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Complementation Rescue of Rous Sarcoma Virus from Transformed Mammalian Cells by Polyethylene Glycol-Mediated Cell Fusion

KATHELYN S. STEIMER AND DAVID BOETTIGER*

Department of Microbiology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

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Polyethylene glycol (PEG) is effective as a fusing agent for the rescue of virus from Rous sarcoma virus-transformed mammalian cells. The procedure for PEG-mediated rescue of virus from virogenic cell lines is described, and the technique is compared with that of Sendai virus-mediated rescue. Virus may be rescued quantitatively from virogenic cell lines by plating mitomycin C-killed transformed mammalian cells with chicken embryo cells, treating the monolayers with 50% PEG and overlaying the monolayers with focus agar. The number of foci that appeared reflected the number of heterokaryons in the fusion mixtures that released infectious virus. PEG gave reproducible results in virus rescue experiments with an efficiency equal to the best Sendai virus preparations. In addition to the description of the technique for PEG-mediated virus rescue from virogenic cell lines, a method for virus rescue from nonvirogenic lines is presented. Preinfection of the chicken embryo cells with helper avian leukosis virus (Rous-associated virus) prior to fusion with mammalian cells transformed by defective viruses complements the virus defect. We examined four nonvirogenic cell lines, and all released infectious virus in the complementation rescue assay.

The induction of tumors in animals and the morphological transformation of cells in tissue culture by Rous sarcoma virus (RSV) has been described for a variety of mammalian species (21, 22). Although the majority of RSV-transformed mammalian cells do not release infectious virus (11, 21), several lines of evidence indicate that the virus persists in these cells. Some of the markers for RSV expression that have been examined include: (1) avian virus group-specific antigens (30); (ii) RSV tumor-specific surface antigens (15); (iii) RSV-specific DNA (25, 26); (iv) RSV-specific RNA (9); and (v) reverse transcriptase-containing particles (6). However, the most convincing evidence for the persistence of the RSV genome is the ability to rescue infectious virus from certain RSV-transformed mammalian cell lines (5, 16, 21, 23, 24).

Even though it has been possible to demonstrate the presence of RSV-specific markers in transformed mammalian cells, it has not been possible to rescue virus from all of these lines. Thus, some lines have been referred to as virogenic and others as nonvirogenic. Virogenic cells generate tumors upon injection into chickens, and virus that has a subgroup similar to the original virus used to transform the cells is recovered (21). Virus rescue may also be demonstrated in vitro by fusion of the transformed mammalian cells with chicken embryo cells (5, 16, 21, 23, 29). Nonvirogenic lines fail to release virus in either of these assays (21, 24).

The failure to rescue virus from some RSV-transformed mammalian cell lines, in spite of their expression of some viral-specific markers, has not been explained. One attractive hypothesis is that the RSV genome in these nonvirogenic lines contains a deletion of viral genes. Thus, any virus rescued would be defective. It should be possible to complement this defect by using a nontransforming avian leukosis virus to supply the missing replication gene(s). This report describes an efficient complementation rescue assay, and we demonstrate that all four nonvirogenic RSV-transformed mammalian cell lines that we have examined can be complemented by avian leukosis virus.

The study of virus rescue from avian sarcoma virus (ASV)-transformed mammalian cells has been limited by the difficulties encountered in hybridizing cells. Sendai virus is effective in inducing cell fusion, but it is difficult to prepare and lacks reproducibility from batch to batch. In addition, Sendai virus-mediated rescue assays are difficult and tedious (2). These problems make it virtually impossible to perform

rescue assays routinely and on a large scale.

Recently, a series of papers on the effectiveness of polyethylene glycol (PEG) in inducing somatic cell hybridization have been published (7, 8, 20). The possibility of using PEG as the fusing agent in virus rescue studies was considered. PEG proved to be quite effective for studying virus rescue. This communication describes the conditions for PEG-mediated virus rescue from RSV-transformed cells, presents data comparing the technique with that of Sendai virus, and describes a method for the rescue of virus from mammalian cells transformed by defective viruses.

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 (31). The medium routinely used for maintenance of avian cells was a Dulbecco modification of Eagle minimal essential medium (E) supplemented with 10% tryptose phosphate broth (T) (Difco Laboratories, Detroit, Mich.), 1% fetal calf serum (F) (Irvine Scientific, Irvine, Calif.), and 1% chicken serum (Grand Island Biological Co., Grand Island, N.Y.). Reaseheath C line (Chf-negative C/AE) fertile eggs were obtained from Houghton Poultry Research Station, Houghton, Huntingtonshire, England. Fertile Chf-negative C/E eggs were obtained from SPAFAS Inc., Norwich, Conn. Quail eggs were obtained from Truslow Farms, Chesterton, Md.

