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Avian Leukosis Viruses of Different Subgroups and Types Isolated After Passage of Rous Sarcoma Virus-Rous-Associated Virus-0 in Cells from Different Ring-Necked Pheasant Embryos

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Avian leukosis viruses of subgroups A and F (RAV-A and RAV-F) arose at a low rate after passage of Rous sarcoma virus-Rous-associated virus-0, which is subgroup E, in cells from ring-necked pheasant embryos. In cells of two embryos, all of the viruses isolated after virus passage were RAV-F. However, in cells of a third embryo, both RAV-A and RAV-F were isolated. In addition, there sometimes were type-specific differences among the different isolates of RAV-A and RAV-F from the cells of single embryos. These results indicate that the RAV-A and RAV-F probably arose by recombination of viral and cellular genes, that different ring-necked pheasant embryo may have different endogenous avian leukosis virus-related nucleotide sequences, and that recombination at different sites in these endogenous sequences might give rise to type-specific differences among the RAV-A and RAV-F.

Many different groups, or species, of ribodeoxyviruses have been described (18, 22). Within each well-studied group of ribodeoxyviruses, there are many different strains of virus. For example, the avian leukosis virus group contains six subgroups, and each of these subgroups contains strains, or types, differing in the type of transformation induced, the antigenicity of the envelope glycoproteins, and/or the extent of replication in cells of fowl other than chickens.

There appear to be two major processes responsible for the origin of the large numbers of different ribodeoxyvirus strains. One variational process is spontaneous mutation, which occurs at a high rate. For example, Zarling and Temin (25) found that an avian leukosis virus, B77 virus, mutates to an ability to infect duck cells approximately once per 50 infected cell generations. The second variational process is recombination. The recombination can be between two viruses or between a virus and cellular genes. Recombination between an avian leukosis virus and cellular genes is probably illustrated by the appearance of Rous-associated virus (RAV)-61 or ring-necked pheasant virus after multiplication of avian leukosis virus in ring-necked pheasant cells (2, 4, 16, 17).

In this paper, we present the results of a study of variation in the envelope properties of avian leukosis viruses after passage in pheasant cells. We have studied the kinetics of appearance of avian leukosis viruses able to infect C/E chicken cells after infection of ring-necked pheasant cells with avian leukosis viruses incapable of infecting C/E chicken cells. In particular, we studied the kinetics of origin of viruses able to infect C/E chicken cells in cells of different pheasant embryos, and we determined whether or not all of the newly isolated viruses able to infect C/E chicken cells were of the same subgroup and type.

We found that, after infection of pheasant cells with subgroup E avian leukosis virus, avian leukosis viruses of subgroups F and A appeared at a low rate. In most cases, all of the viruses arising from cells of a single pheasant embryo were of subgroup F or subgroup A. There was, however, sometimes type-specific variation among the RAV-F or RAV-A from cells of a single embryo. Some of the newly isolated viruses had a cytopathic effect, although the parental viruses were not cytopathic.

MATERIALS AND METHODS

Cells, viruses, and antibodies. Chicken cells were from C/E embryos from Spafas, which were negative for avian leukosis virus group-specific antigen, sedimentable DNA polymerase activity, and chick helper factor. Ring-necked pheasant cells were prepared from embryos from Poynette Game Farm, Wisconsin Division of Natural Resources (pheasant cells W2 and W8) or were a gift of P. K. Vogt,

University of Southern California School of Medicine (pheasant cells C8), and were negative for avian leukosis virus group-specific antigen, sedimentable DNA polymerase activity, and helper factor. DNA from these pheasant cells contained 10 to 20% of the nucleotide sequences of RAV-0 RNA (9; Kang, personal communication). Duck cells were from Muscovy duck embryos obtained from W. Thrun, Madison, Wis. Turkey cells were from Orlopp turkey embryos obtained from the Wilmar Poultry Co., Wilmar, Minn.

