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Virus Envelope Markers in Mammalian Tropism of Avian RNA Tumor Viruses

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Pseudotypes of vesicular stomatitis virus were prepared with avian sarcoma viruses and avian leukemia viruses representing five different subgroups. These pseudotypes display a host range restricted to that of the avian tumor virus when assayed on avian cells and are neutralized by subgroup-specific antisera. The efficiency of penetration of mammalian cells was assayed by using these vesicular stomatitis virus pseudotypes. Pseudotypes of avian tumor viruses belonging to subgroup D and of B77 virus were able to plate on mammalian cells with a high efficiency, whereas pseudotypes of other strains were not. The efficiency of penetration of the vesicular stomatitis virus pseudotypes was 10^2- to 10^3-fold higher than the efficiency of transformation of the corresponding avian tumor virus strain assayed on mammalian cells, suggesting that there are postpenetration blocks to the expression of transformation in these cells.

Mammalian cells derived from a variety of different species have been infected and transformed by several strains of avian RNA tumor viruses (12). Typically, the frequency of transformation of mammalian cells relative to avian cells by the avian RNA tumor viruses is extremely low. It has been demonstrated that the viral envelope is one of the important factors affecting the ability of a particular virus strain to produce tumors in mammalian hosts (4, 5). However, other stages in the virus replication and transformation cycle may also be important (2, 3).

The demonstration that vesicular stomatitis virus (VSV) is capable of phenotypic mixing with avian RNA tumor viruses (21) suggests a method for separating events in the virus replication cycle that are determined by viral envelope properties from events that involve other viral elements. Phenotypically mixed virions, or pseudotypes, can be prepared by selective neutralization or heat inactivation in which only the envelope properties of the avian RNA tumor virus are expressed (10, 21, 22). These pseudotypes may be used to assay the ability of the virus strain which provides the envelope to penetrate various cell types, providing the genome portion of the pseudotype is capable of replicating in that cell type. Since the envelope components of the pseudotype not only provide the attachment function but also allow the genome to penetrate, this system provides a functional definition of penetration. A simple assay for VSV pseudotypes has been developed by Zavada (22) by using the thermolabile VSV mutant tl-17. This mutant is in complementation group V, thought to represent the viral glycoprotein (23). Its infectivity can be inactivated by a factor of 10^{-6} to 10^{-7} by heating at 45 C for 60 min (10, 22). When the mutant is grown in cells producing avian RNA tumor viruses, the thermolabile lesion is phenotypically complemented by the avian RNA tumor virus envelope proteins. The level of this phenotypic complementation can be measured by determining the virus titer before and after heat inactivation. The apparent complementation observed is not genetic, since it is lost in further cycles of viral replication.

We have exploited the phenotypic mixing between avian tumor viruses and VSV mutant tl-17 to assay the susceptibility of mammalian cells to penetration by various strains of avian tumor virus. This system is advantageous because: (i) chicken cells are available which are specifically resistant to penetration by one subgroup of avian tumor virus but are sensitive to other subgroups (11), (ii) VSV grows equally well in avian and mammalian cells, and (iii) some strains of avian tumor virus are more tumorigenic in mammals than are other strains.

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MATERIALS AND METHODS

All experiments involving VSV or phenotypically mixed stocks were conducted under conditions recommended for handling potentially dangerous organisms and were contained within a designated virus isolation laboratory.

Cells and culture methods. The growth of cells, VSV, VSV avian tumor virus pseudotypes, and assay conditions have been described previously (10). Focus assays of Rous sarcoma virus (RSV) and avian sarcoma virus B77 in chicken and normal rat kidney (NRK) cells have been described previously (3). The presence of chick helper factor (chf) was tested by the method of Weiss et al. (19).

Brown Leghorn C/E (chicken cells resistant to subgroup E), Reaseheath C line (chf-negative C/AE chicken cells resistant to subgroups A and E), and Japanese quail (*Coturnix coturnix*) embryos were provided by Houghton Poultry Research Station, Houghton, Huntingdonshire, England. Chf-negative C/E cells were obtained from SPAFAS Inc., Norwich, Connecticut. C/B chf-negative chicken cells were a gift from R. R. Friis. Golden pheasant (*Crysolophis pictus*) embryos were kindly provided by the London Zoological Society's Gardens at Whipsnade, Bedfordshire, England.

