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Assay of Noninfectious Fragments of DNA of Avian Leukosis Virus-Infected Cells by Marker Rescue

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A marker rescue assay of noninfectious fragments of avian leukosis virus DNAs is described. DNA fragments were prepared either by sonication or *Eco*RI-digestion of DNAs of chicken cells infected with wild-type Rous sarcoma virus, with a nontransforming avian leukosis virus, and with a mutant of Rous sarcoma virus temperature sensitive for transformation. Recipient cultures of chicken embryo fibroblasts were treated with noninfectious DNA fragments and infected with temperature-sensitive mutants of Rous sarcoma virus defective in DNA polymerase or in an internal virion structural protein. Wild-type progeny viruses which replicated at the nonpermissive temperature were isolated. Some of the wild-type progeny acquired both the wild-type DNA polymerase and the subgroup specificity of the Rous sarcoma virus strain used for preparation of sonicated or *Eco*RI-digested DNA fragments. Therefore the genetic markers for DNA polymerase and envelope were linked and appeared to be located on the same *Eco*RI fragment of the DNA of Rous sarcoma virus-infected cells.

The infectivity of DNAs of cells infected with several groups of retroviruses (RNA tumor viruses) has provided direct evidence for a DNA intermediate in the replication of this family of viruses (3, 10, 11, 20–22). In addition, assays of DNA infectivity have been used to characterize integrated and unintegrated forms of virus DNAs in virus-infected cells (1, 3, 6, 9, 22) and to study endogenous virus-related DNAs in uninfected cells (4, 21). These assays of infectious virus DNAs have been based on the infectivity of intact virus genomes which contained all of the information required for the production of infectious progeny virus.

Since biological assays of subgenomic DNA fragments could be useful for further characterization of retrovirus DNAs, we have investigated the possibility of assaying noninfectious fragments of avian leukosis virus (ALV) DNAs by marker rescue. In this paper we describe marker rescue experiments in which wild-type progeny viruses were isolated from cells treated with noninfectious fragments of ALV DNAs and infected with temperature-sensitive mutants of Rous sarcoma virus (RSV) defective in DNA polymerase or in an internal virion structural protein.

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. Spafas chicken embryo fibroblasts were C/E, ALV-negative, and chicken helper factor-negative. Fertile line 100 chicken eggs were a generous gift of L. B. Crittenden, U. S. Department of Agriculture, East Lansing, Mich. The line 100 chicken embryo fibroblasts used in these experiments were V^+C/O and produced high titers of Rous-associated virus-O (RAV-O) as a result of exogenous RAV-O infection (4, 5). Fertile Orlopp turkey eggs were obtained from the Wilmar Poultry Co., Wilmar, Minn. Turkey embryo fibroblasts were T/BD, ALV-negative, and helper factor-negative.

Two temperature-sensitive mutants of RSV, *LA335* and *LA3342*, were used in marker rescue experiments. They were generously donated by W. S. Mason, Philadelphia, Pa., and by P. K. Vogt, Los Angeles, Calif. *LA335* is an early mutant of Prague RSV subgroup C (PR-RSV-C) which is defective in DNA polymerase (17, 18). *LA3342* is a late mutant of B77 avian sarcoma virus (B77V) (subgroup C) which is defective in an internal virion structural protein (12, 13). Stocks of these viruses for use in marker rescue experiments were harvested at 6-h intervals from transformed cultures of chicken embryo fibroblasts incubated at 35°C in 100-mm culture dishes which contained 3 ml of ET medium supplemented with 6% calf serum and 3% fetal bovine serum.

LA25 (subgroup C), a temperature-sensitive transformation-maintenance mutant of Prague RSV (29, 30), was obtained from H. M. Temin, Madison, Wis. PR-RSV-C was obtained from W. S. Mason. Schmidt-Ruppin RSV subgroup D (SR-RSV-D), B77V (subgroup C), and Rous-associated virus-49

(RAV-49) (subgroup C) were obtained from H. M. Temin. The SR-RSV-D used in these experiments did not contain excess nontransforming ALV (unpublished observations).