Most of the viruses used have been previously described (8, 12). RAV-61 was originally obtained from H. Hanafusa, Rockefeller University. Bryan high-titer Rous sarcoma virus (RSV)-RAV-0, induced leukosis virus from Reaseheath line I chicken cells (I-ILV), and induced leukosis virus from Regional Poultry Laboratory line 7 chicken cells (L7-ILV) were obtained from P. K. Vogt and R. Weiss, University of Southern California School of Medicine, as infected pheasant cells C2 (23).

Sera from chickens immune to RAV-61, numbers 819 and 822, were a gift of H. Hanafusa. Immune serum to RAV-1, number 102, was previously described (1).

General experimental techniques. General experimental techniques were similar to those used previously in this laboratory. Assay plates containing 6×10^5 chicken, pheasant, duck, or turkey cells were prepared in 60-mm plastic petri dishes (Lux) in medium without serum and were infected after either a 4-h or an overnight incubation. Complete medium consisted of modified Eagle minimal essential medium with 20% tryptose phosphate broth, 2% calf serum, and 2% fetal bovine serum.

Viruses were assayed by inoculation of 0.2 ml of serial 10-fold dilutions of virus in complete medium, and, after 40 min of incubation at 37°C with agitation, by addition of 5 ml of complete medium. The plates were (i) examined for the appearance of transformation, giving titers in focus-forming units, or (ii) examined for the appearance of plaques or generalized cytopathic effects, giving titers in PFU. (The plaques and cytopathic effects appeared within a few days after infection in the absence of agar, neutral red, or preinfection with a temperature-sensitive mutant of RSV. They, thus, are not exactly the same as the avian leukosis virus-induced plaques described by Graf [3] and Kawai and Hanafusa [10]. The cytopathic effect disappeared after one or two passages, similar to what was previously described for spleen necrosis virus-induced cytopathic effect [20].) In addition, (iii) the supernatant media were harvested and concentrated by ultracentrifugation, the sedimented viruses were disrupted, and the amounts of activated calf thymus DNA-directed DNA polymerase activity were determined by using a standard of concentrated spleen necrosis virus, as described previously (19), giving titers in DNA polymerase-forming units; or (iv) the supernatant media were inoculated on cultures of sensitive cells, and these cells were tested for infection by methods (i), (ii), or (iii), giving titers in infectious units.

Virus was "cloned" by end point dilution. The supernatant medium was harvested from the last plate in a serial 10-fold dilution series to show a positive response by any of the assays described above, and it was used as a virus stock.

Complement fixation was by techniques previously described (7).

DNA polymerase neutralization was carried out as previously described (12).

Interference tests were carried out by inoculating assay plates of chicken cells with the viruses to be tested or control medium (mock infection), transferring the cells three times until all the cells were infected and most of any cytopathic effect had disappeared, and then carrying out standard virus assays looking for appearance of cytopathic effects or focus formation. The amounts of interference were determined by comparison of virus titers in the infected and mock-infected cells.

Antibody neutralizations were performed by incubating equal volumes of heat-inactivated immune or normal chicken serum and virus diluted to contain 100 to 1,000 infectious units. After incubation at 37°C for 30 min, the mixtures were diluted and inoculated on chicken cells to determine the amount of virus surviving.

Cell growth curves were carried out by inoculating assay plates with 10^4 to 10^5 infectious units of virus, growing the cells in complete medium with one complete medium change, and, at 6 or 7 days after infection, releasing the cells from the culture dishes with trypsin and counting the cell number with a Coulter counter.

Naming of viruses. The newly isolated viruses were designated by the name of the pheasant cells (W2, W8, C8, or C2), a number designating the virus stock (1, the RSV-RAV-0 used in Fig. 1; 2, the RSV-RAV-0 used in Fig. 2; 3, L7-ILV; 4, I-ILV), and the passage number. For example, virus W2-1-2 was isolated from passage 2 of virus 1 (Fig. 1) in pheasant embryo cells of W2, and C2-3 was isolated from L7-ILV.