NRK cells (normal rat kidney cell line) were obtained from J. Wyke, BHK-21/C13 cells (baby hamster kidney cell line) from I. A. Macpherson, and SIRC cells (rabbit corneal cell line) from the American Type Culture Collection, Rockville, Md.

Viruses. The VSV mutant tl-17 was kindly provided by J. Zavada; after recloning, a highly thermolabile stock was prepared (10). SR-RSV-H was kindly provided by T. Graf. RBA virus was rescued from R(B77) cells obtained from C. Altaner (2). All other stocks of avian RNA tumor viruses were derived from viruses routinely used in this laboratory and previously in P. K. Vogt's laboratory. Nondefective sarcoma virus stocks were prepared from recently cloned viruses.

Antisera. Antisera to RAV-1 (subgroup A), RAV-2 (subgroup B) and RAV-49 (subgroup C) were prepared in Brown Leghorn chickens (19). Chicken antiserum to SR-RSV-D (subgroup D) was kindly provided by P. K. Vogt. Neutralization assays were carried out as described previously (10).

RESULTS

Assay of VSV pseudotypes on avian and mammalian cells. VSV pseudotypes (phenotypically complemented VSV) of a variety of nontransforming avian RNA tumor viruses (Table 1) and several transforming avian RNA tumor viruses (Table 2) were prepared. The level of pseudotypes in each stock was determined by plaque titration on avian cells that are susceptible to the particular strain of avian RNA tumor virus used, both before and after heat inactivation for 60 min at 45 C. In most cases, stocks have been obtained in which the surviving fraction after heating was 3×10^{-2} to 3×10^{-3} relative to the nonheated control. VSV stocks grown in parallel uninfected cultures gave a surviving fraction of $< 3 \times 10^{-5}$. Pseudotypes formed with RAV-50 exhibited about 100-fold lower level of phenotypic complementation. This may represent a structural difference between RAV-50 envelope proteins and those from the other strains tested in terms of their ability to assemble on VSV.

Table 1 represents a composite of several experiments. In some of the early experiments, heat inactivation of the control VSV was incomplete, and an abnormally high heat-resistant fraction was observed. Subsequently, it was found that the heat inactivation was inefficient at low pH (6.8 to 7.0), but nearly complete inactivation could be obtained by adjusting the pH to about 8.0 with bicarbonate before heating.

The pseudotype stocks were also assayed on NRK cells which can be transformed only at a low efficiency by avian RNA tumor viruses. For each virus stock, the assay on avian and NRK cells was done in the same experiment. The sensitivity of the NRK cells to VSV was slightly less than the sensitivity of chicken cells as judged by the t_0 (unheated) virus titer (Table 1). After heat inactivation (t_{60}), only viruses from subgroup D retained any significant level of infectivity. This observation is consistent with those of Hanafusa and Hanafusa (5), who found that only subgroup D viruses produced tumors in hamsters.

Love and Weiss (10) have shown that VSV is capable of forming pseudotypes with the endogenous avian RNA tumor virus glycoprotein in cells positive for chicken helper factor (chf^+ cells) (17, 20) even though no endogenous virus particles are produced. To avoid any contribution of chf to the VSV pseudotypes, they were prepared in chf^- cells (Spafas or Reaseheath C line chicken cells, or golden pheasant cells). However, control VSV grown in uninfected chf^- cells showed some residual plaques after heat inactivation when assayed on chicken cells but none when assayed on NRK cells (Table 1). This discrepancy between the surviving fraction on chicken cells and NRK cells could possibly result from some unknown complementation by the avian cells in which the VSV stock was grown; alternatively, it could result from a differential susceptibility of the chicken and NRK cells to heat-damaged virions. In either case, the lack of a surviving fraction of pure grown VSV when assayed on NRK cells after heat inactivation provides a greater level of

TABLE 1. *Assay of leukosis virus pseudotypes of VSV on avian and mammalian cells*

