Marker Rescue of Endogenous Cellular Genetic Information Related to the Avian Leukosis Virus Gene Encoding RNA-Directed DNA Polymerase

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Endogenous cellular genetic information related to the avian leukosis virus gene encoding RNA-directed DNA polymerase was studied, using a marker rescue assay to detect biological activity of subgenomic fragments of virus-related DNAs of uninfected avian cells. Recipient cultures of chicken embryo fibroblasts were treated with sonicated DNA fragments and were infected with a temperature-sensitive mutant of Rous sarcoma virus that encoded a thermolabile DNA polymerase. Wild-type progeny viruses were isolated by marker rescue with fragments of DNA of uninfected chicken, pheasant, quail, and turkey cells. The DNAs of these uninfected avian cells, therefore, appeared to contain endogenous genetic information related to the avian leukosis virus DNA polymerase gene.

Avian cells appear to inherit endogenous genetic information related to three groups of avian retroviruses. The DNAs of uninfected chicken cells contain nucleotide sequences that are homologous to most of the RNA sequences of Rous-associated virus 0 (RAV-0), an endogenous avian leukosis virus (ALV) spontaneously produced by some inbred lines of chickens (20, 25, 26, 31, 37). Nucleotide sequences that are related to ALV RNA have also been detected in the DNAs of other avian species, including pheasant, quail, and turkey (20, 25, 31, 35). In addition, avian DNAs contain nucleotide sequences related to some sequences of the RNA of avian reticuloendotheliosis viruses, a second group of avian retroviruses which lack nucleic acid homology with viruses of the ALV group (20). Endogenous genetic information related to a third group of avian retroviruses has also been identified in some species of *Chrysolophus* pheasants (3, 9, 16).

In most cases, endogenous virus-related DNA sequences are not expressed to yield infectious virus. Although apparently all chickens contain similar amounts of RAV-0-related DNA sequences (25, 26), only a few inbred lines of V⁺ chickens produce RAV-0 (7, 8, 29, 30, 37). Partial expression of the ALV-related DNAs of non-virus-producing (V⁻) chicken cells has been demonstrated by immunological assays of ALV-related proteins (2, 12, 28) and by the ability of some V⁻ chicken cells to complement replication of Rous sarcoma virus (RSV) strains defective in envelope glycoprotein synthesis (14, 39). Sim-

ilarly, some pheasant cells produce proteins related to ALV internal structural proteins (group-specific antigens) (9, 15) and some pheasant, partridge, and Chinese quail cells produce viral envelope glycoproteins or infectious virus (3, 9, 15, 16). The biological activity of endogenous genetic information related to ALV envelope glycoproteins has also been demonstrated by recombination between exogenous ALV and endogenous envelope glycoprotein genes of chicken (17, 18, 31, 41) and pheasant (9, 15, 31, 32, 34) cells.

Assays of DNA infectivity by transfection were previously used to study the endogenous ALV-related genes of uninfected chicken cells (6). In contrast to the infectivity of RAV-0 proviral DNAs of cells which were exogenously infected with RAV-0, the endogenous RAV-0-related DNAs of uninfected V⁻ and V⁺ chicken cells lacked detectable infectivity in transfection assays (6). Therefore the endogenous RAV-0-related DNAs of uninfected chicken cells appeared to differ from the RAV-0 proviral DNAs of RAV-0-infected cells. Since uninfected chicken cells did not contain infectious RAV-0 genomes, it was of interest to investigate the biological activity of subgenomic fragments of endogenous ALV-related DNAs.

We recently described a biological assay of subgenomic fragments of ALV DNA by marker rescue (4). In these experiments, wild-type progeny RSV were isolated after treatment of recipient cells with subgenomic fragments of ALV DNA and infection with temperature-sensitive

mutants of RSV. We have now assayed DNAs of uninfected avian cells for genetic information related to the ALV gene for RNA-directed DNA polymerase (*pol*) by the marker-rescue technique. Wild-type progeny RSV were isolated by marker rescue of *pol*^{ts} RSV with fragments of DNA of uninfected chicken, pheasant, quail, and turkey cells. These results indicated that DNAs of these uninfected avian cells contained endogenous genetic information related to the ALV *pol* gene.

MATERIALS AND METHODS

Cells and viruses. Cells were grown in Temin-modified Eagle minimal essential medium containing 20% tryptose phosphate broth (ET medium) and supplemented with calf and fetal bovine sera. Fertile chicken eggs were purchased from SPAFAS, Norwich, Conn., and from Hyline, Dallas Center, Iowa. Fertile line 100 chicken eggs were a generous gift of L. B. Crittenden, U.S. Department of Agriculture, East Lansing, Mich. Fertile Orlopp turkey eggs were provided by the Wilmar Poultry Co., Wilmar, Minn. Fertile ringneck pheasant eggs were a kind gift of M. Smith, Apopka, Fla. Fertile Japanese quail eggs were obtained from Life Sciences, Inc., St. Petersburg, Fla. Fertile Pekin duck eggs were obtained from a local farmer. Mouse NIH 3T3 cells were a kind gift of R. A. Weinberg, Massachusetts Institute of Technology, Cambridge.