RESULTS

Initial observations. This study had its genesis in the following two observations. (i) In the course of growing large quantities of RSV-RAV-0 in pheasant cells (8, 13), a cytopathic effect was seen in the infected pheasant cells, and a cytopathic virus capable of replicating in C/E chicken cells was isolated from the supernatant medium of these infected pheasant cells. (The RSV-RAV-0 actually used in the experiments described by Kang and Temin [8] and Mizutani and Temin [13] did not contain this cytopathic virus nor virus capable of replicating in C/E chicken cells.) (ii) Cytopathic viruses capable of replicating in C/E chicken cells were also found in stocks of I-ILV and L7-ILV grown in other pheasant cells.

In both of these cases the original parental viruses, that is, RSV-RAV-0 and ILV, respec-

tively, did not induce a cytopathic effect in pheasant cells and did not replicate in C/E chicken cells.

A preliminary experiment was performed to determine whether or not these observations were reproducible. Passage in pheasant cells of a stock of RSV-RAV-0 that did not contain any virus capable of replicating in C/E chicken cells again led to the appearance of a virus capable of replicating in C/E chicken cells (data not shown).

Kinetics of origin of virus capable of replicating in C/E chicken cells. A large experiment was then performed to determine in pheasant cells from different embryos the kinetics of origin from RSV-RAV-0 of viruses capable of replicating in C/E chicken cells. Two different stocks of RSV-RAV-0, each titering about 10^6 DNA polymerase-forming units/ml and 10^5 focus-forming units/ml and selected to contain no virus capable of replicating in C/E chicken cells, were serially passaged at 11-day intervals in pheasant cells from three different embryos. Eight and 11 days after each infection of the pheasant cells, the supernatant media were inoculated on cultures of C/E chicken cells to test for the presence of a virus capable of replicating in these cells. Such a variant virus was recognized by the formation of foci in the chicken cells, the appearance of a cytopathic effect in the chicken cells, or the appearance of a sedimentable DNA polymerase activity in the supernatant medium of the chicken cells. The results of this experiment are presented in Fig. 1 and 2. Several variant viruses capable of replicating in C/E chicken cells were found. In a control experiment, uninfected pheasant cells were serially passaged, and the supernatant media were inoculated on chicken cells. No virus was detected (data not shown).

With the RSV-RAV-0 stock used in the experiment of Fig. 1, a variant virus capable of replicating in C/E chicken cells appeared in passage 2 or 3 in PH-W2 and PH-W8 cells, respectively. Such a variant virus was not present in the original RSV-RAV-0 stock, as shown by its failure to appear after infection of chicken cells with passage 1 virus (data not shown) and its failure to appear in an early passage in PH-C8 cells. In addition, it will be shown later that the variant viruses isolated from passage 2 in PH-W2 cells and from passage 3 in PH-W8 cells were different.

In the other passage series described in Fig. 1 and 2, a variant virus capable of replicating in C/E chicken cells appeared only after several passages or did not appear at all.

The variant viruses capable of replicating in C/E chicken cells had no apparent selective advantage for replication in pheasant cells, as shown by their failure to persist in some cases of continued passage in pheasant cells (for example, Fig. 1, passage 2 in PH-W2 cells and passage 6a in PH-C8 cells) and their failure to increase markedly in other passages in pheasant cells (for example, Fig. 1, passage 3 in PH-W8 cells, and Fig. 2, passage 6a in PH-C8 cells).

Characterization of newly isolated viruses that replicate in C/E chicken cells: classification in virus species or group. The supernatant media of the cultures of chicken cells positive for variant virus in the experiments described in Fig. 1 and 2 were inoculated on fresh C/E chicken cells to confirm the presence of a virus capable of replicating in these cells. In all cases, a virus was shown to be present by one or more of the assays described above. The viruses were named as described in Materials and Methods by the name of the pheasant cells (W2, W8, or C8), the number of the virus (1 or 2), and the passage number. These viruses were then cloned as described above, and stocks were grown in C/E chicken cells. These stocks had usually undergone only two or three passages in chicken cells after the passages in pheasant cells. In no case did these viruses still cause focus formation.