Virus determining pseudotype			PFU/ml				Penetration ratio[a] [(NRK/chick) $\log_{10}$]
Subgroup	Strain	Host cell	Chicken cells		NRK cells		
			t_0[b]	t_{60}[b]	t_0	t_{60}	
A	RAV-1	GPh[c]	7.3×10^6	5.6×10^4	2.5×10^6	2×10^0	−4.0
	RAV-5	GPh	4.6×10^7	5.3×10^5	NA[d]	NA	
B	RAV-2	GPh	1.0×10^8	7.6×10^5	7.2×10^7	2×10^1	−4.4
	RAV-2	SP[e]	2.1×10^7	7.0×10^4	2.9×10^6	3×10^1	−3.5
	MAV-2	GPh	7.2×10^7	7.7×10^5	3.1×10^7	3×10^0	−5.1
	MAV-2	SP	3.2×10^6	2.0×10^3	5.2×10^5	$<3 \times 10^0$	<-2.0
C	RAV-49	GPh	2.0×10^8	2.8×10^6	8.1×10^7	$<3 \times 10^0$	<-5.7
	RAV-49	SP	7.7×10^7	4.8×10^4	3.5×10^7	$<2 \times 10^2$	<-2.0
	NT-B77	GPh	6.2×10^7	2.6×10^6	2.8×10^7	3.0×10^1	−4.4
	NT-B77	SP	8.0×10^7	3.2×10^5	2.1×10^7	$<3 \times 10^0$	<-4.5
	RAV-7	SP	1.3×10^8	9.0×10^5	2.8×10^7	$<3 \times 10^0$	<-4.8
D	CZAV	GPh	1.2×10^8	4.2×10^6	6.4×10^6	3.6×10^3	−1.8
	CZAV	SP	9.0×10^7	3.4×10^5	1.1×10^7	2.9×10^3	−1.2
	RAV-50	GPh	4.9×10^7	7.4×10^3	6.5×10^6	6×10^1	−1.2
E	RAV-0	GPh	4.6×10^7[f]	1.6×10^6[f]	3.5×10^6	$<3 \times 10^0$	<-4.6
Control		GPh	8.0×10^7	2.3×10^3	8.3×10^6	$<3 \times 10^0$	<-6.9
		SP	2.6×10^7	9.0×10^2	4.2×10^6	$<3 \times 10^0$	<-5.1

[a] Penetration ratio is a measure of the relative complementation on chicken cells (t_{60}/t_0) to the relative complementation on rat cells (t_{60}/t_0) for the particular pseudotype.
[b] t_0, Unheated pseudotype stock; t_{60}, pseudotype stock heated at 45 C for 60 min. PFU per milliliter.
[c] GPh, Golden pheasant.
[d] NA, Not assayed.
[e] SP, SPAFAS Chf(−) chicken.
[f] Assay on quail.

TABLE 2. *Assay of sarcoma virus pseudotypes of VSV on avian and mammalian cells*[a]

Virus determining pseudotype			PFU/ml				Penetration ratio [(NRK/chick) $\log_{10}$]
Subgroup	Strain	Host cell	Chicken cells		NRK cells		
			t_0	t_{60}	t_0	t_{60}	
A	PR-A[b]	SP	3.4×10^6	1.1×10^4	2.3×10^6	$<3 \times 10^0$	<-3.4
C	B77[c]	RC[d]	1.5×10^8	1.4×10^6	9.0×10^6	7.0×10^4	−0.1
	RBA[e]	SP	1.0×10^8	6.8×10^4	9.0×10^6	7.0×10^4	−1.5
D	SR-H[f]	SP	6.7×10^6	1.1×10^4	4.8×10^6	2.0×10^3	−1.6

[a] Symbols as in Table 1.
[b] PR, Prague strain RSV.
[c] B77, Chicken passage of B77 virus derived from virus produced.
[d] RC, Reaseheath C line chicken.
[e] RBA, B77 virus rescued from rat cells (2).
[f] SR-H, Schmidt-Ruppin strain virus from hamster tumor.

significance to the pseudotype stocks that do plate on NRK cells.

Specificity of pseudotypes. Since the pseudotypes were being used to determine the sensitivity of mammalian cells to penetration by various strains and subgroups of avian RNA tumor viruses, it was necessary to demonstrate that the specificity of the particular pseudotype

being tested is the same as its avian tumor virus parent. Three methods have been employed to classify avian RNA tumor viruses with respect to the viral envelope: (i) plating of the virus on chicken embryo cells which are resistant to certain virus subgroups (15), (ii) interference between viruses of the same subgroup (16), and (iii) cross-neutralization by certain antisera (4).