Focus assays of ASV were routinely carried out on third- or fourth-passage chicken or quail embryo cells. The cells were trypsinized with 0.05% trypsin and neutralized with medium plus 5% calf serum. The cells were diluted to 1.25×10^5 to 2×10^5/ml in ET, and 2 ml of cell suspension was seeded in 35-mm tissue culture dishes. Three to five hours after seeding, the medium was changed to standard chicken cell medium, and 0.1 ml of diluted virus was added directly to the medium. After 12 to 14, h, the medium was removed, and 2 ml of focus assay overlay agar was added (25% double-strength E–30.5% F12–10% tryptose phosphate–6% calf serum–1% chicken serum–0.75% Me_2SO–0.6% agar [Difco]). The plates were incubated at 40°C for 5 to 7 days before foci were counted.

Avian leukosis virus (Rous-associated virus [RAV])-infected cells were prepared by adding 0.5 ml of virus to secondary SPAFAS C/E chicken embryo cells (CEC) and passaging the cells twice before use. Viral interference assays (32) were set up at the time that RAV-infected cells were used in rescue experiments. Interference assays were performed by comparing the focus-forming ability of recently cloned stocks of PRA, PRB, PRC, and SRD on C/E cells with the RAV-infected C/E cells in the standard focus assay.

Cells and culture methods for mammalian cells. Rat and hamster cells were maintained in Eagle medium supplemented with 5% fetal calf serum (EF_5). KC cells were maintained in Eagle medium with 10% fetal calf serum (EF_{10}).

NRK/B77 T1 cells are a line of NRK cells derived by transforming NRK cells with B77 sarcoma virus in vitro as described previously (3). B4 cells were obtained from I. A. MacPherson Imperial Cancer Research Fund Laboratories, London. KC cells were a gift of Carlo M. Croce, Wistar Institute, Philadelphia, Pa. These cells are a human glioma cell line transformed in vitro by the Engelbreath-Holm strain of ASV (17). SR 3 and subclones of morphological revertant (SR 3/5, SR 3/11 R, SR 3/4a) and transformed (SR 3/1a) variants, which were derived from Schmidt-Ruppin BHK-21/13 hamster cells (18), were described previously (2, 9). Rat embryo primary cultures from pregnant female inbred Lewis rats (Microbiological Associates) were prepared by trypsinizing 17- to 20-day-old embryos. Cultures were prepared from six individual embryos of the same litter and will be referred to as LR1-LR6. Cultures were passaged every 5 days in EF_5 or frozen as primaries in E with 10% Me_2SO and 15% calf serum.

Isolation of ASV-transformed rat embryo cells. Rat embryo cells at the second or third passage were seeded at 5×10^4 cells per 35-mm tissue culture dish in EF_5 with 2 μg of polybrene per ml. After 4 h, the medium was removed, and 0.1 ml (multiplicity of infection, 4) of Schmidt-Ruppin D ASV was absorbed for 30 min at 38°C. After absorption, the plates were fed with 2 ml of EF_5. After 24 h, the cultures were overlaid with 2 ml of 0.6% agar in E supplemented with 10% tryptose phosphate and 10% calf serum. Fourteen days after virus exposure, foci of transformed cells began to be evident, with an average of one to three foci per 35-mm culture dish. Foci were picked from separate dishes and seeded in 2 ml of medium in 35-mm dishes. Four of ten foci grew to confluence in the 35-mm dishes and were passaged to 60- and finally to 100-mm dishes. These four independent lines of SRD-transformed rat embryo cells will be referred to as LR3/1, LR3/2, LR3/3, and LR5/1.

Agar Cloning. Agar cloning assays were performed as described by MacPherson (19).

Virus rescue. (i) Sendai virus-mediated rescue. Virus was rescued by Sendai virus-mediated fusion as described previously (2).

(ii) PEG-mediated virus rescue. The described procedure was adapted to virus rescue (see below) by a modification of the PEG-monolayer fusion technique of Davidson and Gerald (7). Fifty percent PEG was prepared as described by Pontecorvo (20). Briefly, 10 g of PEG 6000 (molecular weight 6,000 to 7,500; J. T. Baker Chemical Co.) was autoclaved, and 10 ml of E at 37°C (without serum) was added. This solution was mixed well and cooled to room temperature. If crystals appeared, the solution was incubated at 56°C until a crystal-free solution was obtained.

