

Plaque Assay of Avian Sarcoma Viruses Using Casein

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The caseinolytic activity of several strains of Rous sarcoma virus (RSV), conditional and nonconditional mutants of RSV, and nontransforming avian leukosis viruses was investigated. Only those viruses capable of transforming chick fibroblasts *in vitro* induced lysis of casein incorporated into an agar overlay. Lysis produced distinct clear areas in the turbid casein-agar gel which allowed a quantitative "plaque" assay of cell transformation. Casein plaque formation could not be separated from morphological conversion in cultures infected by wild-type RSV strains. In plates infected by mutants temperature sensitive for transformation, the caseinolytic activity appeared to be affected by temperature to a lesser extent than morphological conversion.

Cells transformed *in vitro* by either DNA or RNA tumor viruses produce a factor which, interacting with plasminogen contained in serum, causes the lysis of fibrin (6). Recently Goldberg (2) found that chicken embryo fibroblasts transformed by Rous sarcoma virus (RSV) cause lysis of casein added to nutrient agar medium. Whereas the assay for fibrinolytic activity lends itself to accurate quantitative studies of the average production of plasminogen activator, the caseinolytic assay seems potentially useful as a plaque assay for avian sarcoma viruses. This test could also be useful to select nonconditional or conditional mutants, unable to induce proteolysis, for genetic studies with avian RNA tumor viruses. To investigate these points a study was carried out on the caseinolytic activity of chicken embryo fibroblasts infected with several laboratory strains of RSV, with several temperature-sensitive (ts) mutants derived from these strains, and with nontransforming viruses of the avian leukosis group. We have found that only viruses which transformed cells *in vitro* were able to induce lysis of casein. In cells infected by mutants of RSV, temperature sensitive for cell transformation, caseinolysis was inhibited by temperature to a lesser extent than was morphological conversion. Also, in preliminary attempts to isolate virus mutants which would induce either morphological conversion or caseinolysis, these two characters were always found to be associated.

MATERIALS AND METHODS

Virus strains. Several RSV laboratory strains of different subgroup specificity (7) were used in this

investigation: the Bryan high-titer strain of subgroup A, BH-RSV (RAV-1), of the morph^r and morph^f type of morphological conversion (5); the Schmidt Rupp (SR) strain also of subgroup A; and two Prague strains of subgroups A and B (PRA, PRB). Four ts mutants isolated from PRA (8) (PRA #23, 24, 25, and 29), kindly provided by J. Wyke, and eight ts mutants isolated from BH-RSV (RAV-1), three of which derived from mutagenized stocks of the morph^r variant, were also used. The characteristics of these and other mutants isolated from BH-RSV stocks will be presented separately. The nontransforming Carr Zilber associated virus of antigenic subgroup D and a transformation-defective (td) derivative isolated from the PRB strain and kindly provided by A. Bernstein were included in these studies.

Cells and cell cultures. Secondary cultures of chick fibroblasts from C/E brown Leghorn embryos were used throughout these experiments. Cell maintenance and conventional infectivity assays were carried out as previously described (1).

Caseinolysis assays. Sixty-millimeter plates with 6×10^6 cells were infected with appropriate dilutions of different virus strains. After 45 min of adsorption the cultures were overlaid with a thin layer (2 ml) of agar medium consisting of a mixture of F12 medium (4) and Dulbecco modified Eagle medium in the ratio of 2:1, with 10% tryptose phosphate broth, 5% calf serum, 1.25% chicken serum, 1% dimethyl sulfoxide, 0.7% agar (Difco), and containing as a source of casein, 2% (wt/vol) dried skimmed milk (Marvel-Cadbury Schweppes Food, England). After this medium had solidified, 5 ml of agar medium without casein were added to each plate. For soft agar assays 60-mm plates with 10^6 cells were infected with 1,000 focus-forming units of the appropriate virus strain. After overnight incubation the plates were trypsinized with 1-ml volumes of trypsin (0.05%) in Tris, and 0.2-ml volumes of cell suspension containing approximately 2×10^6 cells and 100 infected cells were mixed with 2 ml of soft agar (agar 0.36%) containing casein and distributed on plates previously prepared with 4 ml of normal nutrient agar medium. These plates were

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usually fed with soft agar medium without casein after 1 week.

Cytotoxic plaque assay. Media and conditions for the plaque assay of transformation defective viruses were as described by Graf (3).

