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Intracellular Restriction on the Growth of Induced Subgroup E Avian Type C Viruses in Chicken Cells

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Subgroup E avian type C viruses produced by bromodeoxyuridine-treated 100 × 7, line 7, or line C chicken cells were restricted in their intracellular growth on K28 chicken cells but not on line 15 chicken cells. Cells from embryos of line 15 chickens bred with K28 chickens did not restrict the growth of the subgroup E induced leukosis viruses (ILVs). This result indicates that the phenotype for the intracellular restriction of the growth of subgroup E ILVs found in K28 cells is recessive. Long-term growth of the subgroup E ILVs in K28 cells resulted in the appearance of subgroup E virus that grew well on K28 cells. No change in growth characteristics was observed for subgroup E ILVs grown in line 15 cells, indicating that appearance of nonrestricted virus occurred only during growth of the subgroup E ILVs on a restrictive host. RAV-0, a subgroup E virus closely related to the ILVs, had the same growth characteristics as the subgroup E ILVs. RAV-60, a subgroup E virus formed by recombination of exogenous avian leukosis virus with endogenous subgroup E virus coat information, grew well on both line 15 and K28 cells.

Three groups of subgroup E virus have been isolated from chicken cells. The first group, referred to as RAV-60s, can be isolated from stocks of avian leukosis virus (ALV) that have been grown in chicken cells (8). RAV-60s are thought to arise from the recombination of genetic information of an infecting ALV with chicken cell information for type-specific E coat antigen (5, 6) to give a subgroup E ALV. The second group of subgroup E viruses, named RAV-0, is found at high titer in the culture medium of chicken embryo fibroblasts (CEF) that are related to U.S. Department of Agriculture line 7 chickens and are susceptible to subgroup E virus (2, 17, 22). RAV-0 is thought to arise by the spontaneous production of infectious subgroup E virus by line 7 genetic information and the subsequent horizontal spread of this virus in surrounding susceptible cells. The third group of subgroup E viruses is referred to as induced leukosis virus (ILV) and has been isolated from the culture medium of subgroup E virus-resistant cells related to line 7 after growth of these cells in bromodeoxyuridine (BUdR) (17).

RAV-60s grow to high titer in chicken cells, whereas RAV-0 has been reported to grow poorly in chicken cells (6, 22). Recently we observed that an ILV of subgroup E grew in line 15 but not K28 chicken cells and that RAV-0 grew more rapidly in line 15 than in K28 cells (17).

The current work describes the growth of RAV-60, RAV-0, and subgroup E ILVs in CEF of K28, line 15, and crosses of K28 and line 15 chickens.

MATERIALS AND METHODS

Cells. Cells were typed for their susceptibility to subgroup A, B, C, D, and E pseudotypes of Bryan rous sarcoma virus (B-RSV), the presence of the group-specific antigen of ALV, and the expression of the type-specific antigen of endogenous subgroup E ALV. Chicken cells that were resistant to a subgroup or subgroups of avian sarcoma virus (ASV) are designated as C/A, -B, -C, -D, or -E, with the letter or letters following the bar indicating the excluded subgroups of virus. Cells that were susceptible to all subgroups are designated as C/0. Turkey and quail cells follow a similar nomenclature, with T (turkey) or Q (quail) substituted for C (chicken). Cells that contained the group-specific antigen of ALV are termed gs^+, and those which did not are termed gs^- The type-specific antigen of subgroup E ALV has been named chick helper factor, *chf* (6, 23, 24). Cells that expressed type-specific E coat antigen are designated as chf^+, and cells which did not are designated as chf^-.

Embryonated K28 eggs (16) were obtained from chickens maintained at Stanford University School of Medicine. Embryonated eggs from line 15, line C, and from crosses of line 100 with line 7 chickens (100 × 7) were kindly provided by Lyman B. Crittenden from the chickens maintained at the U.S. Department of Agriculture Regional Poultry Research Laboratory at Beltsville, Md. Fertile eggs from line 7 subline 2 chickens were purchased from the U.S.

Department of Agriculture Regional Poultry Research Laboratory at East Lansing, Mich. Crosses of line 15 roosters with K28 hens (15 × K28) were performed at Stanford by artificial insemination (16). Line 15 roosters were obtained from the U.S. Department of Agriculture Regional Poultry Research Laboratory at Beltsville, Md. Embryonated Japanese quail eggs were obtained from Glenn Mitchell (Sunnyvale, Calif.), and embryonated eggs of Orlop turkey strain 93 were obtained from Orlop Turkey Research and Breeding Farm (Orosi, Calif.).

Cell culture. Cells were grown in Dulbecco modified Eagle medium supplemented with 10% tryptose phosphate broth, 4% calf serum, and 1% chicken serum. Treatment of cells with BUdR or thymidine was carried out in medium 199 supplemented with 4% calf serum and 1% chicken serum. Medium was changed daily during treatment with BUdR or thymidine.

Chicken cells were found to maintain rapid growth in culture for several months if passed frequently and at high densities. In practice, 100-mm tissue culture plates were seeded with 1.5×10^6 to 2×10^6 cells per plate and passed at 2- to 4-day intervals when cells had reached densities of 6×10^6 to 10×10^6 cells/plates. No variation in growth characteristics or generation time was observed for cells of different pedigrees.

***gs* antigen.** *gs* antigen was measured by complement fixation in reactions of hamster antiserum to the *gs* proteins of avian type C viruses with homogenates (10^8 cells/ml) of chicken, quail, or turkey cells (10). For details see Robinson and Lamoreux (16).