SPAFAS chicken embryo fibroblasts were C/E, V⁻, chicken helper factor (*chf*) negative, and ALV group-specific (*gs*) antigen negative (assayed by complement fixation). Hyline SC line chicken embryo fibroblasts were C/O, V⁻, *chf* negative, and ALV *gs* antigen negative. Line 100 chicken embryo fibroblasts were of either V⁺C/BE or V⁺C/O phenotype. V⁺C/BE line 100 cells spontaneously produced low titers of RAV-0 and, since they were resistant to exogenous RAV-0 infection due to the absence of receptors required for RAV-0 penetration, presumably contained only endogenous RAV-0-related genetic information (6, 8, 33). V⁺C/O line 100 cells produced high titers of RAV-0, presumably due to exogenous infection with RAV-0 as a result of exposure to the RAV-0 that they spontaneously produced (6, 8, 33). Ringneck pheasant cells, turkey cells, Japanese quail cells, and Pekin duck cells were virus negative (assayed by sedimentable DNA polymerase activity in culture fluids), helper factor negative, and ALV *gs* antigen negative.

LA335 (pol^{ts}), an early temperature-sensitive coordinate mutant of Prague RSV subgroup C (PR-RSV-C) with a thermolabile DNA polymerase (21, 22, 36), was obtained from W. S. Mason, Philadelphia, Pa. Schmidt-Ruppin RSV subgroup D (SR-RSV-D), B77 avian sarcoma virus (subgroup C), Rous-associated virus 1 (RAV-1) (subgroup A), and Rous-associated virus 7 (RAV-7) (subgroup C) were obtained from H. M. Temin, Madison, Wis. PR-RSV-A and PR-RSV-C were obtained from W. S. Mason.

Viruses were grown and assayed by focus formation as previously described (4). Viruses were cloned by plating virus-infected chicken embryo fibroblasts in

soft agar medium (10), using feeder layers of mitomycin C-treated turkey embryo fibroblasts.

Preparation of DNA. DNA was extracted either from cultured cells or from embryos as previously described (5, 6). The average molecular weights of DNAs extracted by this method were approximately 50×10^6 (5). Fragments of DNA for marker-rescue experiments were prepared by sonication of the DNA to average molecular weights of approximately 5×10^5 (4).

Assay of infectious DNA by transfection. Cultures of C/O chicken embryo fibroblasts were prepared and treated with high-molecular-weight (unsonicated) DNA, using the calcium method of Graham and Van der Eb (11) as previously described (6). DNA-treated cells were cultured in ET medium supplemented with 2% fetal bovine serum and polybrene (5 μ g/ml) for approximately 4 weeks (four transfers) after DNA treatment. Virus production was determined by assay of sedimentable DNA polymerase activity in culture fluids (6). The assay was quantitated by end point dilution of the donor DNA (5).

Assay of subgenomic DNA fragments by marker rescue. Cultures of C/O or C/E chicken embryo fibroblasts were treated with sonicated DNA fragments and were infected with *LA335 (pol^{ts})* (approximately 0.1 focus-forming unit [FFU]/cell) at the permissive temperature (35°C) as previously described (4). When C/E cells were used as recipients, turkey cells (T/BD) were added after infection with *LA335* to permit replication of subgroup E progeny RSV. After 6 days of incubation at 35°C, supernatant media were harvested and assayed on fresh C/O or T/BD cells at the nonpermissive temperature (41°C) to select for wild-type (*pol⁺*) progeny virus. All 41°C assay plates positive for transformation were harvested, and the supernatant media were reassayed in a second passage at 41°C to distinguish phenotypic leakiness of the parental *LA335* mutant from the presence of wild-type progeny RSV (4).

Thermolability of virion DNA polymerases. Virions were concentrated 100-fold from cell-free supernatant media, suspended in Eagle medium, and disrupted by addition of an equal volume of disruption buffer (0.05 M sucrose–0.001 M EDTA–0.03 M dithiothreitol–0.01 M Tris-hydrochloride [pH 7.4]–0.25% Nonidet P-40). Disrupted virions were incubated at 41°C, and samples withdrawn at different times were kept at 0°C until all samples were collected. Portions of disrupted virions (25 μ l) were then added to 100 μ l of polymerase reaction mixture containing 0.02 M Tris-hydrochloride (pH 8.0), 0.025 M KCl, 0.02 M MgCl₂, 0.4 mM EDTA, 10 mM dithiothreitol, 0.1 mM dATP, 0.075 mM ATP, 2.5 μ g of phosphoenolpyruvate, 0.5 U of pyruvate kinase, 1.25 μ g of poly(dA-dT), and 1.9 μ Ci of [³H]TTP (50 Ci/mmol, Schwarz/Mann, Orangeburg, N.Y.). Samples were incubated at 35°C for 15, 30, and 60 min and 25- μ l portions were assayed for acid-precipitable radioactivity.