Transformed mammalian cells were treated with 10 μg of mitomycin C per ml for 2 h three days after subculture. The cells were trypsinized and seeded in duplicate at 10^3, 10^4, and 10^5 cells per 35-mm tissue culture dish with 5×10^5 to 6×10^5 CEC in EF_5.

Twenty-four hours after seeding, the medium was removed, and 0.5 ml of PEG suspension was added. Care was taken to remove as much medium as possible before the addition of PEG since the effectiveness of PEG is greatly diminished by dilution (8). PEG was diluted with 2 ml of Tris-buffered saline with dextrose (TD) after 1 min and removed by aspiration. The monolayers were washed twice with 2 ml of TD and fed with EF_5. After 16 to 24 h, the cells were overlaid with 2 ml of standard focus agar and incubated at 38°C. Foci of transformed cells were counted 7 to 10 days later. Those plates with 10 to 100 foci were counted, and controls that were not exposed to PEG were routinely included for each cell density. The data reported are the average of duplicate assays and are expressed as rescue frequency per 10^3 mammalian cells.

To further standardize the rescue assay, the CEC and transformed mammalian cells were maintained on a strict transfer schedule prior to setting up the rescue experiments. It has been observed that the rescue frequencies may be affected by the physiological state of the chicken cells as well as the transformed mammalian cells (Boettiger, unpublished data). CEC were routinely passaged upon reaching confluence, and only third-, fourth- or fifth-passage cells were used for virus rescue. Transformed mammalian cells were plated at 2×10^6 cells per 100-mm tissue culture dish, and rescue experiments were set up 2 or 3 days after subculture.

Infectious center assay. (i) Sarcoma virus-infected chicken cells. Chicken embryo cells infected with either PRA or PRB virus were prepared by adding 2×10^6 focus-forming units (FFU) of virus to 2×10^6 freshly transferred CEC in 100-mm tissue culture dishes. Twelve hours after virus exposure, the cultures were killed by incubation with 10 μg of mitomycin C per ml for 2 h. The killed infected cells were trypsinized and absorbed at various densities in 0.1 ml of EC_5 onto fresh monolayers of CEC for 15 min before they were overlaid with standard focus agar. The infected cells were plated early after infection, before they had begun to release free virus, to ensure that foci arising were a direct measurement of the virus-producing cells in the culture.

(ii) Virus-producing heterokaryons. B4 cells (5×10^5 cells) were plated with 2.5×10^6 RAV-infected CEC in 60-mm tissue culture dishes. Four hours after plating, the cells were treated with 5 ml of PEG for 1 min, rinsed three times with TD, and then fed with EC_5 containing 10 μg of mitomycin C per ml. After incubation with mitomycin C for 2 h, the cultures were fed EC_5 (without mitomycin C). After 12 h, the cultures were trypsinized, and the cells were replated on fresh monolayers of indicator chicken cells in 2.0 ml of EC_5. Three hours after seeding, the cultures were overlaid with standard focus agar. Foci resulting on uninfected C/E cells are a direct measurement of the number of infectious virus-producing heterokaryons in the fusion mixture.

RESULTS

PEG-mediated virus rescue from B77 virus-transformed NRK cells. To evaluate the usefulness of PEG for virus rescue studies, it was desirable to begin with an ASV-transformed mammalian cell line that was known to be virogenic. Virus has been successfully rescued from the B77 virus-transformed cell line NRK/B77 T1 by Sendai virus fusion with CEC (3). Monolayers of CEC and mitomycin C-killed NRK/B77 T1 cells were treated with 50% PEG for 1 min, rinsed, fed, and overlaid with focus agar as described above. Eighteen separate PEG-mediated virus-rescue experiments, performed in duplicate over a period of 4 months, resulted in a mean virus rescue frequency of 12.6 FFU/10^3 cells with a standard deviation of 5.4. Controls that were not exposed to PEG were included with every experiment. The mean background rescue frequency was 0.09 FFU/10^3 cells with a standard deviation of 0.09. It should be emphasized that these data include the results of every rescue experiment that has been performed by PEG fusion of NRK/B77 T1 cells with CEC. There was considerably less variation observed than in previous experiments with Sendai virus-mediated rescue. The absolute rescue frequency for a particular cell line may vary by 10-fold for Sendai virus-mediated assays performed on separate occasions (5). The maximum variation observed for PEG-mediated virus rescue of NRK/B77 T1 cells is only fivefold, with the majority of the values (78%) falling in the narrow range of 8 to 15 FFU/10^3 cells.

