Cell Fusion for Genetic Analysis of Two Nonconditional Rous Sarcoma Virus Replication Mutants

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Procedures for characterizing replication-defective viruses in nonpermissive mammalian cells were developed and applied to three nonvirogenic Rous sarcoma virus (RSV)-transformed mammalian cell lines—B4, a line of Bryan virus-transformed hamster cells, and two SRD-RSV transformed rat cell lines, LR3/1 and LR3/2. Cell fusion was used to study virus complementation. The three cell lines (i) fused with helper virus-infected chicken cells and the host range of the rescued virus examined, (ii) tested for complementation by fusion with chicken cells exhibiting various patterns of endogenous virus expression, (iii) fused with chicken cells infected with the temperature-sensitive replication mutant LA334 and assayed for complementation at permissive and nonpermissive temperatures, and (iv) tested for complementation of defective viruses in other RSV-transformed mammalian cell lines by fusing pairs of nonvirogenic cell lines and permissive chicken cells. Based upon these complementation studies, we concluded that B4 virus is defective only in the *env* gene, LR3/1 virus is an absolute mutant in the *gag* and/or *pol* genes, and LR3/2 virus is a leaky *env* mutant. Clones of LR3/1 and LR3/2 virus-infected chicken cells were established, and the results obtained from the characterization of these viruses in permissive avian cells substantiates the conclusions reached in the fusion-rescue studies.

Defective viruses accumulate at a high frequency during Rous sarcoma virus (RSV) replication. These viruses have been shown to be defective in replicative (19) as well as transforming (42) functions. Transformation-defective viruses are readily propagated and as a result have been studied extensively (for review see 12, 44). To characterize replication-defective viruses, they must be cloned free of any replication-competent viruses and, in the case of nonconditional mutants, such as deletion mutants, long-term cultures of virus-transformed cells must be established. This has proven difficult in permissive avian cells since most cultures of transformed chicken cells fail to survive beyond several passages. Consequently, most of the replication mutants that have been characterized are conditional (temperature sensitive) rather than nonconditional. Although quail cells have been used recently for cloning RSV replication mutants (20, 21, 23), we have, for reasons to be discussed, pursued a different approach.

The infection of mammalian cells with non-defective strains of RSV yields transformants that may be divided into two groups depending upon their ability to release infectious trans-

forming virus after cell fusion with permissive avian cells. Cell lines that produce virus in rescue assays are referred to as virogenic, and those that fail to yield virus are referred to as nonvirogenic (34). One possible explanation for the failure of nonvirogenic cell lines to produce virus in rescue assays is that they are transformed by replication-defective viruses. In a previous report we described a method for detecting replication-defective viruses in RSV-transformed mammalian cells that involved infecting permissive avian cells with nontransforming leukosis virus (RAV) before cell fusion with the transformed mammalian cells (36). Since the helper virus supplies a complete set of functional virus replication genes, any mutant that is complemented by phenotypic mixing should be rescued. Transforming virus was recovered from every rescue-negative (nonvirogenic) cell line that was tested when fused with helper virus-infected chicken embryo cells (CEC), suggesting that these cell lines were indeed transformed by replication-defective viruses. Furthermore, it seemed that mammalian cells might serve as suitable hosts for isolating and characterizing nonconditional RSV replication mutants.

This report describes the characterization of the defective replication functions of the viruses

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in two nonvirogenic rat cell lines, LR3/1 and LR3/2 (36). These viruses have been characterized while in the mammalian host by a series of complementation experiments and have been rescued and cloned in permissive chicken cells. The feasibility of using mammalian cells for cloning RSV replication mutants and the advantages of this approach as compared to the permissive avian cell system are discussed.

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MATERIALS AND METHODS

Viruses. Virus stocks were described previously by Boettiger et al. (4).

Avian cells and culture methods. Primary cultures of avian embryo cells were prepared and propagated by standard techniques (46). Avian cells were routinely maintained in Dulbecco modified Eagle minimal essential medium (E) supplemented with 10% tryptose phosphate broth (T; Difco Labs, Detroit, Mich.), 1% fetal calf serum (F; Irvine Scientific, Irvine, Calif.), and 1% chicken serum (Ch; Grand Island Biological Company, Grand Island, N.Y.) (ETF₁Ch₁). Primary cultures were prepared from fertile eggs of the following types: chicken helper factor negative (chf⁻), group-specific negative (gs⁻), (C/E) CEC (SPAFAS Inc., Norwich, Connecticut); Reasheath C, chf⁻ gs⁻ (C/AE) CEC (Houghton Poultry Research Station, Houghton, Huntingdonshire, England); Kf8, chf⁺ gs⁻ (Harriet Robinson, Worcester Foundation for Experimental Biology, Shrewsbury, Mass.); line 6 chf⁺ gs⁺ (U.S. Department of Agriculture Regional Poultry Research Laboratory, East Lansing, Mich.); and Japanese quail (Truslow Farms, Chestertown, Md.). Primary cultures were prepared from individual embryos, and the phenotype of each embryo was confirmed by virus sensitivity testing, chf testing, and assaying for avian gs antigen expression by the COFAL test (see below).

Mammalian cells and culture methods. Rat and hamster cells were maintained in Eagle medium supplemented with 5% fetal calf serum (EF₅). LR3/1, LR3/2, and LR5/1 were obtained by infected Lewis rat embryo cultures with Schmidt-Ruppin D virus as described previously (36). B4 cells were obtained from Ian Macpherson, Imperial Cancer Research Fund Laboratories, London.

Virus assays. (i) **Focus assays for avian sarcoma viruses.** Focus assays of avian sarcoma viruses were routinely carried out on third- or fourth-passage chicken or quail embryo cells. The cells were trypsinized with 0.05% trypsin, suspended in E plus calf serum (EC₅), and plated at 3×10^5 to 5×10^5 cells per 35-mm tissue culture dish. At 3 to 5 h after cell seeding, the medium was changed to standard chicken cell medium (ETF₁Ch₁), and 0.1 ml of diluted virus was added directly to the medium. At 12 to 14 h later, the medium was removed and 2 ml of focus assay agar was added (25% double-strength E, 30.5% HAMS F12, 10% T, 6% C, 1% Ch, 0.75% Me₂SO, and 0.6% agar [Difco]). The

plates were incubated at 40°C for 5 to 7 days before counting foci.