Antibody neutralization of virion DNA polymerases. DNA polymerase neutralizations were performed as described by Mizutani and Temin (23). Concentrated virions were disrupted with detergent and incubated with antibody at room temperature for 30 min. DNA polymerase activity remaining was then

assayed at 37°C. Preparations of immunoglobulin G (IgG) from antisera against RSV(RAV-0) DNA polymerase (IgG-RSV-RAV-0), spleen necrosis virus DNA polymerase (IgG-SNV), chicken cell DNA polymerase α (IgG- α), and chicken cell DNA polymerase β (IgG- β) (23, 24) were generously provided by H. M. Temin.

RESULTS

Marker rescue of LA335 (*pol^{ts}*) with DNA fragments of uninfected avian cells. The biological activity of fragments of cellular DNAs was tested in marker-rescue experiments with LA335 (*pol^{ts}*). Recipient cultures of chicken embryo fibroblasts were treated with salmon sperm DNA or with sonicated fragments of cellular DNAs and were infected with LA335 at 35°C. After 6 days of incubation at 35°C, supernatant media were harvested, and 0.2-ml samples (approximately 5×10^2 FFU at 35°C) were assayed on fresh cells at 41°C. Foci of transformed cells (one to four foci per plate) were induced at 41°C by supernatant media from approximately 5% of the cultures treated with either salmon sperm or cellular DNAs. To determine whether these foci were due to phenotypic leakiness of LA335 or to *pol^{ts}* progeny RSV, the supernatant media of all 41°C assay plates positive for transformation were harvested and reassayed in a second passage at 41°C. Supernatant media harvested from transformation-positive 41°C assay plates of all control cultures treated with salmon sperm DNA and from assay plates of some cultures treated with fragments of cellular DNAs had titers of <20 FFU/ml in the second passage at 41°C, indicating that focus formation in the first assay at 41°C had resulted from phenotypic leakiness of LA335. In contrast, supernatant media harvested from transformation-positive 41°C assay plates of some recipient cultures treated with cellular DNA fragments had titers of 10^2 to 2×10^3 FFU/ml in the second passage at 41°C, indicating the presence of *pol^{ts}* progeny RSV.

None of 435 control cultures treated with salmon sperm DNA and infected with LA335 produced progeny RSV that replicated at 41°C (Table 1), indicating that reversion of LA335 to *pol^{ts}* RSV did not occur at a significant frequency in these experiments. Since approximately 5×10^2 FFU of *pol^{ts}* RSV were assayed per culture, the reversion frequency of LA335 appeared to be less than 5×10^{-6} (Table 1). As previously reported (4), progeny RSV that replicated at 41°C were isolated in marker-rescue experiments with sonicated fragments of DNA of RAV-0-infected V⁺C/O line 100 cells (Table 1). Progeny RSV that replicated at 41°C were also produced by cells infected with LA335 and treated with sonicated fragments of DNAs of

TABLE 1. Assay of subgenomic fragments of cellular DNAs by marker rescue with LA335 (*pol^{ts}*)^a

Donor DNA ^b	Fraction of positive recipient cultures ^c	Frequency of <i>pol^{ts}</i> progeny ^d
Salmon sperm	0/435	$<5 \times 10^{-6}$
Chicken V ⁺ C/O	1/30	1×10^{-4}
Chicken V ⁺ C/BE	2/30	2×10^{-4}
Chicken V ⁻	3/288	2×10^{-5}
Pheasant	2/199	2×10^{-5}
Quail	3/262	2×10^{-5}
Turkey	3/138	4×10^{-5}
Duck	0/177	$<1 \times 10^{-5}$
Mouse	0/208	$<1 \times 10^{-5}$

^a Cultures of chicken embryo fibroblasts were treated with 5 μ g of salmon sperm DNA or sonicated fragments of cellular DNAs and were infected with LA335 (*pol^{ts}*) at 35°C. The formation of *pol^{ts}* progeny RSV that replicated at 41°C was assayed as described in the text.