In a previous study, the rescue frequency for NRK/B77 T1 cells by Sendai virus-mediated cell fusion was observed to 13.6 FFU/10^3 cells (3). This value represents the rescue frequency observed under optimal conditions with maximum Sendai virus-mediated heterokaryon formation. Table 1 compares the results of a Sendai virus fusion performed parallel to a PEG fusion rescue experiment. Sendai virus rescue resulted in 13 FFU/10^3 cells on this occasion, and PEG resulted in 10 FFU/10^3 cells. Thus, the rescue frequencies obtained by Sendai virus-

TABLE 1. *Sendai virus and PEG rescue frequencies for NRK/B77 T1 cells*

Fusing agent	Rescue frequency (FFU/10^3 cells)	Control[a] (FFU/10^3 cells)
Sendai virus[b]	13.0	0.01
PEG[c]	10.0	0.01

[a] Background rescue in the absence of induced fusion.

[b] Sendai virus fusions performed as described previously (2).

[c] PEG fusions by the standard fusion assay method.

mediated cell fusions agree well with those rescue frequencies obtained with PEG-mediated virus rescue.

The standard PEG rescue fusion assay is linear over a wide range of NRK/B77 T1 cell densities. Transformed cells were plated at densities ranging from 10^2 to 10^4 cells with monolayers of 5×10^5 CEC in 35-mm tissue culture dishes. Figure 1 presents the results of three separate experiments, and each point represents the average of duplicate assays. Similar results were obtained for all three experiments. The solid line represents the theoretical relationship for a linear single-hit assay.

The procedure routinely used for PEG-mediated virus rescue combines cell fusion and the focus assay for ASV. For a focus to appear, virus must be released from the mitomycin C-killed transformed mammalian cell-CEC hybrid and must infect the neighboring CEC (28, 29). The background of focus formation by mitomycin C-killed NRK/B77 T1 cells in the absence of fusing agent is most likely due to spontaneous heterokaryon formation during cocultivation. Spontaneous fusion and virus rescue have been observed previously (28). The frequency of virus rescue in the absence of fusing agents is usually 100- (23) to 300-fold (29) lower than that with added Sendai virus. There is a 200-fold increase in rescue frequency with PEG treatment. Presuming that the frequency of spontaneous fusion is 10^{-4} for ASV-transformed mammalian cells cocultivated with a fivefold excess of chicken cells (28), the expected rescue frequency for NRK/B77 T1 cells would be 0.1 to 0.2 FFU/10^3 cells. This agrees well with the observed frequency of 0.09 FFU/10^3 cells in the absence of fusing agents. Furthermore, spontaneous rescue in vitro in the absence of fusing agents was observed for the two other virogenic lines, LR3/3 and LR5/1, described in this communication.

The effect of chicken cell density on the virus rescue frequency was examined by plating 10^4 mitomycin C-killed NRK/B77 T1 cells with various densities of CEC ranging from 1×10^5 to 8×10^5 CEC per 35-mm dish. There were no significant differences observed with the various CEC cell densities. For standardization, the rescue assay was routinely performed with 4×10^5 to 5×10^5 CEC.

To maximize the rescue assay, the optimum times between cell seeding and PEG treatment before agar overlay were determined (data not shown). The frequency of virus rescue decreased by 50% if PEG treatment was carried out 6 h as compared with 24 h after cell plating. Agar overlay at 2 h, but not 6 or 24 h, after PEG treatment also reduced the rescue frequency. For convenience, the monolayers were routinely treated with PEG 24 h after plating the cells, and agar was overlaid 16 to 24 h later.

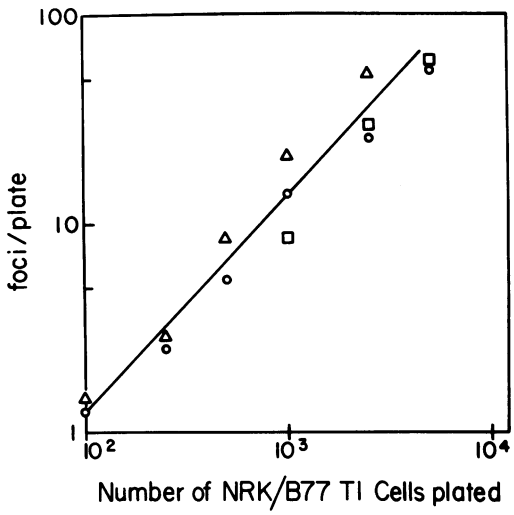

FIG. 1. *Linearity of the PEG-mediated standard fusion-rescue procedure with NRK/B77 T1 cells. Symbols: Trial 1, △; trial 2, ○; trial 3, □.*