Table 3 shows the assay of selected pseudotypes on various sensitive and resistant cell types. In each case, the same pseudotype stock was assayed on both sensitive and specifically resistant cells in the same experiment. All cell types resistant to one subgroup were also tested by a pseudotype belonging to another subgroup (compare VSV[CZAV] on C/A and C/E; VSV[RAV-49] on C/B and C/E[CZAV]) to check the specificity of the resistance. The resistant cells were either pretested for genetic resistance with avian sarcoma viruses, as for C/A, C/B, and C/E cell types, or constructed by infection of C/E cells with a nontransforming avian tumor virus and passaging twice to obtain interfering levels of virus production, as for C/E(RAV-2), C/E(RAV-49), and C/E(CZAV). The t_{60}/t_0 ratios demonstrate a 10^2 to 10^3 greater efficiency of plating of the pseudotypes on sensitive cells than on specifically resistant cells.

Table 4 shows the neutralization of heat-inactivated pseudotypes by antisera to viruses from subgroups A, B, C, and D. The same pseudotype stocks were used as in the assays on resistant cells (Table 3). The controls included neutralization of pseudotype stocks by antisera to different subgroups, i.e., anti SR-D serum for subgroups A and C and anti-RAV-49 for subgroups B and D. Subgroups A and B pseudotypes showed complete neutralization by antisera specific to viruses in those subgroups, but

TABLE 3. *Specificity of VSV pseudotypes: assay on resistant chicken cells*

Subgroup	Pseudotype	Assay cells[a]	t_0[b]	t_{60}[b]	Penetration ratio (t_{60}/t_0) $\log_{10}$
A	RAV-1	C/E	8×10^6	2×10^4	−2.6
		C/A	7.2×10^6	1.0×10^1	−5.8
	PR-A	C/E	3.4×10^6	1.1×10^4	−2.5
		C/A	2.4×10^6	$<3 \times 10^1$	<-5.9
B	RAV-2	C/E	3.9×10^7	8.5×10^4	−2.7
		C/E(RAV-2)	4.8×10^7	4.0×10^1	−6.1
		C/B	4.7×10^7	3.3×10^3	−4.2
	MAV-2	C/E	1.8×10^7	4.0×10^3	−3.7
		C/E(RAV-2)	2.5×10^7	3.0×10^0	−6.9
		C/B	3.3×10^7	9.0×10^1	−5.6
C	RAV-49	C/E	6.7×10^7	4.2×10^5	−2.2
		C/E(CZAV)	5.0×10^7	5.0×10^4	−3.0
		C/B	1.0×10^8	3.0×10^5	−2.5
		C/E(RAV-49)	4.0×10^7	2.1×10^2	−5.3
	B77	C/E	1.2×10^8	7.0×10^5	−2.2
		C/E(RAV-49)	5.4×10^7	8.0×10^2	−4.8
		C/E(CZAV)	5.0×10^7	5.0×10^3	−4.0
D	CZAV	C/E	6.3×10^7	1.2×10^6	−1.7
		C/A	1.3×10^8	4.3×10^6	−1.5
		C/E(RAV-49)	5.3×10^7	7.5×10^5	−1.9
		C/E(CZAV)	5.0×10^7	4.5×10^3	−4.1
	SR-H	C/E	1.3×10^7	5.4×10^4	−2.4
		C/E(CZAV)	3.5×10^6	3.5×10^2	−4.0
E	RAV-0	Quail	4.6×10^6	1.6×10^5	−1.5
		C/E	4.6×10^6	$<3 \times 10^0$	<-6.2

[a] Leukosis virus in parentheses indicates resistance of host cell as a result of viral interference. (Symbols as in Table 1.)

[b] PFU per milliliter.

were not neutralized by heterologous antisera. The subgroup D viruses showed some cross-neutralization by anti-B antiserum as reported by others (4, 18). For the subgroup C and D pseudotypes which plated on NRK cells, a similar degree of neutralization was observed whether the virus was assayed on chicken or NRK cells. Thus, similar viral host range determinants appear to be involved in plating on both avian and mammalian cells.