In addition to stocks of the variant viruses isolated from the experiments described in Fig. 1 and 2, stocks of cloned viruses were prepared in a similar fashion from the preliminary experiments (viruses W8-1-1, W8-1-1b, W8-1-2) and from I-ILV and L7-ILV (viruses C2-4 and C2-3, respectively).

There are two species or groups of ribodeoxyviruses that replicate in chicken cells, the avian leukosis viruses and the avian reticuloendotheliosis viruses (14, 18, 22). The newly isolated viruses were studied to determine the species to which they belonged. The presence of group-specific antigenic proteins and group-specific DNA polymerase was used for this classification.

All of the newly isolated viruses, except W8-2-7a, were grown in C/E chicken cells, and the infected cells were collected and tested by complement fixation for the presence of avian leukosis virus group-specific antigens. All of the infected cells were positive for avian leukosis virus group-specific antigens (data not shown). Therefore, all of the viruses tested belonged to the avian leukosis virus group, or species.

In an additional test, the supernatant medium from cultures of chicken cells infected with all of the newly isolated viruses, except W8-2-7a, were harvested and concentrated, the viruses were disrupted with detergent, and the specificity of the DNA polymerases was tested

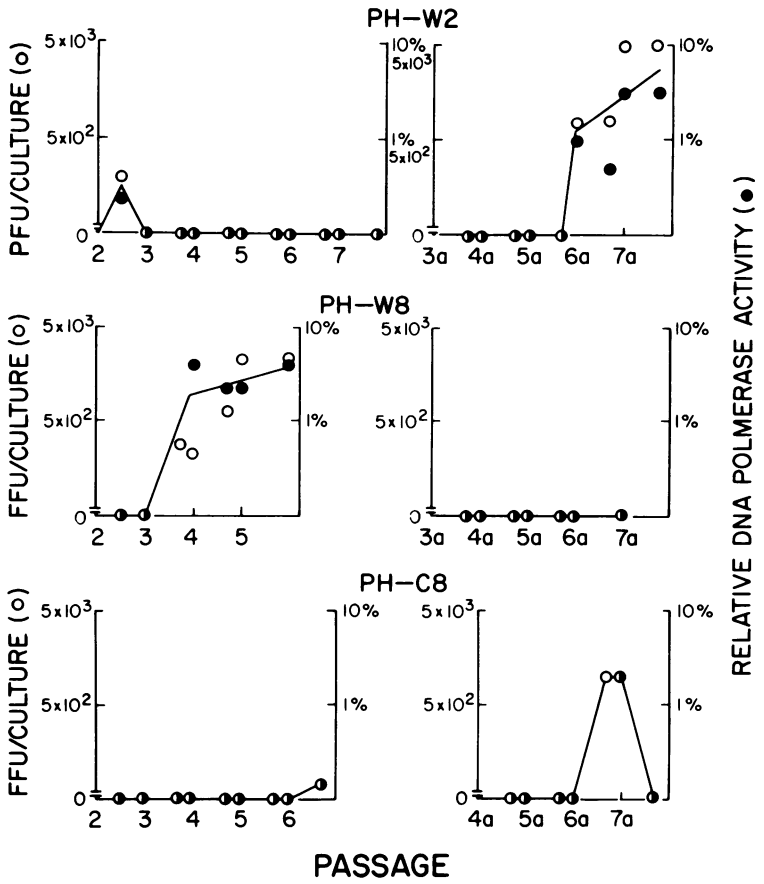

FIG. 1. *Kinetics of origin from RSV-RAV-0-infected pheasant cells of viruses capable of replicating in C/E chicken cells. A stock of RSV-RAV-0 (number 1) was serially passaged in cells from three different pheasant embryos (PH-W2, PH-W8, and PH-C8). The passage inocula were, with three exceptions, 0.2 ml of undiluted media from the 11-day harvests of the previous passage. In addition, media from the 8-day harvests of passage 3 in PH-W2 cells, of passage 3 in PH-W8 cells, and of passage 4 in PH-C8 cells were also used as inocula. The passages continuing, at 11 days after infection, from these 8-day harvests are shown on the right-hand side of the figure and are marked with "a." At each passage, 0.2 ml of undiluted medium was inoculated on cultures containing 6 × 10⁵ pheasant cells. After 40 min of absorption, 5 ml of complete medium was