Viruses were assayed by focus formation. Chicken or turkey embryo fibroblasts were plated in ET medium at 6×10^5 cells per 60-mm culture dish, incubated overnight at 37°C, and inoculated with 0.2-ml portions of virus suspensions. Cultures were incubated for 40 min to allow virus absorption, and 5 ml of ET medium supplemented either with 2% calf serum and 2% fetal bovine serum (for chicken cells) or with 2% fetal bovine serum (for turkey cells) was added. Media were changed 3 to 4 days later and foci were counted 5 to 7 days after infection. In assays of temperature-sensitive RSV, both absorption of virus and subsequent incubation of the cells were either at 35°C (permissive temperature) or at 41°C (nonpermissive temperature).

Viruses were cloned by overlaying virus-infected cells with ET medium supplemented with 1.5% calf serum, 1% fetal bovine serum, and 0.4% agarose (Seakem, Microbiological Assoc., Bethesda, Md.). Individual foci of transformed cells were picked 7 days after infection and used to grow virus stocks.

Preparation of DNA. DNA was extracted from RSV-infected chicken embryo fibroblasts 5 to 7 days after infection (RSV DNA) or from line 100 V$^+$C/O chicken embryo fibroblasts (RAV-O DNA) as previously described (3). Noninfectious fragments of DNA were prepared either by sonication of the DNA or by digestion of the DNA with *Escherichia coli* restriction endonuclease *Eco*RI, which was previously found to inactivate the infectivity of RSV DNA (Cooper and Temin, unpublished data).

DNAs to be sonicated were dissolved in 1× SSC (0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) at a concentration of 200 μg of DNA/ml. Samples of DNA (2 ml) were sonicated on ice for 60 s (two 30-s pulses) using the microtip of a Branson sonifier with an output of 60 W. The molecular weights of sonicated DNAs (within individual preparations) ranged from approximately 2×10^5 to 1×10^6, as determined by comparison of their electrophoretic mobilities in 1.4% agarose gels to the mobilities of marker DNAs prepared by digestion of simian virus 40 DNA with *Haemophilus influenza* restriction endonuclease *Hin*dIII. The molecular weights of the *Hin*dIII fragments of simian virus 40 DNA were approximately 1.2×10^6, 8.1×10^5, 7.4×10^5, 3.8×10^5, 3.1×10^5, and 1.4×10^5 (15).

DNAs to be digested with *Eco*RI were dissolved in buffer that contained 100 mM Tris-hydrochloride (pH 7.4), 50 mM NaCl, 5 mM $MgCl_2$, and 100 μg of gelatin/ml at a concentration of 50 μg of DNA/ml. *Eco*RI (New England Biolabs, Beverly, Mass.) was added (3 to 6 U/μg of DNA) and reaction mixtures were incubated for 20 to 48 h at 37°C. A one-tenth volume of 10× SSC was added and DNAs were stored at −70°C.

Marker rescue assay of noninfectious DNA fragments. Chicken embryo fibroblasts (Spafas) were plated in 5 ml of ET medium at 6×10^5 cells per 60-mm culture dish. Four hours after plating, fetal bovine serum was added to a final concentration of 4%. After overnight incubation at 37°C, the cells were treated with noninfectious DNA fragments using the calcium method of Graham and Van der Eb (7, 8). DNAs were diluted to 10 μg/ml in HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-buffered saline (8). Calcium chloride (1.25 M) was added to a final concentration of 0.125 M, and a precipitate was allowed to form for 10 to 20 min at room temperature. Aliquots (0.5 ml) of the precipitated DNA suspensions were added directly to recipient cell cultures, without removing the culture medium, and the cells were incubated for 4 h at 37°C. Supernatant media were then removed, and the DNA-treated cells were infected at 35°C with *LA335* or *LA3342* at a multiplicity of infection of approximately 0.05 focus-forming units (FFU) per cell. (The sensitivity of focus assays was approximately 10-fold lower than that of end-point dilution assays in which transformation was scored after one transfer of the virus-infected cells; therefore, the multiplicity of infection of 0.05 FFU per cell corresponded to approximately 0.5 infectious units per cell. Higher multiplicities of infection were difficult to obtain due to the lower titers of *LA335* and *LA3342* stocks.) After 40 min of incubation at 35°C, 5 ml of ET medium supplemented with 2% fetal bovine serum was added. In experiments with noninfectious fragments of RAV-O DNA, turkey embryo fibroblasts (4×10^5 cells per culture) were added after virus infection to permit replication of subgroup E progeny.