***chf* assay.** In the induction studies, *chf* was assayed by method 1 of Robinson and Lamoreux (16). In this method, cells are infected at a high multiplicity of infection with B-RSV (RAV-7). Twenty-four hours postinfection, the infected cells are X irradiated (3,000 to 5,000 rads), trypsinized up, and seeded onto lawns of quail embryo fibroblasts (QEF) that are susceptible to B-RSV (*chf*) but not B-RSV (RAV-7) and C/E CEF that are susceptible to B-RSV (RAV-7) but not B-RSV (*chf*). The fraction of chf^+ cells in a culture is the number of infectious centers on QEF divided by the number of infectious centers on CEF. Routine determinations of the expression of *chf* in chicken primary cells were done by method 2 of Robinson and Lamoreux (16).

Virus. RAV-60 was isolated from a stock of RAV-1 grown in chf^+gs^+ CEF (18). Stocks of RAV-0 were obtained by harvesting culture medium from primary 100 × 7 C/0 or C/A CEF. Stocks of ILV were obtained by growing up the virus present in the culture medium of BUdR-treated cells on chf^-gs^- C/C line 15 cells for two or three passages (17). The B-RSV psuedotypes of RAV-60, RAV-0, 100 × 7-ILV, 7-ILV, and C-ILV were prepared by infecting line 15 cells with the desired subgroup E leukosis viruses, superinfecting the infected cells at first passage with B-RSV (RAV-1) at high dilution, and isolating clones of transformed cells (15) that were producing B-RSV in a subgroup E virus coat but not B-RSV in a subgroup A virus coat. The production of stocks of subgroup E virus in chicken cells may result in the contamination of the infecting subgroup E virus with endogenous viral coat protein. Although subgroup E virus coat protein is not expressed in chf^-gs^- line 15 cells either before or after treatment with BUdR (17), the genetic information for subgroup E type-specific antigen does appear to be present in these cells (unpublished observations). Because of the possibility of contamination of exogenous subgroup E coat information with endogenous subgroup E coat information during multiple passages of virus on line 15 cells (9), subgroup E virus stocks and B-RSV pseudotypes of subgroup E virus were harvested from infected cells that had not been passed more than four times. Production of stocks of ILVs on quail or turkey cells that did not appear to express endogenous avian type C virus required long periods of time in culture and resulted in leukosis virus stocks that had different growth properties from the original stocks of subgroup E viruses (unpublished observations). RAV-0 and 100 × 7-ILV were also found to grow poorly on B-RSV-transformed quail cells, with production of moderate titer stocks of B-RSV in a subgroup E coat occurring only after repeated passage of infected cells.

Stocks of B-RSV (RAV-1), B-RSV (RAV-2), B-RSV (RAV-7), and Schmidt-Rupin rous sarcoma virus of subgroup D were originally obtained from H. Rubin or P. Duesberg (University of California, Berkeley). Avian type C viruses of subgroups F and G were a kind gift of D. Fujita (University of California Medical School, San Francisco). Stocks of B-RSV (RAV-7) that contained little or no B-RSV (*chf*) and were suitable for use in *chf* assays were prepared as described by Robinson and Lamoreux (16). These stocks had titers of more than 10^6 focus-forming units of B-RSV (RAV-7) per ml and less than 10 focus-forming units of B-RSV (*chf*) per ml.

Virus assays. All virus infections were done in the presence of 10 μg of polybrene per ml. Assays for focus-forming virus have been described (16).

Two methods based on the development of interference to superinfection by subgroup E virus in infected cells were used to titer the subgroup E leukosis viruses. In both methods, titrations were done on chf^-gs^- C/C line 15 cells. In the first method, actual titers were obtained by infecting triplicate or quadruplicate cultures with serial two-fold dilutions of virus stocks, passing the cultures, and assaying at each passage for the development of interference to subgroup E ASV. When no further cultures were seen to develop interference, the 50% infectious dose was determined by the method of Reed and Muench (14). In the second method, at least three cultures of line 15 cells were infected with 5- to 10-fold dilutions of virus with known as well as unknown titer. Three to four days later, infected cultures were passed and assayed for interference to subgroup E pseudotypes of B-RSV. The initial viral dilutions (abscissa) and focus-forming units of challenge virus per ml (ordinate) were then plotted on log-log graph paper, lines were drawn connecting the points for each virus stock, and the titer was determined by the degree of displacement of the line for the unknown virus stock from the line for the known virus stock (19). Since RAV-60 and 7-ILV gave lines with different slopes in log-log plates

of this assay, unknown viruses were titered against known viruses of similar origin (i.e., a stock of 7-ILV was used to titer stocks of 100 × 7-ILV, RAV-0, or 7-ILV) where no differences in the slopes of the log-log plots were observed. Attempts to develop an XC assay, immunofluorescent assays, or plaque assays for the subgroup E leukosis viruses have been unsuccessful except for our isolate of RAV-60, which plaqued under the conditions of Graf (4).