(ii) **Virus interference assays.** RAV-infected cells were prepared by adding virus (usually 0.5 ml) to secondary chf⁻ gs⁻ CEC and passaging the cells twice before use. Interference assays were performed by comparing the focus-forming ability of recently cloned stocks of PRA, PRB, PRC, and SRD viruses on uninfected and RAV-infected CEC.

(iii) **Infectious center assays of sarcoma virus-infected avian cells.** To test for the production of infectious sarcoma virus, cultures of infected avian cells were killed by incubation with growth medium containing 10 µg of mitomycin C per ml, trypsinized, and plated at various densities on fresh monolayers of 2×10^5 indicator avian cells. The cultures were overlaid with focus agar 2 h after plating. Foci occur only if the infected cells are producing virus capable of infecting the indicator cells.

Assays for endogenous virus expression. (i) **chf assay.** To test for chf expression, CEC were infected with PRB virus (multiplicity of infection = 5), killed with mitomycin C at 12 to 18 h after virus exposure, and plated as infectious centers on SPAFAS C/E CEC and quail cells. The ratio of infectious center formation on quail cells to CEC is generally 10^{-5} for chf⁻ and 10^{-3} for chf⁺ CEC.

(ii) **gs assay (COFAL).** The cells to be tested (either avian or mammalian) were grown to ca. 70 to 90% confluence in 100-mm tissue culture dishes, washed three times with phosphate-buffered saline, scraped into 0.5 ml of phosphate-buffered saline, and frozen at -80°C. Before assay, the cells were thawed and disrupted by sonication. The cells were assayed for avian gs antigen expression by the COFAL test using anti-gs antiserum prepared in pigeons (31). The reagents for this assay were kindly provided by William S. Mason of the Institute for Cancer Research, Fox Chase, Pa.

Soft agar cloning of virus-transformed chicken cells. For cloning infected chicken cells in soft agar, cultures of 7×10^5 CEC were infected with dilutions of virus, trypsinized 12 h after infection, and suspended in agar. The agar suspension was prepared by adding 1.2 ml of cell suspension in EC₁₀ to 1.8 ml of cloning agar (25% double-strength E, 30.5% HAMS F12, 10% T, 10% C, 1% Ch, 0.6% agar [Difco]), and mixing well. The suspensions were layered over 7 ml of solidified cloning agar in 60-mm dishes. The plates were incubated at 37°C and fed with 2 ml of cloning agar every 5 days. Colonies were picked from plates infected with the highest dilution of virus that resulted in colonies. The colonies were resuspended in 2 ml of Eagle cloning medium (10% C, 2% Ch, 10% T, 1% Me₂SO, 77% E) in 35-mm tissue culture dishes. Cultures that grew to confluence in the 35-mm dishes were passaged to 60- and then to 100-mm dishes.

Virus rescue assay. The procedures for using 50% polyethylene glycol (PEG; molecular weight 6,000 to 7,500, J. T. Baker Chemical Co.) as a fusing agent for virus rescue studies have been described (31). These methods were developed from procedures described by Pontecorvo (26) and Davidson et al. (9, 10).

(i) **Infectious center rescue assay.** Mammalian cells (10^6) were plated with 2×10^6 avian cells in 60-

mm dishes, and the cultures were treated with PEG and killed by incubation with 10 μ g of mitomycin C per ml for 2 h. At the end of the 2-h incubation period, the mitomycin C-containing medium was removed, the cultures were fed with 5 ml of cloning medium, and the plates were incubated at 37°C for 24 h. The cultures were then trypsinized and plated at various dilutions on indicator cells. Three hours later the plates were overlaid with focus agar and incubated for 5 to 7 days before counting foci.

(ii) **Harvest of rescued virus from fusions.** To obtain harvests from fusions, the cells were plated and fused exactly as described above. Instead of trypsinizing the cultures, the fusion mixtures were fed Eagle cloning medium and the supernatants were collected from 36 to 60 h after fusion.

RESULTS

Host range of virus rescued from LR3/1 and LR3/2 cells. The two nonvirogenic cell lines, LR3/1 and LR3/2, were obtained by infecting Lewis rat embryo cells with the subgroup D strain of Schmidt-Ruppin virus (36). To test the transforming viruses rescued from these cell lines for subgroup D envelope determinants, mitomycin C-killed heterokaryons of mammalian cells fused with subgroup A helper virus (RAV-1)-infected CEC were plated as infectious centers on C/E and C/AE chicken cells (Table 1). Infectious center formation on C/AE indicator cells should occur only if the transforming virus envelope gene is functional.

The fusion of LR3/1 cells with RAV-1-infected CEC resulted in focus formation on both C/E and C/AE CEC. If LR3/1 cells were fused with uninfected CEC, foci did not occur on either indicator cell type. The results of the LR3/2 fusion studies were complicated by a background of rescue in the absence of helper virus complementation of approximately two foci per 10⁶ LR3/2 cells. This background was detected if

LR3/2 fusions with uninfected CEC were plated on either C/E or C/AE CEC. However, the fusion of LR3/2 cells with RAV-1-infected CEC increased the rescue frequency, as measured by focus formation on C/E and C/AE CEC by 45,500- and 225-fold, respectively.

Fusions of B4 cells, which are Bryan virus-transformed hamster cells, and LR5/1 cells, which are a virogenic SRD-transformed rat cell line, were performed in parallel to provide controls (Table 1). Since B4 cells are transformed by an envelope deletion mutant of RSV, heterokaryons should produce exclusively subgroup A-transforming virus when complemented by RAV-1 (13, 32, 36). As predicted, B4 fusions produced foci on C/E but not C/AE CEC. Since LR5/1 virus does not require helper virus complementation for rescue, the envelope gene of this virus must be functional. As a consequence, foci should occur on C/AE indicator cells when LR5/1 cells are fused with either uninfected or RAV-1-infected CEC. This result was obtained and provided a positive control for this assay.