Since the mutational defects of *LA335* and *LA3342* are expressed at different stages of replication, different procedures were used to select for wild-type progeny in experiments with the two mutants. Cells that were infected with *LA335* were incubated at 35°C, and supernatant media harvested 6 to 9 days after infection were assayed on fresh cells at 41°C to select for progeny able to initiate infection at the nonpermissive temperature. Cells that were infected with *LA3342* were incubated at 35°C for 4 to 5 days and then shifted to 41°C to select for maturation of infectious progeny at the nonpermissive temperature. Supernatant media harvested after 2 to 3 days of incubation of *LA3342*-infected cells at 41°C were assayed on fresh cells at 41°C.

Genetic notation. The following conventions are used to describe the relevant genetic markers of virus strains. The RSV genes for internal structural proteins, DNA polymerase, envelope glycoprotein, and transformation are represented as *gag*, *pol*, *env*, and *src*, respectively. The superscript symbols + and ts are used to represent the genotypes corresponding to wild-type and temperature-sensitive gene expression. Nontransforming ALV are represented as *src*$^-$. The genotypes of subgroup C, D, and E ALV are represented as *env*^{C}, *env*^{D}, and *env*^{E}.

RESULTS

Isolation of wild-type progeny RSV by marker rescue. Recipient cultures of chicken embryo fibroblasts were treated with noninfectious fragments of DNA of wild-type RSV-infected cells and were infected with *LA335* (*pol*^{ts}) or *LA3342* (*gag*^{ts}) at 35°C. Control cul-

tures either were treated with noninfectious fragments of RSV DNA alone or were treated with salmon sperm DNA and infected with temperature-sensitive RSV. Supernatant media were harvested 6 to 9 days after infection and were assayed on fresh cells at 41°C. In order to distinguish the presence of wild-type progeny RSV from leakiness of parental RSV mutants, the supernatant media of all 41°C assay plates which were positive for transformation were harvested and reassayed in a second passage at 41°C. Since both *LA335* and *LA3342* were temperature sensitive for replication, the presence of transforming virus which increased in titer during two passages at 41°C indicated formation of wild-type progeny RSV.

Progeny RSV which replicated at 41°C were produced by approximately 5 to 10% of the cultures treated with sonicated or *Eco*RI-digested fragments of RSV DNA and infected with *LA335* or *LA3342* (Table 1). The supernatant media of these cultures had titers of 10^2 to 10^3 FFU/ml in the second passage at 41°C. In contrast, progeny RSV that replicated at 41°C were not produced by control cultures treated with salmon sperm DNA and infected with *LA335* or *LA3342* (<5 FFU/ml in the second passage at 41°C) (Table 1). Therefore, reversion of the parental RSV mutants did not occur at significant frequency in these experiments. Progeny RSV were also not produced by control cultures treated with sonicated or *Eco*RI-digested fragments of RSV DNA alone (Table 1), indicating that these treatments completely inactivated the infectivity of RSV DNA. In addition, nontransforming ALV were not produced by control cultures treated with sonicated or *Eco*RI-digested fragments of RSV DNA alone, as determined by the lack of cytopathic effect induced by nontransforming subgroup D ALV and by the lack of sedimentable DNA polymerase activity in culture fluids (data not shown).