Assay for particles containing RNA-directed DNA polymerase. Culture medium to be assayed for particles was centrifuged for 5 min at 1,000 rpm in an International PR-4 centrifuge and then for 10 min at 10,000 rpm in a Sorvall RC 2-B centrifuge to remove cells and cell debris. Then up to 6.5 ml of culture medium was layered over 1.5 ml of 45% glycerol in buffer (10^{-2} M Tris, 0.1 M NaCl, and 10^{-3} M EDTA) in thick-walled polycarbonate tubes and centrifuged in a 50Ti rotor at 50,000 rpm for 45 min in a Spinco model L2-B ultracentrifuge to pellet particles with the size and density of avian type C viruses. Pellets were assayed for RNA-directed DNA polymerase in reaction mixtures containing the substrate [^{3}H]dGTP and the template-primer poly(rC)·oligod$G_{(12-18)}$ in a reaction mixture containing 0.01 M Tris, pH 8.3, 0.06 M KCl, 0.005 M dithiothreitol, and 0.01 M $MgCl_2$. 10 μg of template-primer, and 5 μCi of [^{3}H]dGTP (New England Nuclear Corp.; specific activity, 30 Ci/mmol) as described by Robinson and Lamoreux (16). Backgrounds represent count per minute of [^{3}H]dGTP incorporated into acid-insoluble material in parallel assays done on growth medium. This assay appears to give a quantitative measure of RNA-directed DNA polymerase in type C viruses since the amount of [^{3}H]dGTP incorporated into acid-insoluble counts is linear with virus dilution and since the reaction can be completely blocked by preincubation of disrupted viral pellets with antiserum specific to the RNA-directed DNA polymerase of avian type C virus (11).

RESULTS

Three chicken pedigrees that produce subgroup E ILV. In our initial experiments on the production of avian type C viruses by chicken cells grown in BUdR, cells from only one of ten pedigrees tested were found to increase their level of production of endogenous subgroup E virus after growth in BUdR (17). Subgroup E virus-resistant cells from the inducible pedigree, 100 × 7, were found to undergo approximately a 100-fold increase in the fraction of cells expressing viral type-specific E coat antigen (*chf*) and an increased production of virus-like particles containing RNA-directed DNA polymerase, an enzyme characteristic of avian type C viruses (1, 20). Recently cells of two additional chicken pedigrees have been identified that increase their level of expression of endogenous subgroup E virus after growth in BUdR. One of these pedigrees, line 7 subline 2, is closely related to line 100, which is being developed to have the same genes as line 7 except for genes specifying susceptibility to subgroup A and B ASV (2, 17). The other line, Reaseheath line C, is unrelated to line 7 or line 100 (12).

Table 1 presents the expression of subgroup E ALV in 100 × 7, line 7, and line C cells grown in BUdR. In every case the fraction of *chf*$^+$ cells was found to increase approximately 100-fold, and the production of particles containing RNA-directed DNA polymerase was found to increase from levels just over background to levels of from 1,000 to 5,000 counts/min per ml in cultures grown in BUdR. The levels of expression of *chf* and of virus-like particles both before and after BUdR treatment were remarkably similar in BUdR-treated 100 × 7, line 7, and line C cells, except for the production of slightly higher levels (~twofold) of particles containing RNA-directed DNA polymerase by BUdR-treated line C cells than by BUdR-treated line 7 or 100 × 7 cells. The particles containing RNA-directed DNA polymerase in the medium of BUdR-treated 100 × 7 cells have been named 100 × 7-ILV (17). Similarly, the particles produced by line 7 cells grown in BUdR were named 7-ILV, and those produced by line C cells grown in BUdR were named C-ILV.

Growth of subgroup E ILV on cells susceptible to subgroup E virus. Our original isolates of 100 × 7-ILV were found to grow on line 15 *chf*$^-$*gs*$^-$ C/C (17) chicken cells but not in K28 *chf*$^-$*gs*$^-$ C/0 (16, 17) chicken or quail cells. All three of these cell types have a high susceptibility to B-RSV in a *chf* or RAV-60 coat and support the replication of RAV-60 to high titers (17). The data in Table 2 summarize the growth of 100 × 7-ILV, 7-ILV, C-ILV, RAV-0, and RAV-60 on line 15, K28, turkey, and quail cells. In these experiments each cell type was infected with virus containing approximately 1,000 counts/min per ml of RNA-directed DNA polymerase, grown to confluency, and passed three times. Culture medium from the third-passage cells was harvested and analyzed for virus growth by assaying for particles containing RNA-directed DNA polymerase. Under these conditions, the three ILVs as well as RAV-0 grew on line 15 but not on K28, turkey, or quail cells (Table 2). In contrast, RAV-60 grew well on all four cell types.

Demonstration that 7-ILV and C-ILV are subgroup E viruses. Line 15 cells infected with 100 × 7-ILV, 7-ILV, C-ILV, RAV-0, or RAV-60 were next tested for interference to superinfection with subgroup A, B, D, E, F, and G B-RSV

TABLE 1. *Expression of subgroup E ALV in 100 × 7, line 7, and line C cells grown in BUdR*[a]

Pedigree	Susceptibility to ASV	Embryo no.	Cells grown for 48 h in medium containing:	*chf*[+] cells/cells tested	RNA-directed DNA polymerase in particles from culture medium (counts/min per ml)[b]
100 × 7	C/BE	773	10^{-4} M thymidine	0.0006	17
			10^{-4} M BUdR	0.06	1,020
Line 7	C/ABE	1072	10^{-4} M thymidine	0.0006	50
			10^{-4} M BUdR	0.1	1,840
		1075	10^{-4} M thymidine	0.0002	23
			10^{-4} M BUdR	0.06	1,300
Line C	C/AE	984	10^{-4} M thymidine	0.0001	90
			10^{-4} M BUdR	0.008	4,600
		988	10^{-4} M thymidine	0.0001	86
			10^{-4} M BUdR	0.01	3,650

[a] CEF (1.5×10^6) were seeded on multiple 100-mm tissue culture plates in growth medium minus tryptose phosphate broth; 2 to 3 h later, CEF on some plates were counted, and CEF on the remaining plates were changed into growth medium minus tryptose phosphate broth containing the indicated supplements. At 24 h the culture medium was changed. At 48 h, cultures were trypsinized and seeded at 3×10^6 cells/100-mm plate in growth medium. Sixteen hours later, one of the duplicate cultures was used to assay for *chf* according to method 1 of Robinson and Lamoreux (16). Medium from the other culture was harvested 40 h after transfer for assay for RNA-directed DNA polymerase in particles.