RESULTS

Caseinolysis as an infectivity assay. In order to investigate the potential value of the caseinolytic assay as an infectivity test for RSV, secondary cultures of chick fibroblasts were infected with serial dilutions of morph^r or morph^f variants of the Bryan strain of RSV (BH-RSV) of antigenic subgroup A, with PR and with SR virus also of subgroup A. After adsorption, half of the plates were overlaid with casein agar medium as described in Materials and Methods. Control cultures for conventional focus-forming assays were overlaid with 7 ml of agar medium without casein. Plates were incubated either at 36 C or at 41 C.

Within 3 to 5 days, distinct plaques of caseinolysis appeared in plates infected with PR and SR virus, and in plates infected with the morph^r variant of BH-RSV, but not in plates infected with the morph^f variant of the same virus strain. Cells in plates with casein, as well as those in plates without casein, showed no sign of morphological conversion at this time. Within the next few days, however, foci of morphologically transformed cells appeared in plates with or without casein. Table 1 reports the number of foci and plaques observed in an experiment 8 days after infection. In this, as in other experiments, the number of foci in plates without casein was 10 to 15% higher than in plates with casein. Except for morph^f-infected cultures there was a high degree of coincidence of

plaques and foci (Fig. 1). Rare exceptions are noted in the footnote of Table 1. On the other hand, in plates infected with morph^f virus and incubated at 36 C, only about 70% of the foci produced faint caseinolytic plaques, which were much smaller than those produced by foci of cells transformed by the other strains. At 41 C, there was a reduction of foci, all of which developed faint caseinolytic plaques. This in contrast to the other viruses where lytic plaques were larger at 41 C than at 36 C, suggesting that some factor involved in the production of plaques is enhanced by the higher temperature. These experiments also show that RAV-1, the nontransforming "helper" virus present in BH-RSV stocks, does not cause proteolysis. Otherwise a larger excess of caseinolytic plaques would have been observed in plates infected with this virus. On the other hand, in plates infected with BH-RSV or PR virus, occasional well-developed foci without caseinolytic plaques were observed. On the assumption that this character could be due to virus mutations, these "non-caseinolytic" foci were isolated. The cells from individual foci were suspended in a small volume, frozen and thawed once and tested for ability to induce caseinolytic plaques and/or morphological conversion in new cultures. Out of three foci isolated from BH-RSV, two produced wild-type virus able to cause caseinolysis and morphological conversion. One was negative for focus formation and caseinolytic plaques. As foci of cells producing noninfectious virus are occasionally found in plates infected with high dilutions of this virus strain, it is possible that the noninfectious suspension was derived from one such focus. Out of two foci isolated from PR virus-infected plates, one was positive for both characters and one showed partial morphological conversion without caseinolysis. In further purification attempts, however, this isolate also produced foci of transformed cells with caseinolytic plaques similar to those observed with wild-type virus. The reasons for this behavior are not known. Possibly cellular factors could have been responsible for the partial expression of transformation in the earlier tests.

Control of caseinolysis. In an attempt to investigate the role of viral versus cellular factors in the expression of proteolytic activity, experiments were carried out with four temperature-sensitive (ts) mutants of PR virus subgroup A (PRA #23, 24, 25, and 29) and with eight ts mutants isolated from BH-RSV, three of them derived from mutagenized stocks of the morph^f variant.

All ts mutants were tested at 36 C and 41 C.

TABLE 1. Caseinolytic assay of wild-type RSV strains^a

Strain	36 C			41 C		
	No casein		Casein ^b	No casein		Casein ^b
	Foci	Foci	Plaques	Foci	Foci	Plaques
BH morph ^r	123	104	103	137	103	108
BH morph ^f	125	111	70 ^c	77 ^d	60 ^d	60 ^c
PRA	78	65	65	70	61	64
SRA	73	50	43	ND ^e	ND	ND

^a Each result is the average number of foci or plaques in duplicate 60-mm plates infected with 0.2 ml of a 10⁻³ dilution of each virus.

^b At 36 C all plaques coincided with foci. At 41 C a few plaques without foci were present.

^c Very small faint plaques.

^d At 41 C morph^f foci are poorly discernible.

^e ND, Not done.