^b DNAs were extracted from line 100 V⁺C/O and V⁺C/BE chicken embryo fibroblasts, SPAFAS V⁻gs⁻chf⁻ chicken embryo fibroblasts, ringneck pheasant embryo fibroblasts, Japanese quail embryos and Japanese quail embryo fibroblasts, Orlopp turkey embryo fibroblasts, Pekin duck embryo fibroblasts, and mouse NIH 3T3 cells. DNAs were sonicated to average molecular weights of approximately 5×10^6 .

^c Number of cultures that produced progeny RSV that replicated at 41°C over the total number treated.

^d Ratio of the number of *pol^{ts}* progeny RSV isolated to the total FFU of *pol^{ts}* RSV assayed from each group of DNA-treated cultures. Each sample of supernatant medium, assayed at 41°C to detect *pol^{ts}* progeny RSV, contained approximately 5×10^2 FFU of *pol^{ts}* RSV (assayed at 35°C).

uninfected V⁺C/BE and V⁻ chicken cells (Table 1). In addition, progeny RSV that replicated at 41°C were isolated in marker-rescue experiments with sonicated fragments of DNAs of uninfected pheasant, quail, and turkey cells, but not with sonicated fragments of DNAs of uninfected duck or mouse cells (Table 1). The frequencies of *pol^{ts}* progeny RSV produced by cultures treated with fragments of chicken, pheasant, quail, and turkey DNAs were 2×10^{-5} to 2×10^{-4} (Table 1). Thus the frequencies of formation of *pol^{ts}* progeny RSV in cultures treated with these avian cell DNAs were at least 4- to 40-fold greater than the reversion frequency of LA335 in cultures treated with salmon sperm DNA.

Progeny RSV that replicated at 41°C were produced by 1 to 5% of the recipient cultures treated with 5 μ g of each donor DNA active in marker rescue (Table 1). The efficiency of marker rescue with DNAs of uninfected avian cells, therefore, appeared to correspond to approximately 1 to 5 infectious units (IU) per mg

of DNA. This efficiency was two- to fivefold lower than the efficiency of marker rescue previously reported with DNA of RSV-infected cells (4).

Transfection assays of avian cell DNAs. The isolation of progeny RSV that replicated at 41°C in marker-rescue experiments with LA335 (*pol^{ts}*) and sonicated fragments of DNA of uninfected chicken, pheasant, quail, and turkey cells indicated that the DNAs of these uninfected avian cells contained endogenous ALV-related genetic information with detectable biological activity in marker-rescue assays. Therefore, high-molecular-weight (not sonicated) DNAs of uninfected chicken, pheasant, quail, and turkey cells were assayed by transfection to determine if they contained infectious endogenous viral genomes.

As previously reported (6), infectious RAV-0 DNA was obtained from V⁺C/O line 100 cells exogenously infected with RAV-0, but not from uninfected V⁺C/BE or V⁻ chicken cells containing only endogenous RAV-0-related DNA sequences (Table 2). In agreement with previous results (6), the specific infectivity of DNA of V⁺C/O cells, assayed by end point dilution of the donor DNA, was approximately 1 IU/μg of DNA (data not shown). In contrast, the specific infectivities of DNAs of V⁺C/BE and V⁻ cells were less than 0.02 IU/μg of DNA (Table 2). Similarly, the DNAs of uninfected pheasant, quail, and turkey cells were not infectious (<0.04 IU/μg of DNA) (Table 2), indicating that uninfected cells of these heterologous avian species did not contain infectious endogenous proviral DNAs.

Since the DNAs of uninfected chicken, pheasant, quail, and turkey cells were active in marker

rescue but not in transfection, the marker-rescue assay appeared to detect endogenous virus-related DNA sequences that were not part of an infectious provirus.

Characterization of *pol⁺* progeny RSV isolated by marker rescue. Progeny viruses isolated in marker-rescue experiments with LA335 (*pol^{ts}*) were assayed at 35 and 41°C to determine their efficiencies of transformation at the permissive and nonpermissive temperatures. In contrast to the parental RSV mutant, all of the progeny RSV isolates obtained in marker-rescue experiments with DNA fragments of uninfected chicken, pheasant, quail, and turkey cells transformed with similar efficiencies at 35 and 41°C (Table 3). These results indicated that the progeny RSV isolated by selection for transformation and replication at 41°C (Table 1) were *pol⁺*.