An unexpected observation was that the B77 pseudotype was not only neutralized by anti-serum specific to subgroup C but also by antiserum to subgroup D (Table 4). Neutralization of VSV(B77) by anti-SR-D serum was highly significant when assayed on NRK cells, but was of dubious significance when assayed on chicken cells. This result suggests that there may be a common antigenic determinant between virus strains of different subgroups which exhibit a high efficiency of penetration into mammalian cells.

TABLE 4. *Specificity of VSV pseudotypes: antiserum neutralization*[a]

Subgroup	Pseudotype	Antiserum	Log_{10} reduction in titer	
			Chicken cells	NRK cells
A	RAV-1	anti-RAV-1	2.66[b]	
	PR-A	anti-RAV-1	3.38[b]	
	RAV-1	anti-SR-D	0.68	
B	RAV-2	anti-RAV-2	2.77[c]	
		anti-RAV-49	−0.23	
	MAV-2	anti-RAV-2	3.19[b]	
C	B77	anti-RAV-49	3.48[b]	4.24[b]
		anti-SR-D	0.48	1.77
D	CZAV	anti-RAV-2	1.24	1.08
		anti-RAV-49	−0.23	0.08
		anti-D	1.85	1.96
	SR-H	anti-RAV-2	0.66	
		anti-RAV-49	−0.23	
		anti-SR-D	1.70	2.08

[a] Symbols as in Table 1.

[b] Complete neutralization; surviving fraction same level as control.

Sensitivity of different mammalian cells to avian tumor viruses. Since VSV pseudotypes have the same host range on sensitive and resistant avian cells as the avian tumor virus used to construct the pseudotype, the sensitivity of different mammalian cell types to penetration by various strains of avian tumor virus may be assayed with these pseudotypes. Table 5 shows the plating efficiency of the VSV pseudotypes on several mammalian cell types relative to their plating efficiency on chicken cells. To determine the plating efficiencies, the level of complementation of the thermolabile defect (t_{60}/t_0) was determined for each mammalian cell type and for chicken cells. The relative efficiency of penetration is the ratio of the level of complementation on mammalian cells to the level of complementation on chicken cells. This parameter measures the ratio of the plating for the pseudotype corrected for variations in the plating efficiency of pure grown VSV on the different cell types. All pseudotypes show lower efficiencies of penetration of mammalian cells than of chicken cells, though for B77 assayed on rat cells the difference in efficiency of penetration is very small. Avian tumor virus strains which are capable of penetration of one type of mammalian cell appear to be capable of penetrating all the types which were tested. Only B77 virus, RBA virus and subgroup D viruses

TABLE 5. *Relative efficiencies of penetration of VSV pseudotypes on various mammalian cells relative to chicken cells*

Virus subgroup	Virus strain	Relative penetration efficiency[a]					
		NRK rat	Wistar rat embryo	BALB/c mouse embryo	BHK-21/C13 hamster	SIRC rabbit	HeLa human
B	RAV-2	−3.66	−3.57	−3.74[b]	−3.44[b]	−3.89[b]	NT[c]
C	RAV-49	−5.40[b]	NT	−2.66[b]	−2.37[b]	−2.70[b]	−3.15[b]
C	B77	−0.13	NT	−0.77	−1.57	−1.11	−0.68
D	CZAV	−1.77	−1.80	−1.96	−2.31	−2.22	NT

[a] The figures represent log_{10} complementation on mammalian cell (t_{60}/t_0) divided by complementation on chick cell t_{60}/t_0.

[b] Residual plating of control nonpseudotype VSV is not significantly different, i.e., complete resistance of mammalian cell to penetration.

[c] NT, Not tested.

showed significant ability to penetrate mammalian cells.

Efficiency of transformation of NRK cells by avian sarcoma viruses. It has been demonstrated that several strains of avian sarcoma virus are capable of penetrating mammalian cells with a high efficiency (Tables 2 and 5). To compare the efficiencies of transformation of mammalian cells by various strains of avian sarcoma virus, focus assays were carried out on NRK cells and chicken cells (Table 6). There is a good correlation between the ability to penetrate the mammalian cell and the ability to transform NRK cells. PR-RSV-A penetrates and transforms with a relatively low efficiency, whereas B77 and SR-RSV-H penetrate and transform with a relatively high efficiency.

