.

Hybrids were primarily recognized by their resistance to nucleases. The B77V hybrids were also shown to sediment in sucrose at a velocity of about 5-6S and to band at a density characteristic of RNA-DNA hybrid molecules in equilibrium Cs_2SO_4 density gradient centrifugation. Although other hybrids were not characterized in this way, the curves of the melting temperatures of all six of the hybrids tested were sharp with a mid-point of about 80°C in 0.3 M NaCl. Therefore, hybrids with little mismatching appear to have been formed.

ALV. The ALV studied included the strongly transforming B77V, SR-RSV-D, and RSV-RAV-O, the weakly transforming RAV-61, and the nontransforming I-ILV and ILV-UV. Independent of the combination of RNA and DNA studied, over 90% hybridization was found. Furthermore, the melting temperatures of hybrids, homologous or mixed, were similar. Therefore, these results suggest that all ALV have a majority of their nucleotide sequences in common.

In a recent study Hayward and Hanafusa (13) found all RAV-2 sequences in SR-RSV RNA, whereas only 70% of SR-RSV sequences were in

RAV-2. The difference between their results and ours probably relates to the complexity of the DNA products. Haapala and Fischinger (10) found greater differences in nucleotide sequences among RNAs of different strains of murine leukemia viruses.

REV. Our work confirms the previously reported existence of 60-70S RNA in virions of REV-T (19) and extends this finding to virions of TDSNV. Furthermore, all REV RNAs had the same complexity as determined by the kinetics of hybridization in RNA excess. However, we could not confirm the existence of endogenous RNA-directed DNA polymerase activity in REV virions (22). No endogenous actinomycin D-resistant RNase-sensitive DNA polymerase activity was found in disrupted REV virions. Perhaps the DNA polymerase activity previously reported (22) was a result of a contaminating avian leukosis virus. We do not know the explanation for our inability to detect endogenous RNA-directed DNA polymerase activity in TDSNV virions. An extensive study using different reaction conditions (Mizutani, unpublished data) has not detected such activity.

Therefore, TDSNV DNA was made by using purified RSV-RAV-O DNA polymerase and TDSNV RNA. We do not know the complexity of this DNA. However, all four previously classified members of the REV group shared the sequences of the TDSNV DNA. Therefore, this result supports the previous classification.

Chicken fraction. We have confirmed our previous report of hybridization of the DNA product of the chicken endogenous DNA polymerase activity and RNA from the same fraction (14). The higher values reported here for percent hybridization probably relate to the presence of actinomycin D in the DNA polymerase reaction and better conditions for hybridization. Some tissue specificity may exist in this hybridization.

Relationships. No significant relationships were found between the RNAs of ALV, REV, and the template of the chicken endogenous RNA-directed DNA polymerase activity. This result supports the previous classification of REV in a group other than ALV (H. G. Purchase, C. Ludford, K. Nazarian, and H. W. Cox, *J. Nat. Cancer Inst.*, 51:489-499) and the assertion that the chicken endogenous DNA polymerase activity is not viral (14). However, although the ALV DNA products represent all of the nucleotide sequences of ALV RNA, the relative abundance of these sequences varies. Therefore, a few percent of common sequences might not have been detected. In fact, TDSNV DNA hybridized more (3%) to I-ILV and B77V RNAs

than to rat cell RNA. (Further work has confirmed the reproducibility of this low level of hybridization between TDSNV DNA and some ALV RNAs [unpublished data].)

RNA related to the RNAs of ALV and REV was found in the chicken microsome fractions, although this RNA was not a template for the endogenous DNA polymerase activity. As previously reported (13), ALV RNA was absent from ALV group-specific antigen-negative cells and present in pooled embryos; REV RNA was present in both preparations.

Further work is being carried out to define further any relationships among these systems and the chicken cell genome.

ACKNOWLEDGMENTS

We thank V. Kassner, R. Grimstad, and K. Jones for technical assistance, R. Dougherty, T. Hanafusa, A. Levine, R. Nowinski, H. G. Purchase, and P. K. Vogt for materials, and J. Dahlberg, H. Kubinski, and D. Zarling for helpful comments on the manuscript.

This investigation was supported by Program Project grant CA-07175 from the National Cancer Institute. C.-Y.K. is a fellow of the National Cancer Institute of Canada. H.M.T. holds Research Career Development Award CA-8182 from the National Cancer Institute.

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