Progeny RSV that replicated at 41°C were also obtained in marker rescue experiments with sonicated fragments of DNA of cells infected with RAV-O, a nontransforming ALV (*src*$^-$) (Table 2). In these experiments, turkey embryo fibroblasts (T/BD) were added to DNA-treated chicken cells (C/E) to permit replication of progeny virus of either subgroup C (the subgroup of *LA335* and *LA3342*) or subgroup E (the subgroup of RAV-O). Supernatant media were assayed for focus formation at 41°C in turkey embryo fibroblasts. The frequency of isolation of progeny RSV that replicated at 41°C with sonicated fragments of RAV-O DNA was similar to the frequency obtained with sonicated fragments of RSV DNA. The progeny RSV that replicated at 41°C appeared to be

TABLE 1. *Marker rescue with noninfectious fragments of wild-type RSV DNA*[a]

Virus	Fraction of positive cultures[b]		
	Salmon sperm DNA	Sonicated RSV DNA[c]	*Eco*RI-digested RSV DNA[c]
None	ND[d]	0/68	0/48
LA335 (*pol*^{ts})	0/115	5/98	3/60
LA3342 (*gag*^{ts})	0/60	8/74	1/54

[a] Cultures of chicken embryo fibroblasts were treated with 5 μg of salmon sperm DNA, sonicated fragments of RSV DNA, or *Eco*RI-digested fragments of RSV DNA. RSV DNAs were prepared from chicken embryo fibroblasts 5 to 7 days after infection with SR-RSV-D or B77V. After DNA treatment, cultures either were mock-infected, infected with *LA335*, or infected with *LA3342* at 35°C. The formation of progeny RSV which replicated at 41°C was assayed by two passages of supernatant media at 41°C in chicken embryo fibroblasts as described in the text.

[b] Number of cultures that produced progeny RSV which replicated at 41°C over the total number tested.

[c] Noninfectious fragments of RSV DNA were from SR-RSV-D-infected cells, except for 6 mock-infected and 24 *LA3342*-infected cultures which were treated with sonicated B77V DNA. Progeny RSV which replicated at 41°C were produced by 1 of the 24 cultures treated with sonicated B77V DNA and infected with *LA3342*.

[d] ND, Not done.

TABLE 2. *Marker rescue with noninfectious fragments of RAV-O DNA*[a]

Virus	Fraction of positive cultures[b]	
	Salmon sperm DNA	Sonicated RAV-O DNA
LA335 (*pol*^{ts})	0/30	2/30
LA3342 (*gag*^{ts})	0/30	3/30

[a] Cultures of chicken embryo fibroblasts were treated with salmon sperm DNA or with sonicated fragments of DNA of V$^+$ C/O line 100 cells (RAV-O DNA) and were infected with virus as described in Table 1. Turkey embryo fibroblasts were added to permit replication of subgroup E progeny virus. The formation of progeny RSV which replicated at 41°C was assayed by two passages of supernatant media in turkey embryo fibroblasts at 41°C.

[b] Number of cultures that produced progeny RSV which replicated at 41°C over the total number tested.

gag$^+$*pol*$^+$*src*$^+$ recombinants, which acquired the *pol*$^+$ or *gag*$^+$ replication marker of RAV-O (*gag*$^+$*pol*$^+$*src*$^-$) but retained the *src*$^+$ transformation marker of *LA335* (*gag*$^+$*pol*^{ts}*src*$^+$) or *LA3342* (*gag*^{ts}*pol*$^+$*src*$^+$).

Marker rescue experiments were also per-

formed with noninfectious fragments of DNA of cells infected with *LA25*, a mutant of RSV which is temperature sensitive for maintenance of transformation but wild type for replication (29, 30). Supernatant media harvested from cultures treated with noninfectious fragments of *LA25* (*pol*$^+$*src*^{ts}) DNA and infected with *LA335* (*pol*^{ts}*src*$^+$) were passaged twice at 41°C and then assayed for transformation at 35°C. This procedure selected for *pol*$^+$ progeny which replicated at 41°C but did not select for the ability of progeny to transform at 41°C. Therefore, it was possible to study segregation of the *src*^{ts} transformation marker of *LA25* by characterization of the progeny RSV to determine whether they were *pol*$^+$*src*^{ts} or *pol*$^+$*src*$^+$. Progeny RSV that replicated at 41°C were isolated from cultures treated with sonicated or *Eco*RI-digested fragments of *LA25* DNA and infected with *LA335*, but not from control cultures treated with salmon sperm DNA and infected with *LA335* or from control cultures treated with sonicated or *Eco*RI-digested fragments of *LA25* DNA alone (Table 3). Characterization of the *src* allele of the progeny RSV is presented in the following section.