If the relative efficiencies of transformation on NRK cells compared to chicken cells are corrected for penetration (from the values in Table 2), a transformation/penetration ratio can be obtained. In the transformation assay, only one transformed cell was found per 10^2 to 10^3 focus-forming virus particles expected to have penetrated the NRK cells. Since all values are measured in NRK cells relative to the corresponding assay in chicken cells, the efficiency of transformation of NRK cells relative to the number of incoming focus-forming virus particles is much lower than for chicken cells. This implies that some postpenetration events in the viral transformation cycle may be blocked or occur only at a low efficiency in NRK cells.

DISCUSSION

Application of phenotypic mixing between VSV and the avian tumor viruses has demonstrated that VSV can form pseudotypes with a variety of different avian tumor virus strains and that the pseudotypes exhibit the restricted host range of the particular avian tumor virus (10, 19). Assay of the pseudotypes on mammalian cells revealed that only certain viruses, B77 and subgroup D viruses, are capable of penetrating mammalian cells. There findings agree with previously published work (4, 5) that the viral envelope plays a major role in determining whether or not a particular strain of virus will be capable of infecting and transforming a mammalian cell.

The strains of avian tumor virus which exhibit a high efficiency of penetration of mammalian cells may all be derived from strains which have been adapted to mammalian cells by passage through a mammalian host. The D subgroup viruses have all arisen in or been selected from virus stocks originated and maintained in laboratories in which the virus has been used to transform mammalian cells (4). It is difficult to determine whether they all actually arose after passage through mammalian cells, but this would appear to be a likely possibility since no subgroup D field isolates have been reported. The B77 stock (from P. K. Vogt's laboratory) used in the experiments described here was derived from a chicken cell passage of virus released by RB1 rat tumor (4, 7; Altaner, personal communication). The VSV pseudotypes of both this virus and the RBA stock of B77 (recently rescued from rat cells [2] by fusion with chick cells) penetrate NRK cells with a high efficiency. It is remarkable, therefore, that NT-B77, a nontransforming derivative of Vogt's B77 stock (14), appears to have lost the rat-tropic envelope marker in addition to the loss of transforming genes. It should be illuminating to examine the subgroup specificity and tropism for mammalian cells of the original isolate of avian sarcoma virus B77 (13).

Since the assay utilizing phenotypically mixed particles provides a means of measuring penetration, it can also be used to determine if the penetration is the major block to viral replication or if there are additional postpenetration blocks to virus replication or expression. This system has been utilized in the analysis of genetic resistance to murine leukemia virus governed by the *Fv-1* locus (8, 9); *Fv-1* resistance did not render the cells resistant to the VSV(murine leukemia virus) pseudotypes. Similarly, the penetration of mammalian cells by avian RNA tumor viruses is much more efficient than the transformation of the mammalian

TABLE 6. *Efficiency of transformation of chicken and NRK cells by avian sarcoma viruses*

Virus subgroup	Virus strain	Titer[a]		Transformation: NRK/Chicken	Transformation/ penetration[b]
		Chicken	NRK		
A	PR-A	2.0×10^6	$<5 \times 10^0$	$<2.5 \times 10^{-6}$	$<6 \times 10^{-3}$
C	B77	2.7×10^6	4.3×10^3	1.6×10^{-3}	2.0×10^{-3}
D	SR-H	2.2×10^6	2.3×10^3	1.0×10^{-3}	1.4×10^{-2}

[a] PFU per milliliter.

[b] NRK/chick transformation ratio divided by NRK/chicken penetration ratio (from Table 2).

cells, suggesting the presence of additional blocks to viral expression. Recently, it has been found that the infection of NRK cells by B77 virus produces a large proportion of cells which contain the full virus genome in a stable state but which are not transformed (3). These stably infected, nontransformed cells resemble revertants of BHK cells transformed by SR-RSV (D. Boettiger, Virology, in press), which contain very low concentrations of virus-specific RNA (C. Deng et al., Virology, in press). These observations suggest that an additional block to virus replication or to cell transformation occurs at the level of viral RNA synthesis or processing.

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