A number of variations in the standard procedure were also attempted. Varying the number of TD washes or increasing the volume of PEG did not affect significantly the rescue frequency. Only increasing (62%) or decreasing (41%) the PEG concentration or neutralizing the PEG (which is normally slightly acidic when mixed with Eagle medium) resulted in a substantial decrease in the rescue frequency (Table 2). In addition, PEG 1000 or 1540 resulted in rescue frequencies comparable to those observed for PEG 6000, but PEG 600 was only about 10% as effective in the fusion assay (Table 2).

Comparison of clones with a range of virus rescue frequencies. SR3 is a line of Schmidt-Ruppin virus-transformed BHK 21/13 cells which exhibits a high frequency of reversion to the normal nontransformed morphology (18). A series of subclones displaying various degrees of reversion were isolated and compared with the parent line in quantitative virus rescue experiments (2). A direct correlation between morphological reversion and rescue frequency was observed; revertant subclones exhibited low rescue frequencies, and transformed variants exhibited high rescue frequencies. In addition, morphology and virus rescue frequencies reflected the levels of virus-specific mRNA expression in these cells (9).

Virus rescue by PEG-mediated fusion was

TABLE 2. *Effect of variations in PEG concentrations and grades on virus rescue*

PEG concn (%)	Grade	Rescue frequency (FFU/10^3 cells)
50	PEG 6000	10.6
41	PEG 6000	1.6
62	PEG 6000	2.6
50	PEG 1000	9.3
50	PEG 1540	11.2
50	PEG 600	0.8
50	PEG 6000 neutralized	4.5

examined and compared with the previously published results of Sendai virus-mediated rescue for SR3 and its subclones (Table 3). The relative rescue frequencies for these cell lines as measured by PEG and Sendai virus-mediated rescue experiments were essentially identical. The highly transformed subclone SR3/1a displayed a rescue frequency of 3.0 in Sendai virus fusion-rescue experiments and 3.1 in PEG-mediated rescue experiments. The revertant subclones SR3/5, SR3/11R, and SR3/4a also yielded comparable relative rescue frequencies by these methods. It appears that the differences in relative rescue frequencies observed with Sendai virus-mediated cell fusion were reproduced with PEG-mediated cell fusion.

Complementation rescue. The failure to rescue virus from some RSV-transformed mammalian cells may be the result of a deletion or mutation in the genome of the resident transforming virus. If this were the case, then it should be possible to rescue virus by complementation with RAV. To examine the possibility of recovering defective viruses from transformed mammalian cells, a line of Bryan strain RSV-transformed BHK cells isolated by Macpherson (B4 cells) was used. Bryan strain RSV grown in avian cells contains a deletion in the glycoprotein gene (10, 13) rendering the virus non-infectious. Superinfection of Bryan strain-transformed CEC with nontransforming avian leukosis viruses complements this defect, resulting in release of transforming virus with a host range identical to that of the helper virus (12, 33). These properties of Bryan strain RSV lead one to the prediction that if virus may be rescued from B4 cells, it must be strictly dependent upon the presence of helper RAV. Thus, B4 was chosen as a model to establish a procedure for PEG-mediated complementation-rescue of defective RSV from mammalian cells.

Rescue by fusion of mitomycin C-killed B4 cells with monolayers of RAV-infected chicken cells was examined for leukosis viruses of subgroups A, B, and C. RAV-infected cultures were prepared by infecting secondary cultures of CEC with the appropriate virus and passaging the cells twice before use to ensure complete infection. The assay was performed by plating mitomycin C-killed B4 cells with RAV-infected chicken cells, treating the monolayers with PEG, and overlaying with focus agar exactly as described above (Table 4).

As predicted, attempts to rescue virus from B4 cells by fusion with chf^-gs^- CEC failed. Fusions of B4 cells with subgroup A leukosis virus (RAV-1)-infected cells or subgroup C leukosis virus (RAV-7, RAV-49, or NTB77)-infected cells rescued virus at frequencies of 2 to 8 FFU/10^3 B4 cells. However, if the chicken cells are infected with the subgroup B leukosis virus RAV-6, the rescue frequency is reduced to 0.01 FFU/10^3 B4 cells.