Efficiency of transformation and replication of progeny RSV at permissive and nonpermissive temperatures. The efficiency of transformation of progeny RSV isolated in marker rescue experiments with *LA335* (*pol*^{ts}) was determined by titration of each isolate at 35 and 41°C. All progeny RSV isolated in marker rescue experiments with noninfectious fragments of SR-RSV-D or RAV-O DNAs had similar efficiencies of transformation at the two temperatures, in contrast to *LA335* which had a 10^3-fold lower efficiency of transformation at 41°C (Table 4). Therefore the progeny RSV were *pol*$^+$. In addition, the progeny RSV isolated with noninfectious fragments of *LA25* (*pol*$^+$*src*^{ts}) DNA in marker rescue experiments with *LA335* (*pol*^{ts}*src*$^+$) were wild type for transformation at 41°C, whereas both parental viruses were temperature sensitive (Table 4). The progeny RSV isolated with noninfectious fragments of *LA25* DNA were therefore *pol*$^+$*src*$^+$ recombinants which had acquired the *pol*$^+$ allele of *LA25* but had retained the *src*$^+$ allele of *LA335*.

To determine the efficiency of replication of progeny RSV isolated in marker rescue experiments with *LA3342* (*gag*^{ts}), cells were infected with each progeny virus isolate and incubated at 35 or 41°C for 5 days. Supernatant media were harvested and assayed at 35°C to determine the extent of virus replication at the two temperatures. All of the progeny virus isolates from marker rescue experiments with *LA3342* replicated with similar efficiencies at 35 and 41°C (Table 5), indicating that the progeny RSV were *gag*$^+$.

TABLE 3. *Marker rescue with noninfectious fragments of LA25 (pol*$^+$*src*^{ts}*) DNA*[a]

Virus	Fraction of positive cultures[b]		
	Salmon Sperm DNA	Sonicated *LA25* DNA	*Eco*RI-digested *LA25* DNA
None	ND[c]	0/72	0/12
LA335 (*pol*^{ts}*src*$^+$)	0/42	2/51	2/21

[a] Cultures of chicken embryo fibroblasts were treated with salmon sperm DNA or with fragments of DNA of *LA25*-infected cells and were infected with virus as described in Table 1. Supernatant media were passaged twice at 41°C in chicken embryo fibroblasts and then assayed for transformation at 35°C.

[b] Number of cultures which produced progeny RSV which replicated at 41°C over the total number tested.

[c] ND, Not done.

Subgroup specificity of progeny RSV. Progeny viruses isolated in marker rescue experiments with noninfectious fragments of SR-RSV-D (subgroup D) and RAV-O (subgroup E) DNAs were characterized to determine if the progeny viruses had the subgroup specificity of the *LA335* or *LA3342* parents (both subgroup C) or the subgroup specificity of the viruses used for preparation of DNA fragments. All of the progeny viruses were isolated by selection for replication at 41°C without selection for subgroup.

To determine the subgroup of progeny virus isolates, the viruses were assayed on C/E cells, on T/BD cells, and on RAV-49-infected C/E cells [C/E(RAV-49) cells] which were resistant to infection with subgroup C RSV due to interference. All three *pol*$^+$ progeny RSV isolated with *Eco*RI-digested fragments of SR-RSV-D (*pol*$^+$*env*^{D}) DNA in marker rescue experiments with *LA335* (*pol*^{ts}*env*^{C}) were subgroup D (Table 6). Similarly, two of five *pol*$^+$ progeny RSV isolated with sonicated fragments of SR-RSV-D (*pol*$^+$*env*^{D}) DNA in marker rescue experiments with *LA335* (*pol*^{ts}*env*^{C}) were subgroup D, whereas the other three were subgroup C (Table 6). Both of the *pol*$^+$ progeny RSV isolated with sonicated fragments of RAV-O (*pol*$^+$*env*^{E}) DNA in marker rescue experiments with *LA335* (*pol*^{ts}*env*^{C}) were subgroup C (Table 6). These results indicated that some of the progeny RSV selected for the *pol*$^+$ allele of the DNA polymerase gene of *LA335* also acquired the subgroup specificity of the virus used for prepa-

