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NOTES

Transformation of Rat Cells by Fusion-Infection with Rous Sarcoma Virus

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The transformation of a rat cell line, 3Y1, by nonmammalian tropic strains of avian sarcoma virus was tested using cell-virus fusion mediated by Sendai virus or polyethylene glycol. Furthermore, the establishment of several transformed 3Y1 cell clones induced by the Schmidt-Ruppin strain of Rous sarcoma virus (RSV), its derivative mutants, and the Bryan high-titer strain of RSV is reported. The presence and expression of the viral genomes in these cells were examined, and all transformed cell clones tested were found to contain rescuable RSV genomes when they had been fused with normal chicken embryo fibroblast cells or those preinfected with Rous-associated virus type 1. However, the *gag* gene product, pr76, was barely detectable in wild-type RSV-transformed cells, whereas it was produced in considerable amounts in cells transformed by *env*-deleted mutants, the Bryan high-titer strain of RSV and NY8 derived from the Schmidt-Ruppin strain of RSV.

Infection of chicken embryo fibroblasts (CEFs) with avian sarcoma virus induces cell transformation and, at the same time, a release of progeny viruses as a result of complete expression of viral genes. Whereas some mammalian cells can be transformed at low efficiency by mammalian tropic strains of avian sarcoma virus (e.g., the Schmidt-Ruppin subgroup D Rous sarcoma virus [SR-RSV-D], Prague subgroup C RSV [PR-RSV-C], and B77), they usually cannot support virus replication. As the mechanisms leading to abortive avian sarcoma virus infection in mammalian cells may differ among cell systems and virus strains (1, 2, 4-6, 13, 14), an analysis of the mode of expression of the viral genes in mammalian cells may aid in the elucidation of cellular or viral factors controlling gene expression. Moreover, since mammalian cell systems are much more stable than chicken cell systems and can be easily maintained as permanent cell lines, they provide better systems for studies of the synthesis or function of certain viral gene products such as the *src* gene product. However, one disadvantage of mammalian cell systems is their lack of susceptibility to certain widely used virus strains.

To circumvent this problem, mammalian cells have been infected either (i) by direct exposure to pseudotypes of RSVs enveloped with glycoproteins of mammalian tropic viruses (such as avian leukosis viruses of subgroup C or D) or to xenotropic murine leukemia viruses or (ii) by cocultivation with RSV-transformed CEFs (7, 17, 23, 24).

In the present study, a simpler procedure that takes advantage of cell-virus fusion which has been used for CEF infection with RSV mutants lacking viral envelopes (10, 19) is described as a means of transforming a rat cell line, 3Y1 (12), by nonmammalian tropic strains of RSV.

In this system, since cell-virus fusion does not involve a specific reaction between viral envelope glycoproteins and cell receptors, all avian sarcoma viruses of any subgroup, including mutants lacking the *env* gene such as the Bryan high-titer strain of RSV [BH-RSV(−)] and NY8(−), should have the same potential for transforming mammalian cells.

Cell-virus fusion was performed by using two different agents, Sendai virus and polyethylene glycol (PEG). Cell-free culture fluids obtained from cultures of CEFs fully transformed by SR-RSV-A, SR-RSV-B, BH-RSV(−), NY8(−), NY8(td105) (NY8 enveloped with td105, a transformation-defective mutant of SR-RSV-A), NY8α(td105) (polymerase-negative derivative of NY8 rescued by td105), and tsNY68. The viruses used and the preparation of NY8(−)- and BH-RSV(−)-infected cells have been described previously (8-11). 3Y1 cells (5×10^5) suspended in