[b] Counts per minute incorporated during 60 min of incubation. A background of 40 counts/min has been subtracted.

TABLE 2. *Growth at three passages after infection of 100 × 7-ILV, 7-ILV, C-ILV, RAV-0, and RAV-60 on different subgroup E virus-susceptible cells*[a]

Cell being tested for growth of virus	RNA-directed DNA polymerase in particles in culture medium of cells infected with:					
	Growth medium	100 × 7-ILV	7-ILV	C-ILV	RAV-0	RAV-60
K28 (*chf*$^-$*gs*$^-$ C/O)	21–55[b]	74	365	0	0	>50,000
Line 15 (*chf*$^-$*gs*$^-$ C/C)	23–59	>50,000	>50,000	>50,000	>50,000	>50,000
Turkey (*gs*$^-$ T/BC)	13–81	98	268	81	0	>50,000
Quail (*gs*$^-$ Q/BC)	0–75	58	5	5	NT[c]	>50,000

[a] Cells were infected for 1 h with virus containing 1,000 counts/min per ml of RNA-directed DNA polymerase. Culture medium was harvested for assay from confluent cultures at the third passage after infection.

[b] Counts per minute per milliliter incorporated during 60 min of incubation. A background of 30 counts/min has been subtracted for assays on chicken cells, 100 counts/min for assays on quail cells, and 40 counts/min for assays on turkey cells.

[c] NT, Not tested.

(Table 3). In all cases high levels of interference (>10^4-fold) to subgroup E but not other subgroups of virus were found to develop by three passages of the infected line 15 cells. The three ILVs and RAV-0 were found to have identical patterns of interference. In contrast, our isolate of RAV-60 showed high levels (>10^4-fold) of interference to subgroup E virus and intermediate levels of interference (10- to 1,000-fold) with subgroups F and G virus. The high level of interference seen in 100 × 7-ILV-, 7-ILV-, C-ILV-, RAV-0-, and RAV-60-infected cells to subgroup E ASVs indicates that all of these viruses are subgroup E. The intermediate level of interference of RAV-60 with subgroups F and G virus indicates that the coat protein of our isolate of RAV-60 may be related to the coat proteins of subgroup F and G virus (3, 7).

Does B-RSV in a 100 × 7-ILV, 7-ILV, C-ILV, or RAV-0 coat infect K28 cells? Because of the strikingly different patterns of growth of the subgroup E ILVs and RAV-0 on K28 and

TABLE 3. *Interference of 100 × 7-ILV, 7-ILV, C-ILV, RAV-0, and RAV-60 with subgroup A, B, D, E, F, and G ASV*[a]

Challenge virus	FFU[b] of challenge virus on infected CEF/FFU of challenge virus on uninfected CEF				
	100 × 7-ILV[c]	7-ILV	C-ILV	RAV-0	RAV-60
Subgroup A					
B-RSV (RAV-1)	1.0	0.4	1.1	1.0	0.8
Subgroup B					
B-RSV (RAV-2)	0.6	0.4	1.1	0.7	0.6
Subgroup D					
SR-RSV-D[d]	0.1	0.1	0.2	0.2	0.2
Subgroup E					
B-RSV(100 × 7-ILV)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
B-RSV(7-ILV)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
B-RSV(C-ILV)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
R-RSV(RAV-0)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
B-RSV(RAV-60)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
B-RSV(*chf*)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Subgroup F					
B-RSV(RPV)	0.5	0.6	0.5	0.5	0.008
Subgroup G					
B-RSV(GPV)	1.0	0.8	1.4	1.1	0.02

[a] CEF were infected with original isolates of the designated viruses. Cultures were passed at 2- to 4-day intervals and tested for interference at the third and fifth passages. Third- and fifth-passage cells had similar levels of interfering activity for all of the subgroups of virus tested.

[b] FFU, Focus-forming units.

[c] Virus with which line 15 *chf*$^-$*gs*$^-$ C/C CEF were infected.

[d] Schmidt-Rupin Rous sarcoma virus of subgroup D.

line 15 cells, B- RSV pseudotypes of 100 × 7-ILV, 7-ILV, C-ILV, RAV-0, and RAV-60 were made in order to examine the plating efficiency of these viruses on K28 and line 15 cells (Table 4). Subgroup E pseudotypes of B-RSV had a slightly lower plating efficiency (two- to three-fold) on K28 cells than on line 15 cells. No difference in the plating efficiency of subgroup A, B, and D pseudotypes of B-RSV on K28 or line 15 cells was observed.

When the plating efficiency of subgroup B and E virus was examined in the presence of different levels of polybrene or DEAE-dextran (21), K28 and line 15 cells were found to have similar susceptibilities to subgroup B and E viruses in the presence or absence of polycations, with a 10-fold increase in susceptibility occurring in the presence of polycations.