TABLE 4. *Efficiency of transformation of progeny viruses isolated in marker rescue experiments with LA335 (pol^{ts})*[a]

Virus	Titer (FFU/ml)		EOT[b]
	35°C	41°C	
Controls:			
LA335 ($pol^{ts}src^{+}$)	2×10^{3}	2	10^{-3}
LA25 ($pol^{+}src^{ts}$)	2×10^{6}	2×10^{1}	10^{-5}
PR-RSV-C ($pol^{+}src^{+}$)	4×10^{2}	2×10^{2}	0.5
SR-RSV-D ($pol^{+}src^{+}$)	1×10^{5}	3×10^{4}	0.3
Marker rescue progeny:[c]			
*Eco*RI SR-RSV-D DNA			
1	9×10^{3}	2×10^{3}	0.2
2	2×10^{4}	7×10^{3}	0.3
3	2×10^{3}	3×10^{2}	0.2
Sonicated SR-RSV-D DNA			
1	8×10^{2}	6×10^{2}	0.7
2	4×10^{3}	9×10^{3}	2.2
3	1×10^{2}	7×10^{1}	0.7
4	4×10^{3}	8×10^{3}	2.0
5	1×10^{2}	3×10^{1}	0.3
Sonicated RAV-O DNA			
1	3×10^{3}	5×10^{2}	0.2
2	5×10^{3}	1×10^{3}	0.2
*Eco*RI *LA25* DNA			
1	3×10^{2}	1×10^{2}	0.3
2	2×10^{2}	1×10^{2}	0.5
Sonicated *LA25* DNA			
1	1×10^{3}	2×10^{3}	2.0
2	1×10^{2}	4×10^{1}	0.4

[a] Viruses were assayed at 35 and 41°C on chicken or turkey embryo fibroblasts and foci were counted 4 to 6 days after infection. Progeny viruses from marker rescue experiments with RAV-O DNA were assayed on turkey embryo fibroblasts; other progeny viruses were assayed on chicken embryo fibroblasts. *LA335* and PR-RSV-C had similar efficiencies of transformation on both types of cells.

[b] 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 were harvested after two passages at 41°C. The viruses are designated according to the type of DNA fragments used for marker rescue, followed by the isolate number of the progeny virus. Each progeny virus was independently isolated.

ration of DNA fragments. In contrast, all of the gag^{+} progeny viruses isolated in marker rescue experiments with *LA3342* ($gag^{ts}env^{C}$) retained the subgroup C specificity of *LA3342* (Table 7).

To test the genetic stability of the subgroup D progeny viruses, two of the $pol^{+}env^{D}$ progeny RSV isolated with *Eco*RI-digested fragments of SR-RSV-D DNA in marker rescue experiments with *LA335* were cloned under agarose. Four individual foci were picked and used to grow virus stocks. The four subclones all had titers of 10^{3} to 10^{4} FFU/ml on C/E cells and on C/E (RAV-49) cells but were not infectious for T/BD cells (<5 FFU/ml). In addition, the uncloned stocks of these progeny RSV isolates were found not to contain an excess of nontransforming subgroup D ALV (data not shown). Therefore, the env^{D} marker of the progeny RSV appeared to be genetically stable.

DISCUSSION

In this paper we describe marker rescue experiments in which wild-type progeny RSV were isolated from cells which were treated with noninfectious fragments of ALV DNA and infected with temperature-sensitive mutants of RSV. The formation of wild-type progeny RSV in these experiments probably occurred by recombination between DNA fragments and an intracellular form of viral DNA of the mutant RSV. Similar types of marker rescue experiments have been described with bacteriophage (23) and with herpes simplex virus (28).