The relative efficiencies of focus formation on C/AE as compared to C/E CEC by heterokaryons of LR3/1, LR3/2, and LR5/1 cells fused with RAV-1-infected CEC were 2, 0.5, and 7%, respectively. In contrast, infectious centers of SRD virus-infected CEC were 67% as efficient in producing foci on C/AE as compared to C/E indicator chicken cells. If every heterokaryon is capable of producing subgroup D virus, then why is the efficiency of plating on C/AE CEC indicator cells so low? This assay involves fusing nonpermissive mammalian cells with fully infected helper virus-producing chicken cells. If the helper virus were contributing the majority of the host range determinants to the rescued transforming virus, then we might expect this to be reflected as a reduced efficiency of infectious

TABLE 1. Infectious center assay of RAV-1 fusion mixtures on C/E and C/AE CEC^a

Cell line	Fused with:	FFU/10 ⁵ mammalian cells on: ^b		(C/AE)/(C/E) (%)
		C/E CEC	C/AE CEC	
B4	RAV-1-infected CEC	7,200	<0.2	<0.003
B4	Uninfected CEC	<0.2	<0.2	
LR3/1	RAV-1-infected CEC	10,000	210	2
LR3/1	Uninfected CEC	<0.2	<0.2	
LR3/2	RAV-1-infected CEC	8,700	45	0.5
LR3/2	Uninfected CEC	0.2 ^c	0.2 ^c	
LR5/1	RAV-1-infected CEC	42,000	3,100	7
LR5/1	Uninfected CEC	5,200	7,700	148
PRA-infected CEC ^d	Not fused	10,000	<0.2	<0.002
SRD-infected CEC ^d	Not fused	10,000	6,700	67

^a Experimental details described in the text.

^b The results reported are the average of five assays performed in parallel.

^c One focus resulted on one of the five assay plates.

^d The sarcoma virus-infected cells listed in column 1 were plated as infectious centers.

center formation on C/AE indicator cells. Direct evidence for this explanation was obtained by harvesting the virus from fusions of the various mammalian cells with RAV-1-infected CEC and examining the relative proportion of transforming virus exhibiting subgroups A and D host ranges (Table 2). Only 2.6, 0.15, and 0.8% of the virus harvested from LR3/1, LR3/2, and LR5/1 fusions, respectively, exhibited subgroup D host range as measured by focus formation on C/AE CEC. In addition, RAV-1 failed to interfere with the plating of this virus.

From these results, it is clear that the transforming viruses resident in both LR3/1 and LR3/2 cells are capable of contributing subgroup D envelope determinants to rescued virus. It is notable, however, that LR3/2 cells consistently produce less subgroup D virus than LR3/1 cells after fusion with RAV-1-infected CEC. The efficiency of infectious center formation on C/AE indicator cells is 5- to 10-fold lower with LR3/2 heterokaryons (Table 1), and the yield of subgroup D transforming virus in LR3/2 supernatant harvests is at least 20-fold lower than in LR3/1 harvests (Table 2).

Complementation studies with avian cells expressing endogenous virus genes. Virus-free chickens have been shown to contain one to two copies of endogenous virus sequences related to avian RNA tumor viruses per haploid genome (1, 24, 40). Various patterns of expression of these endogenous viruses have been observed. Certain inbred lines of chickens express the endogenous virus glycoprotein (chf) and antigens related to the internal virion *gag* proteins

(*gs*) and are commonly referred to as *chf*⁺ *gs*⁺ birds (15, 49). Other lines of inbred chickens express high levels of *chf* activity but are *gs* negative (*chf*⁺ *gs*⁻) (15, 29). There are also other birds referred to as *V*⁺ that shed infectious endogenous RAV-0 virus (8, 45). The envelope expressed in *chf*⁺ cells is functional. Infection of *chf*⁺ cells results in the release of virus of subgroup E host range in addition to the host range of the infecting virus (48, 49). The available biochemical evidence suggests that the *gag* proteins expressed in *gs*⁺ cells are not functional (11, 35).

To test the viruses in various nonvirogenic cell lines for complementation by endogenous virus functions, cultures of *chf*⁻ *gs*⁻, *chf*⁺ *gs*⁺, and *chf*⁺ *gs*⁻ CEC were fused with mammalian cells and plated as infectious centers on C/E chicken cells and quail cells. For these experiments, K18 (*chf*⁺ *gs*⁻), line 6 (*chf*⁺ *gs*⁺), and SPAFAS (*chf*⁻ *gs*⁻) CEC were employed. The phenotype of each embryo was confirmed by *chf* testing and *gs* testing by complement fixation (see Materials and Methods). The results of these complementation studies are summarized in Table 3.

The complementation of an envelope defect by endogenous *chf* should result in foci on quail cells but not on C/E chicken cells. This was observed when B4 cells were fused with either *chf*⁺ *gs*⁺ or *chf*⁺ *gs*⁻ CEC, a result consistent with the envelope defectiveness of the original Bryan virus used to establish B4 cells.

Fusion of LR3/1 cells with *chf*⁻ *gs*⁻, *chf*⁺ *gs*⁺, or *chf*⁺ *gs*⁻ CEC failed to result in virus rescue. Consequently, this cell line must harbor a virus defective in a replication gene(s) that was not functionally expressed in any of these chicken cells.

TABLE 2. Titer of transforming virus rescued from fusions of nonvirogenic mammalian cells with RAV-1-infected CEC^a

Cell line	Virus titer (FFU/ml) on: ^b			(C/AE)/(C/E) (%)
	C/E CEC	C/AE CEC	RAV-1-infected CEC	
B4	1.7×10^4	<5 ^c	<5 ^c	<0.02
LR3/1	2.7×10^4	4.2×10^2	7.0×10^2	2.6
LR3/2	1.3×10^4	1×10^1 ^d	2×10^1 ^d	0.15
LR5/1	4.0×10^4	3.3×10^2	NT ^e	0.8
LR5/1 × uninfect CEC	2.0×10^2	8.0×10^1	NT	40
PRA virus	2.3×10^6	<5 ^c	<5 ^c	<0.0002
SRD virus	4.5×10^5	1.5×10^5	7.0×10^5	33

^a Cultures were plated and treated with PEG as described for "infectious center rescue assays." At 24 h after PEG treatment, the cultures were fed 5 ml of cloning medium, and the virus was harvested 24 h later.

^b Virus titers are reported as the average of duplicate assays.

^c No foci resulted on either of the assay plates when 0.1 ml of virus inoculum was used.

^d These results were obtained from assay plates with less than 10 foci.

^e NT, Not tested.