Counts per minute of RNA-directed DNA polymerase per infectious unit in different stocks of subgroup E virus. All of our initial studies on growth of subgroup E viruses were done by using sedimentable RNA-directed DNA polymerase as an assay for virus. The next experiments were undertaken to correlate counts per minute of RNA-directed DNA polymerase in particles with infectious units (IU) of virus. To accomplish this, 7-ILV, C-ILV, and RAV-60 were titered in end-point assays on line 15 cells, with infected cultures being distinguished from uninfected cultures by their development of interference to superinfection with subgroup E ASV (see Materials and Methods). Both 7-ILV- and C-ILV-infected line 15 cells were morphologically indistinguishable from uninfected cells. With both of these viruses, cultures at the end point of the virus titration showed maximal levels of interference at three to four passages after infection. In contrast, RAV-60-infected line 15 cells had a larger, more granular appearance than noninfected cells. RAV-60 also grew more rapidly on line 15 cells than did 7-ILV or C-ILV, with cultures at the end point of virus titrations developing maximal levels of interference by the second passage. 7-ILV, C-ILV, and RAV-60 were all found to have approximately 0.05 counts/min of RNA-directed DNA polymerase per IU of virus (Table 5).

Further assays to determine the number of IU per milliliter of stocks of subgroup E viruses were done by comparing the extent of interference to subgroup E ASV at one passage after infection in cells infected with different dilutions of virus of known as well as unknown titer (19; see also Materials and Methods). Using this assay, the number of counts per minute of RNA-directed DNA polymerase in particles per IU was found to be consistently about 0.05 for all of our subgroup E virus stocks.

Effect of multiplicity of infection and time in culture on the growth of subgroup E ILVs

TABLE 4. *Titer of subgroup A, B, C, D, and E pseudotypes of B-RSV on line 15 and K28 cells*

Virus	Titer of virus on:						FFU/ml on line 15 cells/FFU/ml on K28 cells[a]
	Line 15 cells			K28 cells			
	921[b]	922	1087	756	1252	1254	
Subgroup A							
B-RSV(RAV-1)	4.0[c]	2.0	6.5	9.2	4.0	4.0	0.7[d]
Subgroup B							
B-RSV(RAV-2)	inc[e]	inc	5.0	NT[f]	6.0	7.0	0.8
Subgroup C							
B-RSV(RAV-7)				NT	9.0	5.5	
Subgroup D							
SR-RSV-D[g]	9.0	5.0	4.8	NT	7.6	9.0	0.8
Subgroup E							
B-RSV(RAV-0)	2.3	2.5	6.5	1.1	2.9	1.1	2.2
B-RSV(7-ILV)	2.6	1.3	3.0	0.6	1.0	0.5	3.3
B-RSV(C-ILV)	1.1	1.0	1.9	0.6	0.7	0.5	2.2
B-RSV(*chf*)	0.4	1.0	0.6	0.2	0.5	0.5	1.7
B-RSV(RAV-60)	6.0	3.5	8.5	1.5	3.1	3.2	2.3

[a] FFU, Focus-forming units.
[b] Embryo number.
[c] FFU per milliliter $\times 10^{-5}$.
[d] Average FFU per milliliter on line 15 cells/average FFU per milliliter on K28 cells.
[e] inc, Incomplete.
[f] NT, Not tested.
[g] Schmidt-Rupin Rous sarcoma virus of subgroup D.

TABLE 5. *7-ILV, C-ILV, and RAV-60: ratio of physical particles to infectious particles and growth characteristics on line 15 cells*

Virus	RNA-directed DNA polymerase in virus (count/min per ml)	IU/ml	Counts/min of RNA-directed DNA polymerase/IU	Appearance of infected cells	Passage at which end point was reached
7-ILV	4.2×10^{5}[a]	1.3×10^{7}	0.03	Healthy	3–4
C-ILV	2.8×10^{5}	5.1×10^{6}	0.05	Healthy	3–4
RAV-60	5.7×10^{5}	6.5×10^{6}	0.09	Granular	2

[a] Counts per minute per milliliter incorporated during 60 min of incubation. A background of 100 counts/min has been subtracted.

on K28 and line 15 cells. Since the coat protein of 100 × 7-ILV, 7-ILV, and C-ILV all seemed competent to initiate absorption and penetration of avian type C virus into K28 as well as line 15 cells, experiments were next done to look at the growth of 100 × 7-ILV on K28 cells at different multiplicities of infection (MOI) and different times in culture. In this experiment, cultures of K28 and line 15 cells were infected with 2×10^{6} IU (MOI of 1.0; this was the highest practical MOI that could be achieved with our virus stock) and 2×10^{4} IU (MOI of 0.01; this is the MOI used in Table 2, where no virus growth was observed on K28 cells) of 100 × 7-ILV. When the infected cells had grown to confluency, the culture medium was harvested for analysis for the amount of RNA-directed DNA polymerase in virus-like particles, and the infected cells were passed. This protocol was carried out for 12 passages of the infected cells with culture medium saved at three passage intervals after the third passage. From the first to the third passages, 100 × 7-ILV replicated to an intermediate titer in K28 cells infected with 2×10^{6} IU, but did not replicate to detectable levels in K28 cells infected with 2×10^{4} IU of 100 × 7-ILV (Fig. 1). Between the third and sixth passages, however, the level of production of virus by the K28 cells infected with 2×10^{6} IU of 100 × 7-ILV increased approximately 10-fold, whereas the level of production of virus by K28 cells infected with 2×10^{4} IU changed from undetectable to levels approximately fivefold lower than that seen in the medium of the cells infected with 2×10^{6} IU of virus. In contrast, line 15 cells infected with either 2×10^{6} or 2×10^{4} IU of 100 × 7-ILV were producing equally high titers of