3 ml of Eagle minimum essential medium supplemented with 10% tryptose phosphate broth and 5% newborn calf serum (growth medium) were mixed with 1 ml of UV-inactivated Sendai virus containing 2,560 hemagglutinin units, 1 ml of the respective RSV preparation, and 20 μg of Polybrene and seeded in 60-mm plastic dishes. After overnight incubation at 37°C, culture fluids were replaced with fresh growth medium, the cultures were transferred once on day 3 and further incubated for 2 weeks with medium replacement every 4 days. When 3Y1 cells inoculated with UV-inactivated Sendai virus and the RSV preparation were incubated, few to several hundred foci of transformed cells were formed by all virus preparations tested, whereas no foci appeared in the absence of UV-inactivated Sendai virus. The efficiency of focus formation varied with the different virus preparations (Table 1), probably because of a difference in the number of virus particles in the preparations; it also varied from experiment to experiment, possibly because of the physiological condition of the 3Y1 cells at the time of virus inoculation.

BH-RSV induces compact and discrete foci on CEFs, whereas SR-RSV forms diffuse and large foci (22). Similarly, foci of BH-RSV(−)-transformed 3Y1 cells were composed of rather flat and hexagonal cells (Fig. 1A), forming clear boundaries with the surrounding normal cell area, whereas foci induced by SR-RSV (Fig. 1B) and its derivatives consisted of refractile round and spindle-shaped cells.

As PEG-mediated cell fusion has been reported (18, 21), focus formation by cell-virus fusion with PEG was also tested in the present investigation. To adsorb the virus, 5×10^5 3Y1 cells seeded in a 60-mm plastic dish were exposed for 90 min to 1 ml of the respective RSV preparation in the presence of 20 μg of Polybrene, washed with phosphate-buffered saline (21), and then treated for 1 min with 50% PEG (average molecular weight, 1,500). After PEG was removed by several washings with phosphate-buffered saline, growth medium was added and the culture was incubated for 2 weeks. The efficiency of PEG-induced focus formation in RSV-infected cell cultures appeared to be affected by factors such as the physiological condition of the cells, the PEG lot used, and the duration of PEG exposure. However, in general, the efficiency of focus formation by RSVs appeared to be higher in cells treated with UV-inactivated Sendai virus than in those treated with PEG (Table 1).

Several transformed cell clones were isolated from cultures infected with the above-mentioned RSVs, and cell clones expressing temperature-sensitive transformation were also established from tsNY68-infected 3Y1 cells. Furthermore, from uninfected cell cultures containing a focus of round and refractile cells which had inadvertently been left in the incubator for more than 3 weeks without a change in medium, spontaneously transformed cell clones were isolated.

To demonstrate the presence of RSV ge-

TABLE 1. *Focus formation of 3Y1 cells transformed by RSV after cell-virus fusion with UV-inactivated Sendai virus or PEG*

Virus	Titer on CEFs[a]	Focus no.				
		Without fusion[b]	With fusion by:			
			UV-inactivated Sendai virus[c]		PEG[d]	
			Expt 1	Expt 2	Expt 1	Expt 2
SR-RSV-A	4.5×10^6	0, 0	267, 213	79, 56	38, 28	41, 23
NY8(td105)	8.0×10^5	0, 0	26, 17	8, 5	5, 2	ND[e]
NY8(−)	0	0, 0	10, 8	3, 0	3, 1	0, 0
BH-RSV(−)	0	0, 0	48, 40	13, 8	26, 17	11, 4

[a] The virus titers on CEFs were assayed as described previously (20).

[b] The respective virus preparation (1.0 ml) was inoculated onto 5×10^5 3Y1 cells in the presence of Polybrene, and the culture was transferred to three dishes on day 3. Foci were scored on day 15.

[c] 3Y1 cells (5×10^5) mixed with 1.0 ml of the respective virus preparation, 1.0 ml of UV-irradiated Sendai virus, and 20 μg of Polybrene were seeded in a 60-mm dish and incubated at 37°C. On day 3, the culture was divided among three dishes. Transformed cell foci were scored after 2 weeks. Focus number indicates the sum of the numbers in the three dishes.