TABLE 3. Infectious center assays of fusions between nonvirogenic mammalian cells and *chf*⁻ *gs*⁻, *chf*⁺ *gs*⁺, or *chf*⁺ *gs*⁻ CEC^a

Cell line	Expt	FFU/10 ⁵ mammalian cells when fused with:					
		<i>chf</i> ⁻ <i>gs</i> ⁻ plated on:		<i>chf</i> ⁺ <i>gs</i> ⁺ plated on:		<i>chf</i> ⁺ <i>gs</i> ⁻ plated on:	
		C/E CEC	Quail	C/E CEC	Quail	C/E CEC	Quail
B4	1	<0.5	<0.5	<0.5	700	<0.5	800
	2	<0.5	<0.5	<0.5	935	<0.5	355
	3	<0.5	<0.5	<0.5	1,200	<0.5	750
LR3/1	1	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
	2	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
	3	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
LR3/2	1	0.5 ^b	<0.5	30	300	10	400
	2	<0.5	<0.5	6	150	1	810
	3	<0.5	<0.5	<0.5	380	5	1,250

^a Experimental details are described in the text.

^b One focus occurred on one of two assay plates.

LR3/2 cells displayed a pattern of complementation distinct from either LR3/1 or B4 cells. Fusion of LR3/2 cells with $\text{chf}^- \text{gs}^-$ CEC resulted in an occasional focus on C/E indicator cells. Efficient rescue of transforming virus could be demonstrated if LR3/2 cells were fused with either line 6 ($\text{chf}^+ \text{gs}^+$) or K18 ($\text{chf}^+ \text{gs}^-$) CEC. These heterokaryons plated efficiently only on quail cells, indicating that the virus produced was primarily subgroup E pseudotypes. However, there was a slight enhancement of subgroup D virus rescue as demonstrated by an increase of infectious center formation on C/E CEC as compared to the LR3/2 $\text{chf}^- \text{gs}^-$ CEC heterokaryons. It is apparent from these results that the replication defect of LR3/2 virus is complemented by endogenous virus envelope (chf) expression. Therefore, LR3/2 virus must be defective in the envelope gene. However, the complementation studies with RAV-1, the background rescue of subgroup D virus in the absence of complementation, and the slight enhancement of subgroup D rescue in the chf complementation studies all indicate that LR3/2 virus is capable of producing some subgroup D envelope determinants.

Triple fusions. It would be particularly useful in characterizing replication-defective viruses to be able to test for complementation between various nonconditional mutants. The fusion of pairs of cell lines transformed by such viruses should provide a direct test for complementation. Infectious transforming virus should be recovered only if the viruses are defective in different complementation groups. However, to study complementation of defective viruses in nonpermissive cells, the two cell types must also fuse with a permissive avian cell to allow the virus to replicate.

By fusing pairs of mitomycin C-killed mammalian cells with $\text{chf}^- \text{gs}^-$ CEC, we have been able to demonstrate complementation between the viruses resident in nonvirogenic mammalian cells (Table 4). Complementation occurred after fusion of B4 and LR3/1 cells with $\text{chf}^- \text{gs}^-$ CEC. The rescue frequencies were 18 and 80 focus-forming units (FFU)/ 10^5 mammalian cells in experiments 1 and 2, respectively. LR3/1 and LR3/2 viruses also complemented one another efficiently, exhibiting rescue frequencies of 40 and 55 FFU/ 10^5 mammalian cells in the two experiments. This was at least a 40-fold enhancement in experiment 1 and a 55-fold enhancement in experiment 2 over the background rescue observed with LR3/2 cells in the absence of complementation. In contrast, B4 and LR3/2 viruses failed to efficiently complement one another in this assay. The rescue frequencies in

TABLE 4. Triple fusions between pairs of mammalian cells and uninfected $\text{chf}^- \text{gs}^-$ C/E CEC^a

Crosses	FFU/ 10^5 mammalian cells ^b	
	Expt 1	Expt 2
B4 $\times$ CEC	<0.33 (3)	<0.5 (2)
LR3/1 $\times$ CEC	<0.33 (3)	<0.5 (2)
LR3/2 $\times$ CEC	<0.33 (3)	1.0 (2)
B4 $\times$ LR3/1 $\times$ CEC	18 (3)	80 (5)
B4 $\times$ LR3/2 $\times$ CEC	0.66 (3)	1.0 (5)
LR3/1 $\times$ LR3/2 $\times$ CEC	40 (3)	55 (5)

^a The assay was performed by plating 10^5 mitomycin C-killed mammalian cells of each cell type with 3×10^5 CEC and treating the mixture with PEG in the usual manner. Cultures were overlaid with focus agar 24 h after PEG treatment, and foci were counted 7 to 9 days later.

^b The numbers in parentheses indicate the number of assays that were averaged to obtain the reported results. No foci occurred in the absence of PEG treatment.

the two experiments were 0.66 and 1.0 FFU/ 10^5 mammalian cells, values similar to those obtained in the LR3/2 control fusions.

We conclude that LR3/1 and B4 viruses are defective in functions mapping in different complementation groups. Similar conclusions may be drawn from the LR3/1 and LR3/2 complementation studies. In contrast, LR3/2 and B4 viruses must be defective in a function mapping in the same complementation group because they fail to efficiently complement one another in this assay.

Complementation studies with a temperature-sensitive replication mutant. The temperature-sensitive mutant LA334 is defective for both the maintenance of cellular transformation and virus production at the nonpermissive temperature (3, 17, 18). The production of infectious virus is reduced approximately 500- to 1,000-fold at the nonpermissive temperature and is apparently the result of a lesion affecting processing of Pr76, the precursor to the internal virion structural proteins (17). The failure to maintain cellular transformation is due to a separate temperature-sensitive mutation mapping within the *src* gene (3, 18).