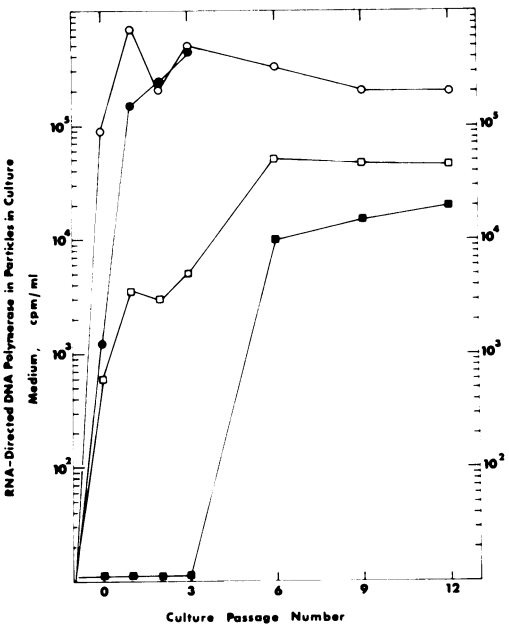

FIG. 1. *Effect of multiplicity of infection and time in culture on growth of 100 × 7-ILV on K28 and line 15 cells. Cells were seeded at 1.5 × 10⁶ cells per 100-mm tissue culture plate and infected 16 to 24 h later with 2 × 10⁶ IU of 100 × 7-ILV, 2 × 10⁴ IU of 100 × 7-ILV, or growth medium. Cultures were passed at 2- to 3-day intervals. RNA-directed DNA polymerase (counts per minute per milliliter) in culture medium of K28 (□) or line 15 (○) cells infected with 2 × 10⁶ IU of 100 × 7-ILV and of K28 (■) or line 15 (●) cells infected with 2 × 10⁴ IU of 100 × 7-ILV. Counts per minute per milliliter incorporated during 60 min of incubation. A background of 30 counts/min has been subtracted.*

virus after two passages in culture. When similar experiments were done with 7-ILV, C-ILV, and RAV-0, similar patterns of growth were observed. In every case, whereas infection with 2 × 10⁴ IU led to low or undetectable levels of virus production for the first three passages in culture on K28 cells (Table 2), by six passages infected cells were producing virus containing 10⁴ to 10⁵ counts/min per ml of RNA-directed DNA polymerase (Table 6).

RAV-60, which grew much more rapidly in K28 cells than did the subgroup E ILVs or RAV-0 (Table 2), grew to higher titer (10- to 40-fold) in the K28 cells than did the subgroup E ILVs or RAV-0 (Table 6). Both RAV-60 and the subgroup E ILVs grew to higher titers (~10-fold) in line 15 cells than in K28 cells (Fig. 1, Table 6).

Assays to determine the counts per minute of sedimentable RNA-directed DNA polymerase per IU of virus grown in K28 or line 15 cells indicated that progeny virus had the same ratio of counts per minute of sedimentable RNA-directed DNA polymerase to IU as parental virus.

TABLE 6. *Growth at six passages after infection of 100 × 7-ILV, 7-ILV, C-ILV, RAV-0, and RAV-60 on K28 and line 15 cells*[a]

Infecting virus	Production of virus by infected cells: RNA-directed DNA polymerase in particles in culture medium (counts/min per ml × 10⁻³)	
	K28 cells	Line 15 cells
100 × 7-ILV	10.0[b]	350.0
7-ILV	44.5	560.0
C-ILV	58.0	640.0
RAV-0	56.0	590.0
RAV-60	264.0	1,950.0

[a] Cells were infected for 1 h with 2 × 10⁴ IU of virus containing 1,000 counts/min per ml of RNA-directed DNA polymerase. Culture medium was harvested for assays from confluent cultures at the sixth passage after infection.

[b] Counts per minute per milliliter incorporated during 60 min of incubation. A background of 40 counts/min has been subtracted.

Growth of ILV that had been passed on K28 cells or line 15 cells on K28 cells and line 15 cells. Because of the dramatic increase in virus production between the third and sixth passage of K28 cells infected with 2 × 10⁴ IU of 7-ILV, C-ILV, or 100 × 7-ILV, sixth-passage virus from K28-infected cells (designated as 100 × 7-ILV-K28 ⑥, 7-ILV-K28 ⑥, and C-ILV-K28 ⑥), and line 15-infected cells (100 × 7-ILV line 15 ⑥, 7-ILV-line 15 ⑥, and C-ILV-line 15 ⑥) were used to infect K28 and line 15 cells with 4 × 10⁵ and 4 × 10³ IU of virus. The infected cells were grown to confluency and passed three times. At the third passage, culture medium was collected and analyzed for RNA-directed DNA polymerase. In every case, passage of an ILV on K28 cells resulted in the production of a stock of virus that grew better on K28 cells than the original ILV stock (Table 7). In contrast, virus grown on line 15 cells had the same growth characteristics as the original isolates of subgroup E ILV, with good growth on line 15 cells but poor growth on K28 cells.