The efficient rescue of transforming virus in fusions of B4 cells with any of the RAV-infected cells in the monolayer fusion assay was unexpected. Chicken cells infected with avian leuko-

TABLE 3. *Comparison of Sendai virus and PEG-mediated virus rescue of Schmidt-Ruppin virus-transformed baby hamster kidney cells and morphological revertants*

Cell line	Description[a]	PEG rescue frequency (FFU/10^3 cells)	Sendai virus rescue frequency (FFU/10^3 cells)
SR3	Parent cell line	1.05	1.0
SR3/1a	Transformed subclone	3.25	3.0
SR3/5	Revertant fibroblast	0.23	0.15
SR3/11R	Revertant fibroblast	0.005	0.01
SR3/4a	More parallel orientation	0.17	0.10

[a] From D. Boettiger (2).

TABLE 4. *PEG-mediated virus rescue from B4 cells by fusion with RAV-infected CEC*

RAV	Fusion assay rescue frequency/10^3 cells
None	0(10)[a]
RAV-6	0.01 (2)
RAV-1	3.3 (6)
RAV-7	2.9 (1)
RAV-49	2.4 (1)
NTB77	7.5 (1)

[a] Numbers in parentheses indicate the number of experiments performed.

sis viruses are resistant to challenge by sarcoma viruses of the same subgroup (32). Assuming that the virus released in the B4 rescue is the same subgroup as the helper virus, secondary infection of the adjacent chicken cells should be prevented. Since at least one cycle of spreading infection is required to allow foci to appear in this assay, the RAV-1 and subgroup C RAV results present a dilemma.

There are two possible explanations for these results: (i) the RAV-infected CEC were not fully infected (in the case of RAV-1 and the C subgroup RAVs) and hence, the interference was incomplete; and (ii) since the heterokaryons are infectious centers which continuously release virus, there may be differences between the various subgroups in resistance to infectious center challenge. These two possibilities were examined by comparing virus challenge and infectious center challenge of cells infected with the various leukosis viruses.

Interference data for RAV-infected cultures challenged with virus are presented in Table 5. Cells infected with RAV-7, RAV-49, and NTB77 restrict focus formation by challenging PRC virus by only 10- to 12-fold. Therefore, the appearance of foci in the subgroup C rescue of B4 virus may be the result of incomplete interference. This weak interference may also be the result of heterogeneity in interference patterns normally observed among the C subgroup viruses (11). The RAV-1-infected cells, however, completely restricted the formation of foci by a PRA virus challenge of 10^6 FFU. RAV-6 infected cells subjected to a similar challenge of PRB virus also completely prevented focus formation. The simple explanation of incomplete infection of the RAV-1 cultures is not adequate to explain the successful RAV-1 rescue of B4 virus. Both the RAV-1 and RAV-6 cultures were completely infected, yet fusions with B4 produced very different rescue frequencies. This suggests that there may be a difference in the ability of A and B subgroup-infected cells to resist challenge by infectious centers.

Sarcoma virus infected cells (PRA or PRB) were plated as infectious centers (see above) on uninfected C/E chicken cells, cells infected with leukosis virus of the same subgroup, and genetically resistant cells (Table 5). PRA cells plated as infectious centers resulted in foci on monolayers of RAV-1-infected cells at an efficiency of approximately two foci per 10^3 infectious centers. The virus released from the PRA cells is subgroup A, as determined by its inability to infect C/A cells. PRB cells, however, did not produce foci when plated as infectious centers on monolayers of RAV-6-infected cells. In addition, no foci were evident when PRB cells were plated as infectious centers on subgroup B genetically resistant quail cells. It appears that although the RAV-1- and RAV-6-infected cultures interfere equally as efficiently with a direct virus challenge, subgroup A sarcoma virus-infected cells are able to overcome superinfection resistance and infect subgroup A leukosis virus-infected cells.

To test directly if the differences in efficiency of interference with virus-producing cells provides a suitable explanation for the RAV-1 and

TABLE 5. *Challenge of genetically resistant and RAV-infected cells by sarcoma virus and infectious centers of sarcoma virus-producing cells*

Challenge virus[a] or cells[b]	Subgroup of challenge	Focus formation on:		
		Sensitive cells[c]	Genetically resistant cells	Cells preinfected with RAV of the challenge subgroup
PRA virus	A	10^6	0[d]	0[e]
PRA-infected cells	A	10^5	0[d]	220[e]
PRB virus	B	10^6	0[f]	0[g]
PRB-infected cells	B	10^5	0[f]	0[g]
PRC virus	C	10^6	NT	7×10^4–9×10^4[h]

[a] Virus challenge was performed with a maximum dose of 10^6 FFU/plate as determined by parallel infection of sensitive cells. Average values for several experiments are reported.