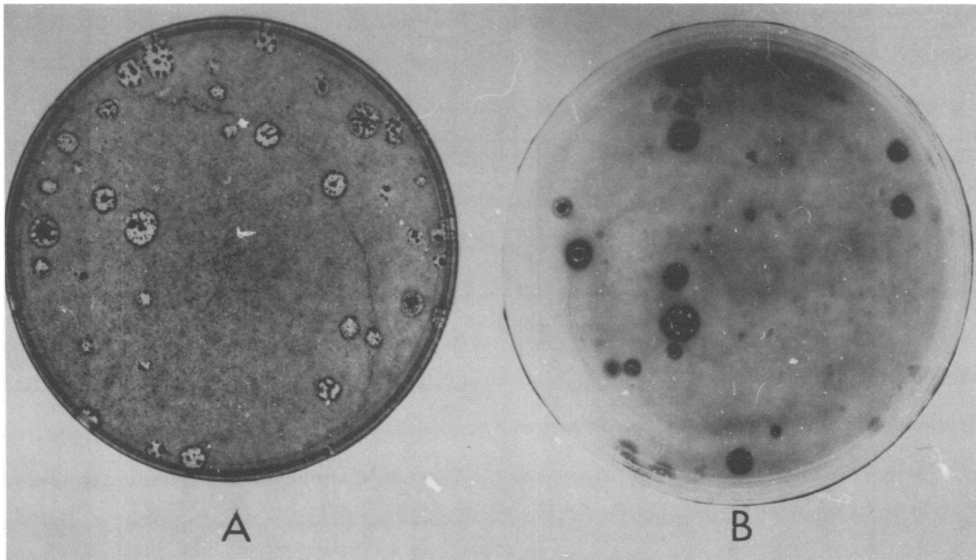


FIG. 1. Plates infected with BH RSV (RAV-1) morph^r and overlaid: A, with agar nutrient medium; B, with casein-containing agar nutrient medium. Plate A received neutral red agar on day 8 postinfection. Pictures were taken the following day.

At 36 C the number of foci caused by the PR ts mutants in plates with or without casein was roughly equal, but several caseinolytic plaques without foci could be observed at this temperature (Table 2). The frequency of these plaques varied between 10 to 30% of all plaques in different experiments. Cells from several such areas of caseinolysis without transformation were isolated in small volumes of medium, frozen, thawed, and tested in new cultures. All suspensions tested produced a 10 to 30% excess of caseinolytic areas over foci of transformed cells as the original ts mutants. This suggests that at 36 C a number of infectious particles fail to cause morphological conversion while still inducing caseinolysis by the infected cells.

At the nonpermissive temperature both focus formation and plaque formation were inhibited by more than 90% (note the difference of inocula at the two temperatures). However, in three out of four cases production of caseinolytic areas was reduced less than was focus formation. For mutant #24, the number of residual caseinolytic plaques was approximately equal to the number of foci. These observations suggest that different ts mutants have different temperature sensitivity for each of the two parameters of transformation considered here, i.e., proteolytic activity and morphological conversion, and that the former property is often more temperature resistant than focus formation.

With the ts mutants of BH-RSV at 36 C all morph^r mutants produced both foci and casein-

olytic plaques with a slight excess of plaques. The three morph^r mutants produced well-developed foci of typically transformed cells with minimal or no caseinolysis. At 41 C both foci and caseinolytic plaques were strongly suppressed in all ts mutants with the exception of ts 38, a morph^r mutant which produced very faint caseinolytic areas and no visible foci. The BH-RSV mutants were also tested in soft agar. For this assay some plates were prepared with casein medium at the time the cells were suspended in soft agar. These plates were incubated either at 36 C or at 41 C. To other plates casein medium was added 24 h after a shift in temperature from 36 to 41 C. The temperature shift was made after visible colonies had already appeared. In soft agar plates at 36 C the five morph^r mutants produced compact, spherical colonies surrounded by caseinolytic areas. The morph^r mutants produced dispersed, fluffy colonies of which about one-third showed faint caseinolytic areas. In plates incubated at the nonpermissive temperature both colony formation and caseinolysis were strongly inhibited. Finally, as it would be expected from the reported temperature lability of the plasminogen activator (6), little or no caseinolysis was observed in the temperature shift experiment. Taken all together these experiments indicate that the continuous expression of virus genes is required for both growth in soft agar and caseinolysis.