We have taken advantage of the fact that this virus is a double mutant in designing complementation studies. The various nonvirogenic cell lines were fused with LA334-infected CEC, the cultures were incubated at 35 and 41°C, and the virus harvested from each of these cultures was titered at 41°C. Virus capable of focus formation at 41°C reflects the level of rescue of transforming virus from the mammalian cells since the

LA334 *src* gene is temperature sensitive, preventing LA334 alone from producing foci at 41°C. Efficient complementation of all of these viruses was expected when the nonvirogenic cell lines were fused with LA334-infected CEC at the permissive temperature. In contrast, efficient rescue at the nonpermissive temperatures should have occurred only if the virus in the mammalian cells was defective in a function that was not temperature sensitive in LA334 virus. A nonvirogenic cell line harboring a virus defective in a replication function(s) similar to LA334 should exhibit a ratio of rescue at the two temperatures (41°C:35°C) similar to the leakiness of the LA334 replication lesion. A cell line transformed by a virus with a replication defect distinct from the LA334 temperature-sensitive lesion should exhibit a 41°C:35°C ratio greater than that observed for the LA334 control. The results of these complementation studies are summarized in Table 5.

Fusions were performed either at 41°C (experiment 1) or 35°C (experiment 2) before transfer to the two temperatures to eliminate any artifacts that might be generated due to differential sensitivity of the various mammalian cells to elevated temperatures (see footnotes to Table 5 for experimental details). The results of both experiments were qualitatively similar. In experiment 1, the ratio of virus produced by control LA334-infected cultures at the two temperatures was 0.001. Both B4 and LR3/2 viruses were complemented at the nonpermissive temperature, resulting in 41°C:35°C rescue ratios of 2.1 and 0.128, respectively. In contrast, LR3/1 virus

was complemented only at the permissive temperature. The ratio of virus rescued at the two temperatures was 0.002, essentially the same ratio of virus production as the control LA334-infected cultures. In experiment 2, the ratio of virus produced by control LA334-infected cultures at the two temperatures was 0.01, and efficient complementation of B4 (41°C:35°C ratio, 0.96) and LR3/2 (41:35 ratio, 1.35) viruses but not LR3/1 virus (41:35 ratio, 0.06) occurred at the nonpermissive temperature.

It is difficult to draw any conclusions from the quantitative differences in the data obtained in experiments 1 and 2. In experiment 1, LR3/2 virus is complemented by LA334 virus at the nonpermissive temperature, but not as efficiently as B4 virus. However, in experiment 2, LR3/2 and B4 viruses are complemented equally as efficiently at the nonpermissive temperature by LA334 virus. In experiment 1, the fused cultures are incubated at 41°C before transfer to the permissive and nonpermissive temperatures. However, in experiment 2, the cultures are incubated at 35°C before transfer to the two temperatures. By comparison, the conditions of experiment 1 are more restrictive than those of experiment 2. It is possible that experiment 1 reflects quantitative differences in the ability of replication function(s) that complement LA334 virus in heterokaryons. However, the complexity of these assays prevent any meaningful conclusions in the absence of additional supporting evidence.

Complement fixation assay (COFAL) for avian *gs* antigen expression. The results of

TABLE 5. Rescue of virus from nonvirogenic mammalian cells by fusion with LA334-infected CEC^a

Cell line	Virus rescue (FFU/ml)				41°C rescue/35°C rescue ratio		Virus production (FFU/ml)				41°C production/35°C production ratio	
	Expt 1		Expt 2		Expt 1	Expt 2	Expt 1		Expt 2		Expt 1	Expt 2
	35°C	41°C	35°C	41°C			35°C	41°C	35°C	41°C		
Mammalian ^a												
B4	1.5 × 10 ⁴	3.1 × 10 ⁴	2.5 × 10 ⁴	2.4 × 10 ³	2.1	0.96						
LR3/1	3.2 × 10 ⁴	5.0 × 10 ⁴	5.5 × 10 ³	3.3 × 10 ²	0.002	0.06						
LR3/2	3.5 × 10 ³	4.3 × 10 ²	3.7 × 10 ³	5.0 × 10 ³	0.128	1.35						
Control ^b (LA334-infected CEC)							1.5 × 10 ⁶	1.4 × 10 ⁴	5.0 × 10 ⁶	4.9 × 10 ⁴	0.001	0.01

^a 1 × 10⁶ mammalian cells were plated with 2 × 10⁶ LA334-infected CEC in 60-mm tissue culture dishes. The mixed cultures were killed with mitomycin C and treated with PEG. The fused cultures were incubated at 41°C (experiment 1) or 35°C (experiment 2) for 24 h and then trypsinized and plated on two fresh 60-mm dishes of uninfected C/E CEC. Immediately after transfer, one plate was incubated at 35°C and one plate was incubated at 41°C. Virus was harvested from these cultures in the interval from 24 to 48 h after transfer. These harvests were titrated at 41°C to determine the rescue of non-temperature-sensitive virus from the mammalian cells. These values are expressed as "virus rescue at 35°C" and "virus rescue at 41°C" in the table.

^b Control cultures of LA334-infected CEC were treated in parallel as described in footnote ^a above. Harvests obtained at the two temperatures were titrated at 35°C to obtain an estimate of the restriction of the LA334 replication lesion at the nonpermissive temperature.

fusion studies with LA334-infected CEC suggested that LR3/1 cells may be transformed by a virus defective in the *gag* gene. To determine if there was a complete absence of *gag* proteins in these cells, extracts were prepared and assayed for the presence of avian *gs* antigens by the COFAL test (Table 6). As controls, avian cells expressing various patterns of endogenous virus proteins, uninfected rat embryo fibroblasts, and the two cell lines, B4 and LR3/2, that complemented the LA334 replication lesion were also tested. As expected, SPAFAS $\text{chf}^- \text{gs}^-$ CEC and uninfected rat embryo cells (LR3) did not express avian *gs* antigens. In addition, K18 $\text{chf}^+ \text{gs}^-$ cells did not express avian *gs* antigens detectable by the COFAL test. In contrast, $\text{chf}^+ \text{gs}^+$ line 6 CEC and the three RSV-transformed mammalian cell lines all expressed detectable avian *gs* antigens. Thus, although the transforming virus resident in LR3/1 cells may be defective in the *gag* gene, the lesion is not due to a complete absence of *gag* proteins.

Cloning of defective viruses rescued from nonvirogenic mammalian cells in permissive avian cells. The conclusion that LR3/1 and LR3/2 cells were transformed by replication-defective viruses was based upon the observation that efficient rescue occurred only if the appropriate helper function was supplied. From the results of the various fusion rescue assays, the nature of the defect of each of these presumptive replication-defective viruses may be hypothesized. However, to confirm that these cell lines harbor defective viruses and that the results of the rescue assays reflect this defectiveness, it was necessary to clone and characterize these viruses in permissive avian cells.