To test the genetic stability of the *pol⁺* prog-

TABLE 3. Efficiency of transformation of progeny viruses isolated by marker rescue with LA335 (*pol^{ts}*)^a

Virus	Titer (FFU/ml)		EOT(41/ 35°C) ^b
	35°C	41°C	
Controls			
LA335 (<i>pol^{ts}</i>)	3 × 10 ⁴	<5	<2 × 10 ⁻⁴
PR-RSV-C (<i>pol⁺</i>)	9 × 10 ²	1 × 10 ²	0.1
B77V (<i>pol⁺</i>)	1 × 10 ⁷	1 × 10 ⁷	1.0
Marker-rescue progeny ^c			
Chicken V ⁺ C/O			
-1	4 × 10 ²	3 × 10 ¹	0.1
Chicken V ⁺ C/BE			
-1	1 × 10 ³	2 × 10 ²	0.2
-2	4 × 10 ²	4 × 10 ¹	0.1
Chicken V ⁻			
-1	1 × 10 ³	2 × 10 ²	0.2
-2	5 × 10 ²	1 × 10 ²	0.2
-3	3 × 10 ²	2 × 10 ²	0.7
Pheasant			
-1	6 × 10 ²	5 × 10 ²	0.8
-2	2 × 10 ³	3 × 10 ³	1.5
Quail			
-1	1 × 10 ²	1 × 10 ²	1.0
-2	6 × 10 ³	1 × 10 ⁴	1.7
-3	3 × 10 ³	1 × 10 ³	0.3
Turkey			
-1	2 × 10 ³	4 × 10 ²	0.2
-2	2 × 10 ³	5 × 10 ²	0.3
-3	3 × 10 ³	3 × 10 ³	1.0

^a Viruses were assayed for transformation at 35 and 41°C, and foci were counted 4 to 6 days after infection.

^b EOT, Efficiency of transformation: ratio of virus titer at 41°C to the titer at 35°C.

^c Stocks of progeny viruses from marker-rescue experiments (Table 1) were harvested after two passages at 41°C. The progeny virus isolates were designated by the source of DNA fragments used in marker rescue followed by the isolate number of the progeny virus.

TABLE 2. Assay of infectivity of donor DNAs by transfection^a

Donor DNA ^b	Fraction of positive recipient cultures ^c
Chicken V ⁺ C/O	7/8
Chicken V ⁺ C/BE	0/25
Chicken V ⁻	0/25
Pheasant	0/11
Quail	0/12
Turkey	0/12

^a Cultures of chicken embryo fibroblasts were treated with 5 μg of high-molecular-weight (unsonicated) DNA per culture. Virus production was assayed by sedimentable DNA polymerase activity in culture fluids.

^b The donor DNAs were as described in Table 1. The average molecular weights of unsonicated DNAs were approximately 50 × 10⁶.

^c Number of cultures that produced virus over the total number treated.

eny RSV, representative isolates obtained with fragments of DNA of uninfected V⁻ chicken cells, pheasant cells, and quail cells were cloned in soft agar at 35°C. Individual colonies of each *pol*⁺ progeny RSV isolate were picked and passaged at 35°C to grow virus stocks, which were then assayed for transformation at 35 and 41°C. All clones of each of the *pol*⁺ progeny RSV isolates transformed with similar efficiencies at the two temperatures (Table 4). Therefore, the *pol*⁺ marker of the progeny RSV isolated in marker-rescue experiments was stable to cloning and passage at the permissive temperature.

Some of the *pol*⁺ progeny RSV previously isolated in marker-rescue experiments with LA335 (*pol*^{ts}, subgroup C) and fragments of DNA of *pol*⁺ subgroup D RSV-infected cells acquired the subgroup D envelope specificity as well as the *pol*⁺ marker of the RSV strain used for preparation of DNA fragments (4). Therefore the subgroup of each of the progeny RSV isolates obtained in the present experiments with fragments of DNA of uninfected cells was determined. Progeny viruses were assayed on C/O cells, C/E cells, T/BD cells, and C/E cells infected with RAV-1 (subgroup A) or with RAV-7 (subgroup C). All of the *pol*⁺ progeny RSV isolates were infectious for C/O, C/E, T/BD, and RAV-1-infected C/E cells, but were not infectious for RAV-7-infected C/E cells (data

obtained with representative isolates only are presented in Table 5). Therefore, all of the *pol*⁺ progeny RSV isolates obtained by marker rescue with fragments of DNA of uninfected avian cells retained the subgroup C envelope specificity of LA335.