[d] 3Y1 cells (5×10^5) which had been seeded in a 60-mm dish were exposed for 90 min to 1 ml of the respective virus in the presence of Polybrene, and then the culture was treated for 1 min with 50% PEG. After PEG was removed, the culture was incubated with medium. On day 3, the culture was divided among three dishes. Transformed cell foci were scored after 2 weeks. Focus number indicates the sum of the number in the three dishes.

[e] ND, Not done.

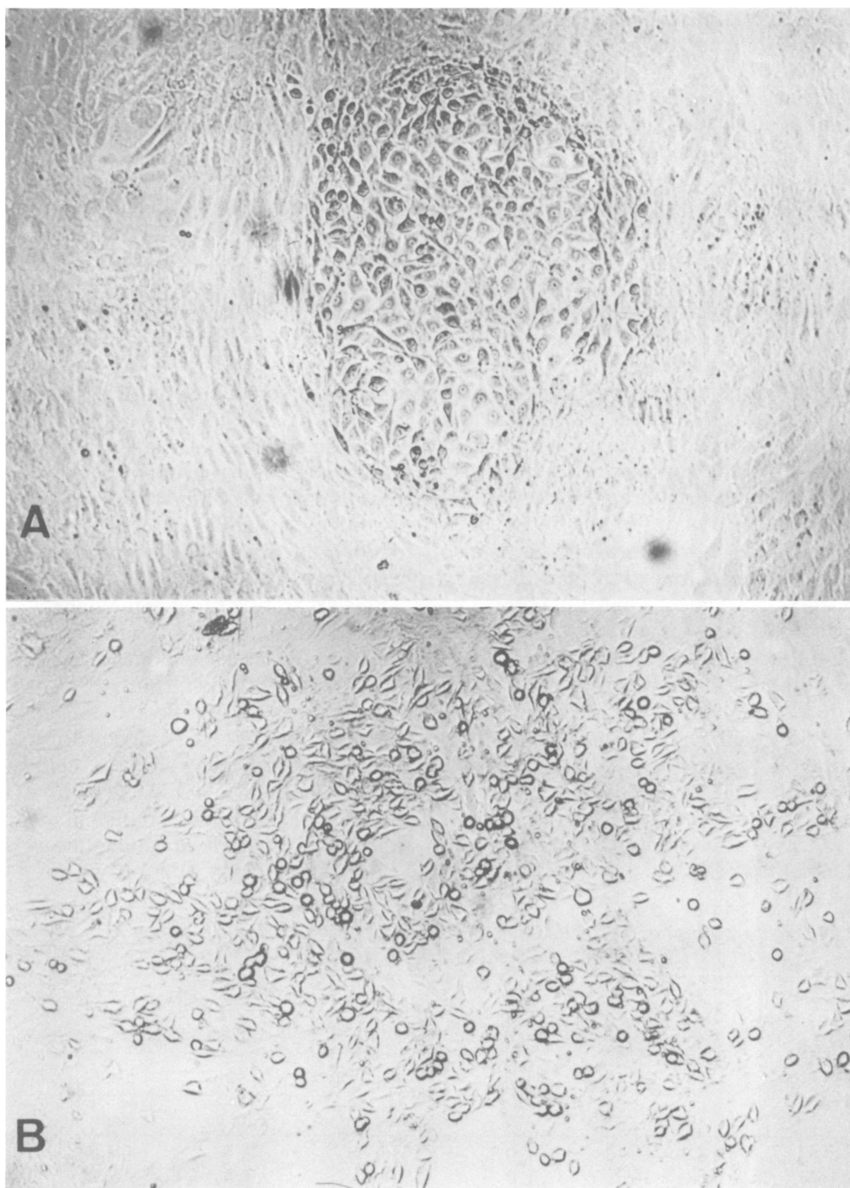

FIG. 1. *Morphology of transformed cell foci. (A) BH-RSV(−)-induced focus; (B) SR-RSV-B-induced focus.*