In attempting to establish such clones, several obstacles were encountered. Avian RNA tumor viruses recombine at a high frequency (2, 44). To minimize the possibility of cloning recombinants, the harvests from mitomycin C-killed fusion mixtures (Table 2) were used as a source of

transforming virus. Assuming that recombination occurs in later rounds of infection and not in the initial multiply infected cells (50), we reasoned that the virus harvested from mitomycin C-killed heterokaryons should contain the original parental virus genomes but not recombinants. Preliminary attempts to clone either LR3/1 or LR3/2 viruses in SPAFAS CEC (C/E), which are sensitive to viruses of the subgroups of both the transforming virus rescued from the mammalian cell and the helper virus, yielded only multiply infected clones. Six clones of SPAFAS CEC transformed by LR3/2 and five clones of SPAFAS CEC transformed by LR3/1 virus were established using the harvests described in Table 2. All of these clones produced high titers of transforming virus ranging from 3.8×10^5 to 1.0×10^6 FFU/ml of culture supernatant. This virus exhibited both subgroup A (helper virus) and subgroup D (original transforming virus) host ranges (data not shown). Since all of the clones were multiply infected by the helper virus and the virus rescued from the mammalian cells, the titer of helper virus must be in vast excess over that of the transforming virus.

Both of these viruses were finally cloned free of helper virus by using C/AE CEC as hosts for establishing transformed clones. Such chicken cells are resistant to infection by viruses of the helper virus subgroup but sensitive to the subgroup of the original transforming viruses. With the exception of subgroup D pseudotypes of the helper virus, the nontransforming RAV-1 virus should be excluded from C/AE cells.

(i) Clones of LR3/2 virus-transformed Reasheath C line CEC. Colonies of transformed Reasheath C line CEC were removed from agar with Pasteur pipettes and grown into cultures. Three clones of LR3/2 virus-infected C/AE cells were established, and the supernatants were tested for the presence of infectious transforming virus by the focus assay on C/E CEC, RAV-1-infected CEC, C/AE CEC, and quail cells (Table 7). All three of these clones produced low levels of infectious transforming virus. The titer of virus, as measured by focus formation on C/E CEC, ranged from 1.8×10^2 to 1.7×10^3 FFU/ml of culture supernatant. Control cultures of C/AE CEC infected by SRD virus produced 10^6 FFU/ml of culture supernatant (data not shown). Thus the yield of virus was reduced approximately 1,000-fold below that of wild-type virus. In addition, the virus produced by these clones plated efficiently on C/E, C/AE, and RAV-1-infected CEC but did not plate efficiently on quail cells (Table 7). This was similar to the plating pattern of control SRD virus on the four cell types.

TABLE 6. COFAL test for avian *gs* antigen expression by transformed mammalian cells

Cells tested	gs antigen at:			
	0 ^a	1/ 2 ^a	1/ 4 ^a	1/ 8 ^a
SPAFAS ($\text{chf}^- \text{gs}^-$)	-	-	-	-
K18 ($\text{chf}^+ \text{gs}^-$)	-	-	-	-
Line 6 ($\text{chf}^+ \text{gs}^+$)	+	+	+	-
LR3 (uninfected rat embryo fibroblasts)	-	-	-	-
LR3/1	+	+	+	-
LR3/2	+	+	+	+
B4	+	+	+	+

^a Dilution of extract.

TABLE 7. *Titer of virus produced by clones of Reasheath C CEC transformed by LR3/2 virus*

Clone designation	Virus titer on:			
	C/E CEC	C/AE (RC) CEC	RAV-1-infected CEC	Quail cells
3/2 RC4	1.8×10^2	3.8×10^2	9.0×10^1	<5
3/2 RC5	4.5×10^2	1.1×10^3	3.1×10^2	<5
3/2 RC6	1.7×10^3	2.5×10^3	1.2×10^3	<5
SRD virus ^a	5.5×10^3	5.0×10^3	1.5×10^3	6.5×10^1
PRA virus ^a	8.0×10^6	10	2.5×10^2	1.1×10^6

^a Control SRD and PRA viruses were grown on SPFAF5 chf⁻gs⁻ CEC.

The absence of quail plating virus in harvests from these clones had several implications. If the clones were doubly infected by both the subgroup D transforming virus rescued from the mammalian cells and the subgroup A helper virus, then subgroup A pseudotypes of transforming virus capable of infecting quail cells (which are resistant to subgroup D but sensitive to subgroup A viruses) should have been produced. In addition, any complementation of the envelope defectiveness of LR3/2 virus by the inducible subgroup E endogenous virus of Reasheath C line chicken cells (28) should have been detected as quail plating virus. The failure to detect quail plating virus in these harvests eliminated either double infection or endogenous virus complementation as an explanation for the production of low levels of infectious virus by these clones. Instead, it suggested that this property was a characteristic of the LR3/2 virus mutation.

(ii) **Clones of LR3/1-virus transformed Reasheath C line CEC.** By using the harvest described in Table 2 to infect Reasheath C line C/AE CEC, two clones of LR3/1-virus transformed cells were established. In contrast to LR3/2 clones, cultures of LR3/1 virus-infected Reasheath C line chicken cells did not release any infectious virus (Table 8). The supernatants from the two cultures of LR3/1 virus-infected C/AE cells, designated 3/1RC1 and 3/1RC2, were tested for focus formation on C/E CEC, C/AE CEC, RAV-1-infected CEC, and quail cells, all of which failed to detect any transforming virus. In addition, interference assays did not detect any nontransforming viruses in the supernatants from 3/1RC1 or 3/1RC2 cultures (data not shown). However, if these clones were superinfected with helper virus, infectious transforming virus was recovered (Table 8).

These results are in agreement with the conclusions reached in the fusion-rescue studies. LR3/1 virus is a replication-defective virus, and the lesion is an absolute rather than a leaky mutation.