To characterize the DNA polymerases of *pol*⁺ progeny RSV, the thermolabilities of the virion DNA polymerases of representative *pol*⁺ progeny RSV isolates were studied. Preparations of disrupted virions were incubated for different times at 41°C, and the amount of remaining DNA polymerase activity was determined by assay at 35°C. The DNA polymerase of disrupted virions of wild-type PR-RSV-A (*pol*⁺) was inactivated at 41°C with a half-life of approximately 50 min (Fig. 1). In contrast, the DNA polymerase of disrupted virions of LA335 (*pol*^{ts}) was inactivated threefold more rapidly at 41°C, with a half-life of approximately 17 min (Fig. 1). The rates of inactivation at 41°C of the DNA polymerases of representative progeny RSV isolated in marker-rescue experiments with DNA fragments of V⁻ chicken cells, quail cells, and turkey cells were indistinguishable from the rate of inactivation of the PR-RSV-A (*pol*⁺) DNA polymerase, and the rate of inactivation of the DNA polymerase of the representative progeny RSV isolate obtained with fragments of pheasant cell DNA was only slightly more rapid (half-life, 36 min) than that of the PR-RSV-A (*pol*⁺) DNA polymerase (Fig. 1). Control experiments in which disrupted virions of LA335 were mixed with disrupted virions of PR-RSV-A or of the *pol*⁺ progeny RSV isolate obtained with fragments of pheasant cell DNA indicated that the differences in the thermolabilities of the DNA polymerases of LA335 and *pol*⁺ RSV were not due to diffusible factors in the virion preparations (data not shown). The thermolabilities of the DNA polymerases of the *pol*⁺ progeny RSV isolated in marker-rescue experiments, thus, appeared similar to that of the DNA polymerase of wild-type (*pol*⁺) RSV.

The antigenic specificities of the DNA polymerases of *pol*⁺ progeny RSV were characterized by neutralization tests of the DNA polymerases of disrupted virions. DNA polymerases of all of the *pol*⁺ progeny RSV isolates and of PR-RSV-A were neutralized (over 90%) by antibody against ALV DNA polymerase (50 µg of IgG-RSV-RAV-0), but not by antibody against reticuloendotheliosis virus DNA polymerase (50 µg of IgG-SNV), chicken cell DNA polymerase α (50 µg of IgG-α), or chicken cell DNA polymerase β (200 µg of IgG-β) (data not shown). In addition, the kinetics of neutralization of the DNA polymerases of *pol*⁺ progeny RSV by IgG-RSV-RAV-0 did not differ significantly from

TABLE 4. Efficiency of transformation of progeny virus isolates cloned at 35°C^a

Virus	Titer (FFU/ml)		EOT (41/ 35°C) ^b
	35°C	41°C	
Controls			
LA335 (<i>pol</i> ^{ts})	3 × 10 ³	<5	<2 × 10 ⁻³
B77V (<i>pol</i> ⁺)	5 × 10 ⁵	1 × 10 ⁶	2.0
Progeny virus clones ^c			
Chicken V ⁻			
-3a	5 × 10 ²	1 × 10 ³	2.0
-3b	5 × 10 ³	3 × 10 ³	0.6
-3c	2 × 10 ³	2 × 10 ³	1.0
Pheasant			
-2a	3 × 10 ⁴	3 × 10 ⁴	1.0
-2b	2 × 10 ²	5 × 10 ²	2.0
Quail			
-2a	5 × 10 ³	5 × 10 ³	1.0
-2b	5 × 10 ³	5 × 10 ³	1.0
-2c	1 × 10 ⁴	2 × 10 ⁴	2.0

^a Progeny viruses isolated from marker-rescue experiments (Table 1) were cloned in soft agar at 35°C, passaged once at 35°C, and assayed for transformation at 35 and 41°C.

^b EOT, Efficiency of transformation: ratio of virus titer at 41°C to the titer at 35°C.

^c Progeny viruses from marker-rescue experiments were named as described in Table 3. Clones of progeny virus isolates are designated by a letter following the isolate number.

TABLE 5. Subgroup of progeny viruses isolated by marker rescue with LA335 (*pol⁺env^r*)

Virus	Titer (FFU/ml) ^a				
	C/O	C/E	T/BD	C/E(RAV-1)	C/E(RAV-7)
Controls					
PR-RSV-A	2×10^4	4×10^4	1×10^5	<5	1×10^4
PR-RSV-C	5×10^2	5×10^2	1×10^3	5×10^2	<5
SR-RSV-D	2×10^5	2×10^5	<5	1×10^5	1×10^5
Marker rescue progeny ^b					
Chicken V ⁺ C/BE-2	2×10^2	2×10^2	1×10^2	1×10^2	<5
Chicken V ⁻ -1	5×10^2	5×10^2	1×10^2	1×10^2	<5
Pheasant-2	1×10^3	1×10^3	2×10^3	2×10^2	<5
Quail-3	4×10^3	4×10^3	4×10^3	5×10^2	<5
Turkey-1	3×10^2	3×10^2	1×10^2	1×10^2	<5

^a Viruses were assayed for focus formation on cultures of C/O chicken embryo fibroblasts, C/E chicken embryo fibroblasts, T/BD turkey embryo fibroblasts, and C/E chicken embryo fibroblasts which were passaged three times after infection with RAV-1 [C/E (RAV-1) cells] or with RAV-7 [C/E (RAV-7) cells].