nomes, the rescue of infectious RSV from 3Y1 cells that had undergone RSV transformation was examined by fusion with normal CEFs or CEFs preinfected with Rous-associated virus type 1. When fused with normal CEFs, SR-RSV-A-transformed cells produced SR-RSV-A (Table 2). Infectious virus was recovered as expected from NY8(−)- and BH-RSV(−)-transformed cells when CEFs preinfected with Rous-associated virus type 1, but not normal CEFs, were fused with the cells. The recovery of infectious virus from NY8(td105)-transformed cells fused with normal CEFs showed that the transformed cells had been doubly infected with NY8(td105) and td105. In the transformed cells both td105 and NY8(−) genomes were present. This result was somewhat unexpected because the efficiency of infection, as determined by the number of transformed cell foci per input virus, would appear to be too low (at most 10^{-4}) to allow such double infection if td105 had the same infection efficiency as NY8(td105) and the infection by the two viruses took place independently. Another independent clone of NY8α(td105)-trans-

formed cells was found to contain the helper virus genome. This high frequency of double infection could be due to infection by aggregates of the two viruses, and also the possibility that only a small fraction of the rat cells are sensitive to infection cannot be ruled out. However, these findings may suggest that virus penetration and the integration of the virus genome occur in a greater number of cells than is estimated from the transformation frequency and that most of the integrated genomes do not express their transforming function. This possibility is now under investigation.

TABLE 2. *Recovery of infectious RSV from RSV-transformed 3Y1 cells*[a]

Clone	RSV yield (FFU/ml)	
	CEF	CEF(RAV-1)
Sp.Tr.-3Y1 no. 1[b]	0	0
SR-A-3Y1 no. 1	1.6×10^4	ND
NY8(−)-3Y1 no. 1	0	3.3×10^2
BH(−)-3Y1 no. 4	0	5.7×10^2
NY8(td105)-3Y1 no. 1	1.0×10^4	10

[a] Cells (5×10^5) of a transformed 3Y1 clone were cocultured with 8×10^5 cells of uninfected CEFs or CEFs preinfected with Rous-associated virus type 1 in the presence of UV-irradiated Sendai virus. The culture fluids obtained on day 4 were assayed on CEFs. FFU, Focus-forming units; ND, not done.

[b] Spontaneously transformed 3Y1 clone.

The gene expression in these and other cells transformed by SR-RSV-D, PR-RSV-C, and B77 (these mammalian tropic strains of avian sarcoma virus were obtained from H. Hanafusa, The Rockefeller University) was examined by using immune sera obtained from mice repeatedly immunized with ^{60}Co-irradiated RSV-transformed mouse cells. The sera were found to contain antibodies against the *src* gene product of SR-RSV and against *gag* gene products (S. Kawai, K. Segawa, and T. Yamamoto, manuscript in preparation).

[^{35}S]methionine-labeled extracts of transformed cells were incubated with mouse immune sera, and the immunoprecipitated polypeptides were analyzed by polyacrylamide gel electrophoresis (3). p60^{src} was detected clearly in the extract of cells transformed by SR-RSV-A, SR-RSV-D, and NY8(−) (Fig. 2E, H, and B, respectively)