added. At approximately 8 and 11 days after each infection, the supernatant media were harvested and frozen. A 0.2-ml amount of undiluted medium from all of the 8- and 11-day harvests was inoculated on chicken cells. After absorption, 5 ml of complete medium was added, and the chicken cells were examined for foci (focus-forming units [FFU]) or cytopathic effects (PFU). (The abscissas are marked FFU or PFU depending on whether foci or plaques were observed.) At approximately 7 or 8 days after infection of the chicken cells, media were harvested, concentrated by ultracentrifugation, and assayed for DNA polymerase activity. In a few cases where there were many FFU or PFU, the DNA polymerase activity was not determined. The DNA polymerase activity is expressed relative to a standard of concentrated spleen necrosis virus, which incorporated approximately 20,000 cpm (1.25 pmol) of [³H]TMP in 15 min. The figure presents the results of the assays in chicken cells (ordinate) plotted against the passage number (2 to 6 or 3a to 7a) and days after infection of the pheasant cells that the media were harvested (8 or 11 days) (abscissa).*

with antibodies to DNA polymerases of RSV-RAV-0 and of spleen necrosis virus (a reticuloendotheliosis virus). (Representative data are presented in Table 1.) The DNA polymerases of all of the newly isolated viruses tested were neutralized by antibody to RSV-RAV-0 DNA polymerase, and no DNA polymerase was neutralized by the antibody to spleen necrosis virus DNA polymerase, which neutralizes all reticuloendotheliosis virus DNA polymerases (13).

These two tests demonstrated that the newly isolated viruses were members of the avian leukosis virus group, or species, and not of the

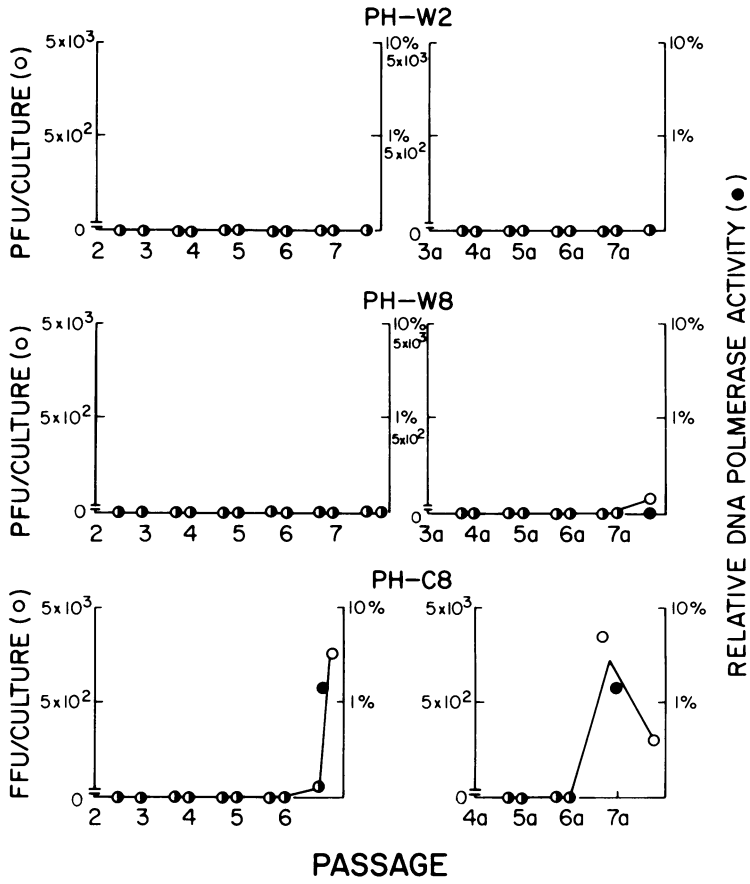

FIG. 2. *Kinetics of origin from RSV-RAV-0-infected pheasant cells of viruses capable of replicating in C/E chicken cells. Another stock of RSV-RAV-0 (number 2), different from that used in the experiment described in Fig. 1, was serially passaged as described in the legend to Fig. 1. (No foci were seen in chicken cells infected with supernatant media from PH-W8.) FFU, Focus-forming units.*

avian reticuloendotheliosis virus group, or species. Therefore, the viruses are referred to as RAV.