^b Progeny viruses from marker-rescue experiments were harvested and designated as described in Table 3.

FIG. 1. Thermolability of virion DNA polymerases of progeny RSV. Virions of LA335 (*pol⁺*) (●), PR-RSV-A (*pol⁺*) (○), and representative progeny virus isolates from marker-rescue experiments with DNA fragments of V⁻ chicken cells (Ch, x), pheasant cells (Ph, +), quail cells (Q, □), and turkey cells (T, Δ) were disrupted with Nonidet P-40 and incubated for different times at 41°C. The remaining DNA polymerase activity was then assayed at 35°C. The progeny viruses from marker-rescue experiments were the isolates designated chicken V⁻-3, pheasant-2, quail-2, and turkey-3 in Table 3. The DNA polymerase activities of each of the disrupted virions before incubation at 41°C (100% activity) were between 5,000 and 20,000 cpm of [³H]TMP incorporated in 60 min at 35°C.

FIG. 2. Neutralization of virion DNA polymerase activity of progeny RSV. Virions of PR-RSV-A (●), *pol⁺* progeny RSV isolate pheasant-1 (○), and *pol⁺* progeny RSV isolate turkey-1 (x) were disrupted with Nonidet P-40 and incubated with IgG-RSV-RAV-O for 30 min at room temperature. Remaining DNA polymerase activity was assayed at 37°C. The 100% activity for each of the viruses was between 1,500 and 3,000 cpm of [³H]TMP incorporated in 30 min at 37°C.

neutralization of PR-RSV-A DNA polymerase. (Representative data are presented in Fig. 2; similar kinetics of neutralization were also observed with *pol⁺* progeny RSV isolates pheasant-2, quail-1, quail-3, turkey-2, and turkey-3.) The DNA polymerases of *pol⁺* progeny RSV, therefore, appeared to have antigenic determinants similar to those of wild-type RSV DNA polymerase.

DISCUSSION

Wild-type (*pol⁺*) progeny RSV were isolated in marker-rescue experiments with LA335 (*pol⁺*)

and fragments of DNA of uninfected V⁺C/BE chicken cells, V⁻gs⁻chf⁻ chicken cells, pheasant cells, quail cells, and turkey cells, but not with fragments of DNA of uninfected duck or mouse cells. The *pol*⁺ progeny RSV isolates did not appear to be revertants of LA335, since no *pol*⁺ progeny RSV were isolated from more than 400 control cultures treated with salmon sperm DNA. Since approximately 5×10^2 FFU (at 35°C) per culture were assayed at 41°C to detect *pol*⁺ progeny, the frequency of *pol*⁺ revertants in the progeny of salmon sperm DNA-treated control cultures was less than 5×10^{-6} . A similar reversion frequency of LA335 was previously noted by Linial and Mason (21), who found that none of 25 LA335 clones that they isolated as foci at 41°C were produced by *pol*⁺ revertants. Since the efficiency of focus formation by LA335 at 41°C was approximately 5×10^{-4} (21), the reversion frequency of LA335 in the experiments of Linial and Mason (21) appeared to be less than 10^{-5} . In contrast to the absence of *pol*⁺ revertants in the progeny of salmon sperm DNA-treated control cultures, 14 *pol*⁺ progeny RSV were isolated from cultures treated with fragments of chicken, pheasant, quail, and turkey DNAs. The frequencies of *pol*⁺ RSV in the progeny produced by cultures treated with these avian cell DNAs were 2×10^{-5} to 2×10^{-4} . Since these frequencies were at least 4- to 40-fold greater than the reversion frequency of LA335 in control cultures, it appeared that formation of *pol*⁺ progeny RSV probably occurred by recombination between LA335 and donor DNA fragments of the uninfected avian cells. In previous experiments with fragments of DNA of RSV-infected cells, *pol*⁺ progeny RSV were obtained when recipient cells were infected with LA335 within 6 h after treatment with DNA fragments, but not when the cells were infected with LA335 either 24 to 48 h before DNA treatment or 24 h after DNA treatment (4; unpublished observations). These observations are consistent with the possibility that recombination in marker-rescue experiments might occur between donor DNA fragments and newly synthesized unintegrated DNA of mutant RSV.

The *pol*⁺ progeny viruses isolated by marker rescue were genetically stable and had virion DNA polymerases with thermostabilities similar to that of wild-type RSV DNA polymerase. The *pol*⁺ progeny thus appeared to represent genetically stable recombinants of RSV with cellular genetic information homologous to the ALV *pol* gene. Neutralization assays of the DNA polymerase activities of disrupted virions indicated that the DNA polymerases of *pol*⁺ progeny RSV were serologically similar to the DNA polymer-

ase of wild-type RSV. However, more sensitive serological or biochemical characterizations of purified DNA polymerases might detect altered properties of the enzymes, possibly related to cellular genetic information.