[b] Infectious center challenge was performed with a maximum dose of 10^5 infectious centers per plate as determined by parallel assay on sensitive cells. Average values for several experiments are reported.

[c] C/E chf$^-$ gs$^-$ chicken cells.

[d] Reasheath line C C/A cells.

[e] RAV-1-infected cells.

[f] Quail cells.

[g] RAV-6-infected cells.

[h] Range of values represents different subgroup C RAVs: RAV-7, RAV-49, and NTB77 were used.

RAV-6 rescue results, fusion mixtures were replated as infectious centers on sensitive and resistant cells (Table 6). As was observed with PRA-infected cells, the RAV-1 × B4 heterokaryons, plated as infectious centers, were able to infect RAV-1-infected cells. The efficiency of focus formation on RAV-1-infected cells is approximately 2×10^{-3}. No foci arose on genetically resistant C/A cells. RAV-6 × B4 infectious centers did not result in foci when plated on RAV-6-infected CEC or quail cells (genetically resistant to subgroup B). The ability of cells producing subgroup A viruses to overcome superinfection resistance and to infect resistant cells appears to explain the RAV-1 rescue results. Subgroup B leukosis virus-infected cells, however, are not infected by association with subgroup B sarcoma virus-producing cells with up to 10^5 cells plated as infectious centers.

PEG-mediated virus rescue from other ASV-transformed cell lines. Five additional ASV-transformed mammalian cell lines were examined in PEG-mediated rescue experiments (Table 7). Virus could not be rescued from KC cells, which are a human glioma cell line transformed in vitro by RSV, by fusion with C/E CEC. This was an unexpected result considering the report that group-specific antigen is expressed in these cells and that virus may be rescued upon injection of KC cells into chickens (17). By fusing KC cells with RAV-1-infected CEC, virus was rescued, however, confirming the presence and expression of at least a portion of the viral genome in these cells.

Virus rescue was examined in four lines of transformed rat embryo cells established by treating rat embryo cells with Schmidt-Ruppin D RSV in vitro (see above). Of the four lines, only LR3/3 and LR5/1 released infectious virus upon fusion with C/E CEC. Virus was rescued from the other two lines LR3/1 and LR/3 only in complementation rescue assays.

TABLE 6. *Assay of B4 × RAV heterokaryons as infectious centers on sensitive and resistant cells*

Challenge virus[a] or cells[b]	Subgroup of challenge	Focus formation on:		
		Sensitive cells[c]	Genetically resistant cells	Cells preinfected with RAV of the challenge subgroup
PRA virus	A	10^6	0[d]	0[e]
B4 XCh (RAV-1)	A	10^5	0[d]	210[e]
PRB virus	B	10^6	0[f]	0[g]
B4 XCh (RAV-6)	B	10^5	0[f]	0[g]

[a-g] See Table 5 for footnotes.

TABLE 7. *Virus rescue from ASV-transformed cell lines by C/E or RAV-1-infected C/E CEC in the standard rescue assay*

Cell line	C/E rescue (FFU/10^3 cells)	C/E (RAV-1) rescue (FFU/10^3 cells)
B4	<0.01	3.3
KC	<0.01	0.3
LR 3/1	<0.01	3.2
LR 3/2	<0.01	0.32
LR 3/3	20.2	NT
LR 5/1	11.3	NT

PEG-mediated fusion has been used to detect virus in both virogenic and nonvirogenic cell lines. Of four SRD-transformed rat embryo cell lines isolated, two proved to be nonvirogenic upon fusion with C/E cells. Thus, the isolation of nonvirogenic lines from which transforming virus may be rescued by the complementation-rescue procedure may be a common event. The properties of the virus rescued from nonvirogenic cells is currently under investigation in an attempt to determine which function(s) is defective.

DISCUSSION

Although Sendai virus is an effective fusing agent in the rescue of RSV from transformed mammalian cells, it suffers from certain technical drawbacks. The fusing capacity may vary from batch to batch, it is somewhat difficult to prepare, and it has a limited storage life. These problems assume major importance if one is planning to do rescue experiments only occasionaly or on a large scale. PEG-mediated cell fusion induces reproducible heterokaryon and somatic cell hybrid formation (7, 8). Therefore, it seemed likely that PEG might serve as a suitable substitute for Sendai virus in rescue experiments. PEG gave reproducible results in virus rescue assays with an efficiency equal to the best Sendai virus preparations. The ease with which this reagent may be prepared and used in virus rescue assays will allow a more widespread use of quantitative virus rescue assays.