TABLE 8. *Assay for virus production by clones of Reasheath C CEC transformed by LR3/1 virus*

Clone designation	Assayed for virus production on: ^a			
	C/E CEC	C/AE CEC	RAV-1-infected C/E CEC	Quail
3/1 RC1	—	—	—	—
3/1 RC1 + NTB77 ^b	+	+	+	NT ^c
3/1 RC2	—	—	—	—
3/1 RC2 + NTB77 ^b	+	+	+	NT

^a The various cultures were tested for the production of infectious transforming virus by adding 1.0 ml of culture supernatant to each of two plates seeded with 3×10^5 of the indicated cell type. One plate was overlaid with focus agar 24 h after inoculation and examined for foci of transformed cells 6 to 8 days later. The other plate was passaged three times and examined for transformed cells at each passage. The positive cultures (superinfections) exhibited numerous foci in the agar-overlaid cultures. Negative cultures did not exhibit foci in the agar-overlaid cultures or after repeated transfer of indicator cultures.

^b Clones were superinfected with 0.1 ml of NTB77 helper virus, and the supernatants were harvested 48 h later.

^c NT, Not tested.

DISCUSSION

The transformation of mammalian cells by nondefective strains of RSV yields cell lines that may be divided into two classes based upon their ability to produce infectious transforming virus in virus rescue assays. Virogenic cell lines release infectious transforming virus when fused with permissive avian cells (6, 22, 37) and induce sarcomas if injected into susceptible birds (34). In contrast, nonvirogenic cell lines fail to release infectious virus detectable by either of these assays (34, 38). In a previous report (36) we demonstrated that transforming virus could be recovered from nonvirogenic cells if the permissive chicken cells were infected with helper virus (RAV) before cell fusion with the mammalian cells. To explain this observation, we hypothesized that these cell lines were transformed by replication-defective viruses. In this paper, we present evidence that the transforming viruses in two nonvirogenic cell lines, LR3/1 and LR3/2, are indeed defective. The specific replication defects of these viruses have been analyzed by a series of complementation-rescue assays, and these viruses have been cloned in permissive avian cells with a retention of their genetic defects.

Table 9 summarizes our interpretation of the results of the various fusion-rescue complementation assays. The defective transforming virus in B4 cells, a line of Bryan virus-transformed BHK-21 cells, was characterized in parallel to

TABLE 9. *Summary of complementation rescue assays*

Cell line	<i>gag</i>	<i>pol</i>	<i>env</i>	<i>src</i>
B4	+	+	—	+
LR3/1	(—) ^a	? ^b	+	+
LR3/2	+	+	—	+

^a Biochemical experiments (Steimer and Boettiger, manuscript in preparation) demonstrate that LR3/1 virus is defective in the synthesis of the *gag* gene products. From the data presented in this communication, however, we cannot definitively conclude that LR3/1 virus is defective in the synthesis of functional *gag* proteins.

^b The results presented in this paper do not allow us to make any conclusions about the functional expression of *pol* by LR3/1 virus.

LR3/1 and LR3/2 analysis, to provide controls for the various assays. Obviously, all three cell lines were transformed by viruses capable of expressing functional *src* gene product(s) because (i) they were all selected as viral transformants and (ii) the transforming virus genome was recovered in complementation rescue assays. In fusions with helper virus-infected CEC, both LR3/1 and LR3/2 cells released infectious transforming virus, exhibiting the host range of the original transforming virus (subgroup D) as well as that of the helper virus (subgroup A) (Tables 1 and 2). In contrast, the virus rescued from B4 cells, which were transformed by an envelope deletion mutant of RSV, exhibited exclusively the helper virus host range. Thus, both LR3/1 and LR3/2 cells, unlike B4 cells, contribute functional envelope determinants to rescued virus.

Virus has been rescued from B4 and LR3/2 cells by fusion with chicken cells expressing endogenous virus genes (Table 3). B4 cells fused with either *chf*⁺ *gs*⁺ or *chf*⁺ *gs*⁻ CEC released infectious transforming virus that plated on quail cells but not on C/E CEC. Thus, B4 virus exhibited only the endogenous virus host range when complemented by *chf* expression. LR3/2 virus is also complemented in *chf*⁺ *gs*⁺ and *chf*⁺ *gs*⁻ fusions and must, therefore, also be an envelope mutant. This appears to contradict the results of the helper virus complementation studies, demonstrating the rescue of subgroup D virus from LR3/2 cells (Tables 1 and 2). This problem is resolved by a closer examination of the LR3/2 complementation studies described in Tables 1 to 3. LR3/2 fusions with helper virus-infected CEC consistently produced a lower proportion of subgroup D-transforming virus than either LR5/1 (a virogenic cell line) or LR3/1 fusions (Tables 1 and 2). Fusions with *chf*⁻ *gs*⁻ CEC resulted in an occasional focus on C/E

CEC in the absence of complementation (Table 3). In addition, although most of the transforming virus in *chf* complementations of LR3/2 virus exhibited the endogenous virus host range, a proportion also plated on C/E CEC and is therefore, most likely subgroup D virus. All of these observations indicate that, although LR3/2 virus is defective in the envelope, it is most likely a leaky mutant rather than an absolute mutant like Bryan virus.

From the complementation studies with chicken cells expressing endogenous virus genes, we may conclude that both B4 and LR3/2 viruses are not defective in their *gag* or *pol* genes. Since there is no functional polymerase expressed in *chf*⁺ *gs*⁺ CEC (14), complementation by these chicken cells could only occur if the polymerase gene of a defective virus was functional. In addition, the *gag* genes of these viruses must be functional because virus was rescued in fusions with *chf*⁺ *gs*⁻ CEC which do not express *gs* proteins detectable by either the COFAL test (Table 6) or radioimmunological assay (K. Steimer, unpublished results), and virus was rescued in fusions with *chf*⁺ *gs*⁺ CEC, in which, according to biochemical studies (11, 35), the *gs* proteins are not functional. The failure of LR3/1 virus to be complemented in fusions with either *chf*⁺ *gs*⁺ or *chf*⁺ *gs*⁻ CEC demonstrates that this virus is defective in a virus replication gene that is not functionally expressed (i.e., *gag*, or *pol*, or both) in either of these cell types.

The results of the triple fusion experiments (Table 4) are consistent with the conclusions discussed above. B4 and LR3/2 viruses fail to complement one another, demonstrating that they are defective in the same function (i.e., *env*). LR3/1 virus complements both LR3/2 and B4 viruses, suggesting that it expresses functional envelope proteins and must therefore be defective in the *gag* or *pol* gene, or both.