The uninfected avian cells used as donors of DNA did not contain DNA proviruses of endogenous avian retroviruses that were infectious in transfection assays (6; Table 2). Therefore, the marker-rescue assay appeared to detect noninfectious forms of endogenous virus-related DNAs, indicating that uninfected chicken, pheasant, quail, and turkey cells contained endogenous DNA sequences sufficiently related to at least a portion of the ALV *pol* gene to yield *pol*⁺ recombinants. However, the activity of uninfected cell DNAs in marker-rescue experiments does not necessarily indicate that the endogenous *pol*-related genetic information represents a complete *pol* gene.

Although the endogenous RAV-0 DNA of uninfected V⁺C/BE chicken cells was not infectious in transfection assays, the cells spontaneously produced a low titer of RAV-0 and therefore appeared to contain a complete endogenous RAV-0 genome, including a biologically active ALV *pol* gene (6, 8, 33). Therefore, the DNA fragments of V⁺C/BE cells active in marker rescue may have been derived from the *pol* gene of the endogenous RAV-0 genome.

In contrast, uninfected V⁻ chicken cells do not produce RAV-0, although they appear to contain DNA sequences homologous to most of the nucleotide sequences of RAV-0 RNA (20, 25, 26, 31). Some V⁻ chicken cells produce proteins related to ALV internal structural proteins and envelope glycoproteins (2, 12, 14, 28, 39), but they apparently do not contain a DNA polymerase similar to ALV DNA polymerase (42), nor are uninfected V⁻ chicken embryo fibroblasts able to complement or recombine with mutants of RSV defective in *pol* (13, 40). However, particles containing RNA-directed DNA polymerase activities serologically distinct from ALV DNA polymerase have been reported in chicken embryos (19) and in the allantoic fluid of uninfected chicken eggs (1). In addition, competition radioimmunoassays have detected antigenic material related to ALV DNA polymerase in extracts of uninfected V⁻ chicken embryo fibroblasts (27) and weak serological relationships between ALV DNA polymerase and chicken cell α and β DNA polymerases have been reported (24). The isolation of *pol*⁺ progeny RSV by marker rescue with fragments of V⁻ chicken cell DNA indicates that V⁻ chicken cells contain genetic information related to at least a portion of the ALV *pol* gene. The lack of for-

mation of *pol*⁺ recombinants after infection of chicken embryo fibroblasts with *pol*⁻ (13) or *pol*^{ts} (40) RSV may indicate that cell-virus recombination in virus-infected cells is dependent on transcription of the endogenous virus-related genes (40, 41). The absence of complete transcripts of the ALV *pol* gene in the RNA of uninfected V-gs⁺chf⁺ chicken cells (38) is consistent with this hypothesis.

Uninfected pheasant, quail, and turkey cells appear to contain DNA sequences homologous to a fraction of the nucleotide sequences of RAV-0 RNA, whereas the DNAs of uninfected duck and mouse cells lack detectable sequence homology with RAV-0 (20, 25, 31). The DNA of pheasant cells appears to hybridize to 10 to 20% of the RNA sequences of RAV-0 (20, 25, 31). Kang and Temin (20) reported that DNAs of uninfected quail and turkey cells hybridized, respectively, to 15 and 10% of the sequences of RAV-0 RNA, although others have reported that the DNAs of uninfected quail (25, 31) and turkey (31) cells hybridized to less than 5% of the RNA of RAV-0. Uninfected pheasant, quail, and turkey cells, but not duck cells, also contain DNA sequences homologous to approximately 10% of the RNA of reticuloendotheliosis viruses (20). Biological activity of endogenous retrovirus-related genetic information in pheasant cells, partridge cells, and Chinese quail cells has been demonstrated by complementation and recombination between ALV and endogenous envelope glycoprotein genes (3, 9, 15, 34). In addition, infectious endogenous viruses have been isolated from cells of several species of pheasants (3, 15) and from Chinese quail (3). The endogenous viruses isolated from *Chrysolophus* pheasants represent a third group of avian retroviruses unrelated by immunological cross-reactivity or nucleic acid hybridization to the avian leukemia or reticuloendotheliosis virus groups (16). The endogenous *pol*-related genetic information we have detected in marker-rescue experiments with the DNAs of heterologous avian species could correspond to endogenous ALV-related DNA sequences detected by nucleic acid hybridization, to endogenous genetic information related to the reticuloendotheliosis virus or *Chrysolophus* pheasant virus groups, or to genetic information related to cellular DNA polymerases. Further characterization of the *pol*⁺ progeny virus isolates may elucidate the nature of the endogenous *pol*-related genetic information in uninfected avian cells.

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