When RAV-0 produced by primary C/A 100 × 7 cells (RAV-0-100 × 7 ⓪) and the same C/A 100 × 7 cells that had been passed 12 times before harvest of a virus stock (RAV-0-100 × 7 ⑫) was analyzed for growth on K28 and line 15 cells, both RAV-0-100 × 7 ⓪ and RAV-0-100 × 7 ⑫ were found to be restricted in their growth on K28 cells (Table 7). This result indicates that during growth of RAV-0 in 100 × 7 cells there is no selection for virus that grows well on K28 cells and implies that 100 × 7 cells

TABLE 7. *Growth at three passages after infection of subgroup E ILVs and RAV-0 passed in K28, line 15, or 100 × 7 cells on K28 and line 15 cells*

Infecting virus	Production of virus by infected cells: RNA-directed DNA polymerase in particles in culture medium (counts/min per ml × 10^{-3})			
	K28 cells infected with:		Line 15 cells infected with:	
	4 × 10^5 IU	4 × 10^3 IU	4 × 10^5 IU	4 × 10^3 IU
100 × 7-ILV-K28⑥	63.0[a]	112.0	320.0	260.0
100 × 7-ILV-15⑥	24.0	0.1	315.2	300.0
7-ILV-K28⑥	78.0	30.0	602.0	530.0
7-ILV-15⑥	19.4	2.4	333.2	246.0
C-ILV-K28⑥	108.0	31.0	364.0	292.0
C-ILV-15⑥	35.6	2.8	290.0	276.0
RAV-0-100 × 7⓪	11.9	0.3	252.0	218.0
RAV 0-100 × 7⑫	15.9	1.0	436.0	322.0

[a] Counts per minute per milliliter incorporated during 60 min of incubation. A background of 75 counts/min has been subtracted.

do not have the restriction for the growth of the subgroup E ILV found in K28 cells.

When virus produced at an intermediate level by K28 cells two passages after infection with 2 × 10^6 IU of 100 × 7-ILV (Fig. 1) was analyzed for growth on K28 and line 15 cells, it was found to be restricted in its growth on K28 but not line 15 cells. This result indicates that virus with the growth characteristics of parental virus was produced by cultures of K28 cells infected with 2 × 10^6 IU of 100 × 7-ILV for the first few passages after infection. However, by six passages after infection, a time that correlated with a sharp increase in the amount of virus produced by the infected K28 cells (Fig. 1), K28 cells infected with 2 × 10^6 IU of 100 × 7-ILV were producing virus that was no longer restricted in its growth on K28 cells. Similarly, second-passage K28 cells infected with 2 × 10^6 IU of 7-ILV or C-ILV were found to be producing intermediate titers of 7-ILV and C-ILV that had the growth characteristics of the parental virus, whereas by sixth passage such cells were producing high titers of virus that grew well on K28 cells.

When 100 × 7-ILV-K28⑥-, 7-ILV-K28⑥-, and C-ILV-K28⑥-infected line 15 cells were analyzed for the development of interference to subgroups A, B, D, E, F, and G ASV, the interference pattern of these viruses was found to be the same as that observed for 100 × 7-ILV, 7-ILV, and C-ILV (Table 8). This result indicates that the change from restricted virus to nonrestricted virus does not involve the subgroup E virus coat protein.

Growth of 100 × 7-ILV in cells of embryos of crosses of line 15 and K28 chickens. The inheritance of the restriction on the growth of the induced subgroup E viruses in K28 cells was next examined by analyzing cells of 10-day embryos of crosses of line 15 and K28 chickens for the growth of 100 × 7-ILV. Because much of our work on the growth of the induced subgroup E viruses on K28 and line 15 cells had been done with cells of only a few embryos of K28 and line 15 lineage, cells from 18 embryos of K28 origin as well as cells from the 4 *chf*$^-$*gs*$^-$ line 15 embryos available to us were also tested for their support of growth of 100 × 7-ILV. In these experiments cells were infected with 2 × 10^6 IU, 2 × 10^4 IU, and no 100 × 7-ILV, grown to confluence, and passed two or three times. Culture medium was then harvested for analysis for particles containing RNA-directed DNA polymerase. Growth of 100 × 7-ILV to high titer (10^5 to 10^6 counts/min per ml) in cells infected with 2 × 10^6 as well as 2 × 10^4 IU of virus indicated that the cells being tested did not restrict the growth of 100 × 7-ILV. Growth of 100 × 7-ILV to intermediate titer (10^4 to 10^5 counts/min per ml) in the cells infected with 2 × 10^6 IU and low titer (<10^3 counts/min per ml) in cells infected with 2 × 10^4 IU of virus indicated that the cells being tested restricted the growth of 100 × 7-ILV.

In all cases, cells of K28 pedigree were found to restrict the growth of 100 × 7-ILV, whereas cells of line 15 embryos and of 15 × K28 embryos were found to support rapid growth of 100 × 7-ILV. These results indicate that the phenotype for restricted growth of subgroup E ILVs is found in all K28 cells, that the phenotype for nonrestricted growth of subgroup E ILV may be found in all line 15 *chf*$^-$*gs*$^-$ cells, and that in 15 × K28 cells the phenotype of K28 for the nonrestricted growth of subgroup E ILVs is recessive.

DISCUSSION

Subgroup E ILVs isolated from the culture medium of BUdR-treated 100 × 7, line 7, and line C cells and RAV-0 isolated from the culture

TABLE 8. *Interference of 100 × 7-ILV-K28⑥, 7-ILV-K28⑥, and C-ILV-K28⑥ with subgroup A, B, D, E, F, and G ASV*[a]

Challenge virus	FFU of challenge virus on infected CEF/FFU of challenge virus on uninfected CEF[a]		
	100 × 7-ILV-K28⑥[b]	7-ILV-K28⑥	C-ILV-K28⑥
Subgroup A			
B-RSV(RAV-1)	1.3	1.0	1.7
Subgroup B			
B-RSV(RAV-2)	0.6	0.7	0.9
Subgroup D			
SR-RSV-D[c]	0.5	0.6	1.0
Subgroup E			
B-RSV(RAV-0)	<0.0001	<0.0001	<0.0001
Subgroup F			
B-RSV(RPV)	0.3	0.4	0.2
Subgroup G			
B-RSV(GPV)	0.8	1.1	1.1

[a] Interference was measured at the third and fifth passages after infection of line 15 cells with 4.0×10^5 IU of virus.