The marker rescue assay of noninfectious fragments of ALV DNAs was much less efficient than the infectivity assay of infectious ALV DNAs. Wild-type progeny RSV were isolated by marker rescue from 5 to 10% of recipient cultures treated with 5 μg of noninfectious fragments of ALV DNA per culture. Therefore, one infectious unit of ALV DNA fragments assayed by marker rescue corresponded to approximately 100 μg of DNA of ALV-infected cells. In contrast, one infectious unit of ALV DNA assayed by infectivity corresponded to 0.1 μg of DNA of RSV-infected cells (3) and to 1.0

TABLE 5. *Efficiency of replication of progeny viruses isolated in marker rescue experiments with LA3342 (gag^{ts})* [a]

Virus	Titer (FFU/ml)		EOR[b]
	35°C	41°C	
Controls:			
LA3342 (gag^{ts})	5×10^3	2	4×10^{-4}
B77V (gag^+)	2×10^6	2×10^6	1.0
Marker rescue progeny:[c]			
*Eco*RI SR-RSV-D DNA			
1	5×10^3	2×10^4	4.0
Sonicated SR-RSV-D DNA			
1	3×10^4	9×10^4	3.0
2	1×10^4	4×10^4	4.0
3	9×10^4	1×10^4	0.1
4	8×10^4	2×10^4	0.25
5	7×10^3	1×10^4	1.4
6	2×10^4	1×10^4	0.5
7	8×10^3	2×10^4	2.5
Sonicated B77V DNA			
1	4×10^3	4×10^3	1.0
Sonicated RAV-O DNA			
1	2×10^3	4×10^3	2.0
2	5×10^2	7×10^2	1.4
3	3×10^4	2×10^4	0.7

[a] Cultures of chicken or turkey embryo fibroblasts were exposed to virus (approximately 0.001 FFU/cell) and were incubated at 35 or 41°C. Supernatant media were harvested 5 days after infection and were assayed on fresh cells at 35°C to determine the extent of virus replication at the two temperatures. Turkey embryo fibroblasts were used for growth and assay of progeny viruses from marker rescue experiments with RAV-O DNA; chicken embryo fibroblasts were used for other viruses. *LA3342* and B77V had similar efficiencies of replication on both types of cells.

[b] Efficiency of replication; ratio of the titer of virus produced at 41°C to the titer of virus produced at 35°C.

[c] Progeny viruses from marker rescue experiments were harvested and named as described in Table 4.

TABLE 6. *Subgroup of progeny viruses isolated in marker rescue experiments with LA335 ($pol^{ts}env^C$)*

Virus	Titer (FFU/ml)[a]		
	C/E cells	T/BD cells	C/E(RAV-49) cells
Controls:			
LA335 ($pol^{ts}env^C$)	3×10^3	3×10^3	<5
B77V (pol^+env^C)	1×10^6	1×10^6	<5
SR-RSV-D (pol^+env^D)	3×10^4	<5	5×10^3
Marker rescue progeny:[b]			
*Eco*RI SR-RSV-D DNA			
1	2×10^4	<5	1×10^3
2	4×10^4	<5	2×10^3
3	3×10^3	<5	3×10^3
Sonicated SR-RSV-D DNA			
1	5×10^2	<5	2×10^2
2	5×10^3	<5	1×10^3
3	5×10^1	1×10^2	<5
4	6×10^2	6×10^2	<5
5	2×10^2	1×10^2	<5
Sonicated RAV-O DNA			
1	1×10^3	1×10^3	<5
2	2×10^3	1×10^3	<5

[a] Viruses were assayed on cultures of chicken embryo fibroblasts (C/E cells), turkey embryo fibroblasts (T/BD cells), and chicken embryo fibroblasts which had been passaged three to four times after infection with RAV-49 [C/E(RAV-49) cells]. Foci were counted four to six days after infection.

[b] Progeny viruses from marker rescue experiments were harvested and named as described in Table 4.