As the caseinolytic activity of ts mutant-infected, nontransformed cells seems to repre-

TABLE 2. Caseinolytic assay of PRA ts mutants^a

Strain	36 C				41 C			
	No casein		Casein ^b		No casein		Casein ^b	
	Foci	Foci	Plaques	Foci/plaques ^c	Foci	Foci	Plaques	Foci/plaques
PRLA #23 A	38	39	49	0.80	5	3	17	0.17
PRLA #24 A	37	22	33	0.67	5	14	16	0.88
PRLA #25 A	25	32	36	0.89	0	0	22	0.05
PRLA #29 A	32	41	62	0.66	2	5	44	0.11

^a Plates incubated at 36 C were infected as in Table 1. Plates incubated at 41 C received 10-fold more virus. Each result is the average of duplicate plates.

^b In plates with casein each focus coincided with a plaque.

^c Calculated from plates with casein in each case.

sent a partial expression of transformation with a lower temperature sensitivity than morphological conversion, the possibility was considered that these two parameters of transformation could be separately controlled by the transforming information of avian sarcoma viruses. In an attempt to clarify this point, experiments were carried out to investigate whether td derivatives of avian sarcoma viruses would retain the ability to induce caseinolysis. Sets of plates were infected with appropriate dilutions of a stock of PRB virus, known to contain a large amount of td virus or with a td virus isolated from this stock, or with a stock of Carr Zilber associated virus a leukosis virus of subgroup D. After adsorption, duplicate plates of each set were overlaid with casein-containing agar medium or with medium which reveals areas of cell lysis upon infection with td of subgroups B and D.

Plates containing casein medium were observed at 7 and 13 days and the number of caseinolytic plaques with foci of transformed cells, plaques without foci, and foci without plaques were recorded. Areas of cell lysis in the cytotoxic plaque assay and foci appearing on these plates were counted at day 13. The results of such an experiment (Table 3) show that for PRB virus caseinolytic plaques generally appear before foci, but by day 13 all foci of transformed cells are surrounded by areas of caseinolysis. Parallel plates overlaid with medium used in the cytotoxic assay showed that the number of cytolytic plaques in this experiment was about 10-fold higher than the number of foci. The plates infected with several dilutions of the td derivative isolated from PRB and those infected with Carr Zilber associated virus showed no caseinolytic plaques while the titer of cytotoxic plaques was 6×10^7 and 3×10^7 for the td from

TABLE 3. Caseinolytic and cytotoxic assay of PRB RSV^a

Day	Caseinolytic assay		Cytotoxic assay		
	Plaques	Plaques + Foci	Foci	Plaques	Foci
7	34	24	1	ND ^b	ND
13	0	64	0	620	52

^a Plates were infected with appropriate dilutions of PRB RSV and overlaid with casein medium or with medium for the cytotoxic assay. The results are the sum of counts on triplicate plates.

^b ND, Not done.

PRB and Carr Zilber associated virus, respectively.

DISCUSSION

These results show a very high correlation between morphological conversion and the proteolytic activity of virus-infected cells. Neither viruses which naturally do not transform cells in vitro (leukosis viruses) nor td derivatives of sarcoma viruses (ts mutants of the PR strain or of the replication-defective BH-RSV strain, and td variants of PRB) induce caseinolytic activity. This correlation is unaffected by the group specificity (A, B, or D) of the virus.

The caseinolytic plaque assay could be used as an infectivity assay for RSV and possibly for other avian viruses able to transform cells in vitro, such as the avian myeloblastosis virus (C. Moscovici, L. Gazzolo, and M. G. Moscovici, personal communication), although it offers little or no advantage over the conventional focus assay. In the case of ts mutants, however, the caseinolytic assay revealed infectious virus unable to cause clear morphological con-

version. It seems that the assay could be useful for agents that naturally do not produce easily detectable foci. Both in the case of BH-RSV morph^f and in three of the four ts mutants of PRA RSV morphological conversion seems to be more temperature labile than caseinolysis. In the other ts mutant, however, both activities were approximately equally reduced at 41 C. Even in this case both activities were markedly reduced by the mutation.

Occasionally, at 36 C, foci were found which were not accompanied by caseinolytic plaques, but when virus was isolated from such transformed foci it had the property of inducing caseinolysis. Taken all together, the simplest interpretation of these results is that a single virus gene is responsible for both morphological conversion and caseinolytic activity as well as for the ability to grow in soft agar. The expression of these characters, however, is altered to a somewhat different extent by temperature, and also perhaps by undefined host cell functions.

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