It is not possible to conclude from the complementation studies with LA334 virus (Table 5) that LR3/1 virus is defective only in the *gag* gene. The processing of Pr180, which is a translation product of both the *gag* and *pol* genes and is the presumptive polymerase precursor (25), may also be temperature sensitive during LA334 replication. The particles produced by LA334-infected CEC at the nonpermissive temperature are reduced in their polymerase content, implying that the LA334 lesion also affects the polymerase precursor (30). However, this evidence is purely circumstantial and, until complementation studies of LA334 virus and known polymerase mutants have been completed, we may only conclude from the data presented in Table 5 that LR3/1 virus is defective in the *gag* or *pol* gene, or both.

A preliminary characterization of clones of avian cells transformed by these viruses allows us to conclude that these viruses are truly defective, and the results of the fusion-rescue assays reflect this defectiveness. LR3/1 virus, as predicted in the fusion-rescue studies, is an absolute mutant. Superinfection of LR3/1-infected CEC converts them from nonproducers to producers (Table 8). From the fusion-rescue studies we predicted that LR3/2 virus would be a leaky envelope mutant. The production of low levels of infectious virus by LR3/2 avian clones agrees with this prediction.

Detailed biological and biochemical studies of LR3/1 and LR3/2 virus-infected avian cells have been completed and will be published separately (K. S. Steimer and D. Boettiger, manuscript in preparation). The results of these studies are in complete agreement with the data reported here. Biochemical studies demonstrate that LR3/2 virus is an envelope mutant and LR3/1 virus is defective in the *gag* gene. Experiments are in progress to determine if LR3/1 virus is also defective in the polymerase gene. In accordance with the proposed system for numbering avian leukosis and sarcoma viruses (47), LR3/2 virus will be assigned the number rdPN3/2SR-D (abbreviated PN3/2) and LR3/1 virus will be assigned the number rdPN3/1SR-D (abbreviated PN3/1).

In addition to characterizing these two defective viruses, this study demonstrates the feasibility of using nonpermissive mammalian cells as hosts for cloning replication-defective mutants of RSV. We have also systematically employed cell fusion for studying RNA tumor virus replication mutants. The use of cell fusion is, in fact, the only possible means for studying complementation between nonconditional mutants. By introducing the virus components from two mutants into a common cell cytoplasm, complementation may be tested directly. We have used this approach for studying complementation between defective viruses in nonpermissive mammalian cells, which requires that the two transformed cell partners also fuse with a permissive avian cell (Table 4). We have also been able to demonstrate complementation by fusing pairs of permissive cells transformed by nonconditional replication-defective viruses (Steimer and Boettiger, manuscript in preparation). This approach might also be used to analyze other retrovirus mutants such as the recently isolated Moloney murine leukemia virus replication defectives (27, 33).

We have been referring to the various rescue assays as demonstrating complementation. There are several reasons for assuming that we

are measuring complementation rather than recombination. In these assays, all partners in the fusions are killed with mitomycin C before cell fusion. Mitomycin C does not affect virus production if the infection is established before adding the drug, but prevents infection if the cells are treated before or very soon after virus exposure (41). It presumably renders cells refractory to infection because host DNA synthesis is essential for the establishment of RSV infection (5, 39). If we assume that recombination does not occur in the first round of mixed infection but rather in later rounds (50), it is unlikely that recombination is occurring in the mitomycin C-killed heterokaryons. The fact that we are able to clone these defective viruses from mitomycin C-killed heterokaryon virus harvests (Tables 7 and 8) and that all of the clones analyzed to date have been either defective virus transformed or doubly infected by helper virus and the defective virus argues against recombination as an explanation for virus rescue in the fusion assays.

Since the cloning procedure involves secondary infection and avian RNA tumor viruses recombine at a high frequency (2, 43, 44), we would expect that a proportion of the clones obtained would be transformed by recombinants if these viruses were capable of recombining with helper RAV. Of the limited number of singly infected clones examined to date (three clones of LR3/2 virus and two clones of LR3/1 virus-infected CEC), none have been recombinants. We are currently testing additional clones in an attempt to isolate both defective and nondefective recombinants.

The generation of nonvirogenic cells at such high frequencies, usually 20 to 50% of the isolates (16, 36), has not been explained. Since the transformation of mammalian cells by RSV only requires the transforming gene of the virus to be expressed and does not depend upon the ability of a virus to replicate, the frequency of generation of nonvirogenic cells may reflect the proportion of replication-defective viruses in a particular virus stock. Previous reports that nonproducer avian cells are generated after single infection by nondefective strains of RSV would tend to support this conclusion (19, 23). It is also possible that there is something peculiar about the establishment of infection in nonpermissive mammalian cells that generates a high proportion of defective transforming proviruses. Both explanations are purely speculative and will remain so until a direct comparison of the frequency of generation of nonproducer avian cells and nonvirogenic mammalian cells has been made.

The use of mammalian cells for isolating and characterizing replication mutants of RSV may seem to be a rather complicated and indirect approach. Quail cells offer several of the advantages that we have attributed to mammalian cells. They are also easily established as long-term cultures after RSV transformation, and restriction enzyme analysis of integrated viral genomes is simplified due to an absence of endogenous virus sequences (with the exception of *src*) related to the chicken viruses. Since quail cells are permissive, biochemical studies may be carried out directly on the infected cells without the necessity of transferring the defective viruses as is required with defective viruses in mammalian cells. However, there are several important advantages that are unique to mammalian cells. Mammalian cells are nonpermissive for virus replication, eliminating problems with secondary infection and increasing the chances of cloning defectives. Certain classes of mutants such as leaky mutants like LR3/2 virus might be overlooked in quail cells. It has also been demonstrated that mammalian cells, specifically NIH3T3 cells, may be transformed directly by proviral DNA whereas avian cells require additional rounds of virus replication before transformed cells are observed (7). This finding implies that it should be possible to transform mammalian cells with fragments of proviral DNA. Using restriction enzymes, viral genomes lacking specific segments could be constructed in vitro and used to transform NIH3T3 cells. The results presented here provide a methodology for the genetic analysis of defective viruses in nonpermissive mammalian cells and the procedure for rescuing and cloning these viruses in permissive avian cells for biochemical characterization.

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