TABLE 7. *Subgroup of progeny viruses isolated in marker rescue experiments with LA3342 ($gag^{ts}env^{C}$)*[a]

Virus	Titer (FFU/ml)		
	C/E cells	T/BD cells	C/E(RAV-49) cells
Controls:			
B77V (env^{C})	1×10^{6}	1×10^{6}	<5
SR-RSV-D (env^{D})	3×10^{4}	<5	5×10^{3}
Marker rescue progeny:			
*Eco*RI SR-RSV-D DNA			
1	1×10^{4}	2×10^{4}	<5
Sonicated SR-RSV-D DNA			
1	3×10^{4}	4×10^{4}	<5
2	6×10^{3}	6×10^{3}	<5
3	1×10^{3}	1×10^{2}	<5
4	1×10^{3}	1×10^{3}	<5
5	1×10^{3}	1×10^{3}	<5
6	1×10^{3}	1×10^{3}	<5
7	5×10^{3}	5×10^{3}	<5
Sonicated RAV-O DNA			
1	3×10^{3}	3×10^{3}	<5
2	1×10^{3}	5×10^{2}	<5
3	5×10^{3}	6×10^{3}	<5

[a] The subgroup of progeny viruses was determined as described in Table 6.

μg of DNA of RAV-O-infected cells (4). We have not been able to increase the efficiency of marker rescue by variation of the size of sonicated DNA fragments, by changes in the relative times of DNA treatment and virus infection, by increases in the multiplicity of infection with mutant RSV, or by the use of infectious center assays to detect formation of wild-type progeny (unpublished observations).

In spite of its low efficiency, marker rescue provides an assay of the biological activity of subgenomic fragments of ALV DNA. The assay may therefore contribute to the characterization of different forms of ALV DNA present in either infected or uninfected cells. Similar marker rescue assays have recently been used in studies of the virion DNAs of papova and herpes simplex viruses (16, 19, 28).

The properties of the progeny RSV isolated by marker rescue indicated that they were recombinants between noninfectious fragments of ALV DNAs and mutant RSV. Progeny RSV isolated with fragments of RAV-O ($gag^{+}pol^{+}src^{-}$) or *LA25* ($gag^{+}pol^{+}src^{ts}$) DNAs in marker rescue experiments with *LA335* ($pol^{ts}src^{+}$) or *LA3342* ($gag^{ts}src^{+}$) acquired the pol^{+}or gag^{+} replication allele of RAV-O or *LA25* but retained the src^{+} transformation gene of *LA335* or *LA3342*. Similarly, gag^{+} progeny RSV isolated with fragments of RAV-O ($gag^{+}env^{E}$) or SR-RSV-D ($gag^{+}env^{D}$) DNAs in marker rescue experiments with *LA3342* ($gag^{ts}env^{C}$) retained the subgroup C envelope specificity of *LA3342*. In contrast, some of the pol^{+} progeny isolated with fragments of SR-RSV-D ($pol^{+}env^{D}$) DNA in marker rescue experiments with *LA335* ($pol^{ts}env^{C}$) acquired the subgroup D envelope specificity together with the wild-type DNA polymerase of SR-RSV-D. This result indicates linkage of the genes for DNA polymerase and subgroup D envelope specificity in the SR-RSV-D DNA fragments. In addition, since genetic markers for DNA polymerase and subgroup specificity were co-transferred in *Eco*RI-digested fragments of SR-RSV-D DNA, these markers appeared to be located on the same fragment of *Eco*RI-digested RSV DNA.

The RSV genes for internal structural proteins (*gag*), DNA polymerase (*pol*), envelope glycoprotein (*env*), and maintenance of transformation (*src*) have been mapped on the RNA of RSV by analysis of large RNase T1 oligonucleotides of the RNAs of recombinants and deletion mutants of RSV (2, 14, 24–27). The gene order of RSV RNA deduced from these studies is, in the 5′ to 3′ direction, *gag-pol-env-src*. The linkage of *pol* and *env* in noninfectious fragments of DNA of RSV-infected cells is therefore consistent with the gene order determined for the virion RNA of RSV.

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