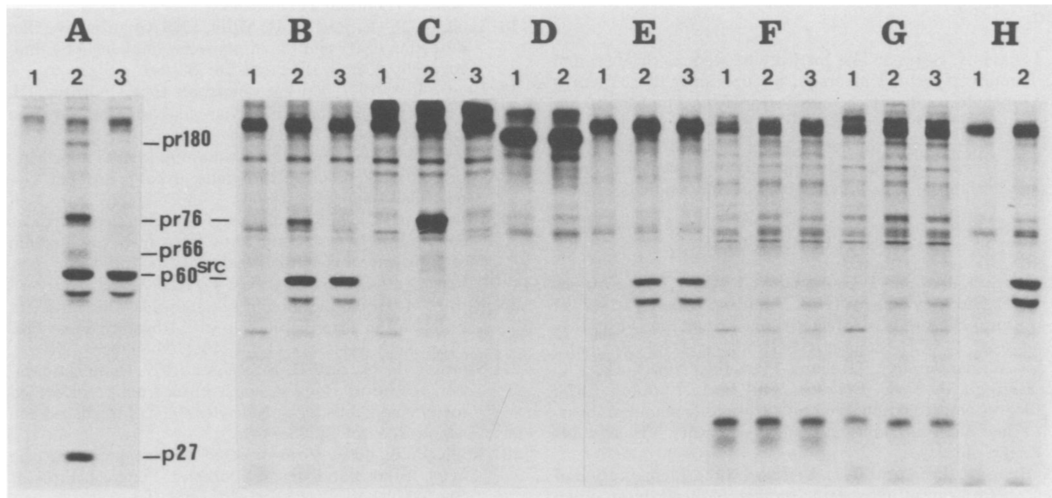

FIG. 2. *Immunoprecipitation of virus gene products from RSV-transformed CEFs and 3Y1 cells. Ten hours after seeding 3 × 10⁶ cells per 60-mm dish, the cells were labeled for 12 h at 37°C with 40 μCi of [^{35}S]-methionine in 3 ml of minimal essential medium supplemented with 5% dialized newborn calf serum. Cells were lysed with 1.5 ml of RIPA buffer (150 mM NaCl, mM Tris-hydrochloride [pH 7.2], 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate, and 2 mM phenyl sulfonyl fluoride), and the lysates were centrifuged at 35,000 rpm in a Beckman type 65 rotor. Portions of the lysates were incubated for 1 h at 4°C with 10 μl of (1) non-immune serum, (2) antiserum, or (3) antiserum preabsorbed with 50 μg of purified Rous-associated virus type 2 lysed in 10 μl of RIPA buffer. A 4% suspension (0.5 ml) of Formalin-fixed Staphylococcus aureus was added, and the mixtures were incubated further for 1 h. The immune complexes were collected by centrifugation, washed four times with RIPA buffer, and heated at 95°C for 1 min in 150 μl of sample buffer (0.07 M Tris-hydrochloride [pH 6.8], 11.2% glycerol, 3% sodium dodecyl sulfate, 0.01% bromophenol blue, 5% mercaptoethanol). The supernatants (100 μl each) were electrophoresed through 10% polyacrylamide gels prepared by the method of Laemmli (15). Gels were processed for fluorography (16) and exposed to Fuji RXS films. (A) CEFs transformed by SR-RSV-A, (B) 3Y1 cells transformed by NY8(−), (C) 3Y1 cells transformed by BH-RSV(−), (D) normal 3Y1 cells, (E, F, G, and H) 3Y1 cells transformed by SR-RSV-A, B77, PR-RSV-C, and SR-RSV-D, respectively.*

but very faintly in other cell extracts, suggesting that the mouse antisera were highly specific for SR-RSV p60^{src}. As for viral structural proteins, pr76 was found to be produced in NY8(−)- and BH-RSV(−)-transformed 3Y1 cells (Fig. 2B and C) and NY8(td105)-transformed 3Y1 cells (data not shown) at levels comparable to that in SR-RSV-A-transformed CEFs (Fig. 2A). Interestingly, in spite of the production of pr76, pr180, a readthrough product of *gag* and *pol* genes, which is the precursor of polymerase, appeared not to be produced in these cells. Furthermore, pr66, an intermediate, and p27, p19, p15, and p12, the final products of *gag* gene, were not detected in these cells (data not shown except for p27). This finding indicates that cleavage of the *gag* precursor proteins does not take place in 3Y1 cells. Compared with these mutant-transformed 3Y1 cells, [^{35}S]methionine-labeled pr76 were barely detectable in cells transformed by SR-RSV-A, SR-RSV-D, or other wild-type RSVs (Fig. 2E through H). Whether the increased synthesis of pr76 in NY8(−)-, NY8(td105)-, and BH-RSV(−)-transformed cells is correlated with a lack of the *env* gene in the genome of these viruses remains to be studied.

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