[b] Virus with which line 15 *chf⁻gs⁻* C/C CEF were infected.

[c] Schmidt-Rupin Rous sarcoma virus of subgroup D.

medium of subgroup E virus susceptible 100 × 7 cells were found to grow poorly on K28 chicken cells, turkey cells, and Japanese quail cells (Table 2) but to grow well on line 15 chicken cells (Table 2). B-RSV in a 100 × 7-ILV, 7-ILV, C-ILV, or RAV-0 coat had a high plating efficiency on both K28 and line 15 cells (Table 4), indicating that the restriction on the replication of these viruses in K28 cells was intracellular.

At early passages after infection, K28 cells infected at an MOI of 1.0 with the subgroup E ILVs produced intermediate titers of virus, whereas cells infected at a lower MOI (0.01) produced low to undetectable levels of virus (Table 2, Fig. 1). The virus produced by K28 cells infected at an MOI of 1.0 had the growth characteristics of parental virus. By six passages after infection, K28 cells infected at an MOI of either 1.0 or 0.01 had begun to produce high titers of subgroup E virus (Fig. 1, Table 6). When this virus was tested for growth on K28 cells, it was found to grow well on K28 cells at both high and low MOI (Table 7). Virus produced by comparably infected and passaged line 15 cells had the growth characteristics of parental virus, indicating that selection for nonrestricted virus occurred only during growth of virus on a restrictive host (Table 7). By analogy, the observation that RAV-0 did not change its growth properties on K28 cells after prolonged passage in 100 × 7 cells suggests that 100 × 7 cells are a nonrestrictive host (Table 7). The change from restricted to nonrestricted virus was not accompanied by a change in the ratio of physical particles to infectious particles or by a change in the coat properties of ILV stocks (Table 8).

Cells of crosses of line 15 and K28 chickens were found to support rapid growth of subgroup E ILVs and RAV-0 (Table 9). This result indicates that the K28 phenotype for the restricted intracellular replication of subgroup E ILVs is recessive. The observation that the intracellular restriction on the growth of subgroup E ILV in chickens is recessive indicates that this restriction is different from the dominant intracellular restriction on the growth of ecotropic murine viruses expressed by the Fv-1 genetic locus of mice (13).

The two chicken pedigrees we have found that appear to be permissive for the intracellu-

TABLE 9. *Growth of 100 × 7-ILV in cells of K28, line 15, and line 15 × K28 10-day embryos*[a]

Pedigree of 10-day embryo	Growth of 100 × 7-ILV	
	Restricted	Nonrestricted
K28	18/18[b]	0/18
Line 15	0/4	4/4
K28 × line 15	0/21	21/21

[a] Cells from each embryo were seeded on three 100-mm tissue culture plates at 3×10^6 cells/plate. Sixteen to twenty-four hours later these plates were infected with 2×10^6 IU of 100 × 7-ILV, 2×10^4 IU of 100 × 7-ILV, and growth medium. Cells were grown to confluency and passed. At the second or third passage, culture medium was harvested and analyzed for RNA-directed DNA polymerase in particles. Embryos in which 100 × 7-ILV had grown to high titer at both multiplicities of infection were classified as nonrestrictive, and embryos in which 100 × 7-ILV had grown 10- to 100-fold better at the higher than the lower multiplicity of infection were classified as restrictive.

[b] Number of embryos/number of embryos tested.

lar growth of subgroup E ILVs, line 15 and 100 × 7, are both inducible for endogenous avian type C virus (17). Both the phenotype for induction of endogenous virus and that for support of rapid growth of subgroup E ILVs are dominant (17; Table 9; unpublished observations). Both phenotypes occur in line 15 and in 100 × 7 cells, whereas neither is found in the cells of other pedigrees that we and others have tested for growth of RAV-0 (6, 17, 22; M. Linial, personal communication). Taken together, these results raise the possibility that the phenotypes for rapid intracellular growth of subgroup E ILV and for increased production of endogenous virus after growth in BUdR reflect the expression of the same genetic locus.

Our isolate of RAV-60 was found to have different growth characteristics (Tables 2, 5, and 6), and a different pattern of interference (Table 3), than the subgroup E ILVs or RAV-0. The characteristics that our isolate of RAV-60 and the subgroup E ILVs had in common were a high level of interference to subgroup E ASV (Table 3), the same relative plating efficiency of their B-RSV pseudotypes on K28 and line 15 cells (Table 4), and a similar ratio of counts per minute of RNA-directed DNA polymerase per IU (Table 5).

In closing, I would like to point out the striking similarities in growth properties (Tables 2 and 6), interference properties (Table 3), and levels of production in BUdR-treated and untreated cells (Table 1) of 100 × 7-ILV, 7-ILV, and C-ILV. In no case was I able to distinguish these viruses from each other or RAV-0, a result suggesting that the genes for inducible subgroup E virus in line 100, line 7, and line C are highly related if not identical. The observation that RAV-60 (18) but not the subgroup E ILVs interfered with subgroup F and G virus (Table 3) raises the possibility that the noninducible subgroup E viral coat protein of chickens (17) is more closely related than the inducible subgroup E virus coat proteins of chickens (17) to the subgroup F and G viral coat proteins of pheasants (3, 7).

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