The procedure for the rescue of virus from mammalian cells transformed by defective viruses is only slightly more complicated than the method for rescue from virogenic cells. Preinfection of the CEC with subgroup A or C leukosis virus prior to fusion with nonvirogenic cells resulted in successful virus rescue from every nonvirogenic line that we examined. With the

exception of Bryan virus-transformed mammalian cells (14, 29), previous attempts to rescue virus from nonvirogenic cell lines by complementation have failed (24). This reflects the increased efficiency of our complementation-rescue technique. The nonvirogenic cell lines examined by Svoboda et al. (24) may be transformed by viruses that are too defective to rescue, as they suggest. However, it is likely that their assay, which involves titrating the transforming virus in the supernatant from nonvirogenic cells fused with leukosis virus-infected chicken cells, lacks the sensitivity to detect transforming virus among the vast excess of RAV that is present. It will be interesting to screen other nonvirogenic lines in our complementation-rescue assay to determine if actual rescue-negative RSV-transformed mammalian cell lines exist.

The fusion-rescue assay requires that the virus produced from the heterokaryons of transformed mammalian cells and CEC reinfects the neighboring CEC to produce a focus. B4 cells fused with normal chicken cells produce no infectious virus but would produce foci if a round of infection was unnecessary. The production of foci by complementation with RAV-1 (and the subgroup C leukosis viruses), however, presents a dilemma. The virus produced from the heterokaryons should have an RAV-1 envelope and host range. Since all of the neighboring CEC are infected with RAV-1 and resistant to superinfection, the complementation rescue assays should not work. Several experiments have been done in an attempt to resolve this paradox.

CEC producing PRA virus and killed with mitomycin C are incapable of producing foci on C/A genetically resistant cells, but do give rise to a reduced number of foci on RAV-1-infected cells (Table 5). B4 cells, fused with RAV-1-infected cells and plated as infectious centers on RAV-1-infected cells, also give rise to foci (Table 6). The efficiency of infection of cells resistant to subgroup A viruses (due to interference) by contact with either PRA virus-producing chicken cells or heterokaryons-producing B4 (RAV-1) pseudotypes is approximately 2×10^{-3} of the actual virus-producing cells. The efficiency of infection of subgroup B leukosis virus-infected cells by subgroup B sarcoma virus-producing cells is less than 10^{-5}. With 10^5 PRB or infectious RAV-6 $\times$ B4 heterokaryons plated as infectious centers on monolayers of RAV-6-infected cells, no foci resulted. Considering these results, it is evident that the successful rescue of virus from B4 cells by fusion with RAV-1-infected CEC in the monolayer rescue assay is due to the ability of cells producing subgroup A viruses to overcome subgroup-specific interference. The RAV-6 complementation in the monolayer rescue was 100-fold less efficient and may be explained on the basis of the apparent inability of subgroup B virus-producing cells to efficiently infect RAV-6-infected cells.

The complementation rescue procedure has been applied to several other RSV-transformed mammalian cell lines. Although this method is much less sensitive than replating fusion mixtures as infectious centers on uninfected cell monolayers, it does provide a means for rapid screening of cell lines for the presence of defective viruses. In addition to B4 cells, a line of RSV-transformed human astrocytes (KC cells [17]) and two lines of SRD-transformed Lewis rat embryo cells (LR3/1 and LR3/2), all of which fail to release infectious virus in rescue assays with uninfected CEC, have been tested. All four of these rescue-negative RSV-transformed cell lines register as rescue positive upon fusion with RAV-1-infected CEC.

The procedure for rescue of virus from nonvirogenic cell lines has been referred to as a "complementation rescue assay." We have not yet determined if our results are due to complementation, recombination, or both. In the case of B4 cells, the virus rescue is most likely due to complementation and not recombination. It is known that Bryan virus does not recombine with helper virus to form nondefective transforming progeny virus. It is unlikely that recombination is necessary for the release of infectious virus from the heterokaryons in the rescue of virus from other nonvirogenic lines. Both the RAV-infected CEC and the transformed mammalian cells may be killed with mitomycin C before fusion without affecting the rescue frequency (not shown). It has been established that mitomycin C-killed chicken cells are not capable of being infected by virus but that infected cells continue to release virus after killing. Unless recombination is occurring at the RNA level, recombination is not likely to be a prerequisite for rescue of defective virus from transformed cells. This does not, however, imply that recombination with helper virus does not occur in later rounds of infection. We are currently examining the virus rescued from the various cell lines for the presence of recombinants.

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