Classification in subgroup, or subspecies, of avian leukosis viruses. The newly isolated RAV were then classified as to subgroup by tests of host range, interference, and neutralization of infectivity.

Since RAV-61, which belongs to subgroup F, grows well in chicken, pheasant, and duck cells, the newly isolated RAV were titered in C/E chicken, Ph/BD pheasant, and D/ABDE duck cells (Table 2). Almost all of the viruses fell into two groups. The members of one group of seven viruses, W2-1-2, W2-1-5a, C8-2-6, C8-2-6a, C8-1-6, C8-1-6a, and C2-3, were like RAV-61 in having approximately equal titers in chicken, pheasant, and duck cells and in being cytopathic. This result and the origin of these viruses from avian leukosis virus-infected pheasant cells indicated that these viruses were probably of subgroup F, like RAV-61.

Four other viruses, W8-1-4, W8-1-1, W8-1-2, and W8-2-7a, did not replicate in duck cell and were not cytopathic. W8-2-7a had a low titer that was not increased by passage. This low titer prevented carrying out many experiments with W8-2-7a. W8-1-1b was cytopathic and had a very low titer in duck cells, whereas C2-4 was only cytopathic at low dilutions of virus and had a very low titer in duck cells. C2-4 and the viruses from W8, therefore, appeared to be different from the other seven RAV.

To determine if W8-1-1b and C2-4 were mixtures of viruses, the parental viruses were "recloned," and the viruses isolated from infection of duck cells were titered on chicken and duck cells. W8-1-1b, after recloning or passage through duck cells, gave viruses with properties generally similar to those of the parental

TABLE 1. *Neutralization of DNA polymerase activity*[a]

Virus	% Neutralization by antibody to DNA polymerases	
	IgG-RSV-RAV-O	IgG-SNV
SNV	0	56
RSV-RAV-O	97	0
W2-1-2	100	0
C8-2-6a	95	0
W8-1-4	100	0
W8-1-2	95	0
C2-4	95	0

[a] Cultures of chicken cells were infected with 10^4 to 10^6 DNA polymerase-forming units of the indicated viruses. The infected cells were transferred two times and plated in 100-mm petri dishes. A 10-ml amount of medium was harvested from each culture on each of 3 successive days; the media from each culture were combined and centrifuged; the pellets were incubated with detergent and the indicated antibody to RSV-RAV-O DNA polymerase (immunoglobulin G [IgG]-RSV-RAV-O), antibody to spleen necrosis virus (SNV) DNA polymerase (IgG-SNV), or control serum; and the DNA polymerase activity remaining was measured as described in Materials and Methods. See text for virus designations.

virus, with the virus isolated from duck cells having a higher plating efficiency on duck cells.

In contrast, two very different viruses were isolated from C2-4. One was like W2-1-2, W2-1-5a, C8-2-6, C8-2-6a, C8-1-6, C8-1-6a, and C2-3. The other was like W8-1-4, W8-1-1, and W8-1-2. These viruses are called C2-4-F and C2-4-A, respectively, for reasons that will become apparent later.

Interference tests were performed to determine the subgroup of the cytopathic viruses. W2-1-2, W2-1-5a, C8-2-6, C8-1-6, C8-1-6a, C2-3, C2-4-F, and W8-1-1b were inoculated (approximately 10^4 PFU) on chicken cells chronically infected with RAV-61. No cytopathic effects were seen (Table 3). (The control experiments with RAV-2, Schmidt-Ruppin-RSV, and spleen necrosis virus showed that there was only avian leukosis virus subgroup-specific interference.) The reciprocal experiment was performed with C8-2-6a. Cells chronically infected with C8-2-6a were resistant to the cytopathic effects of RAV-61 and W8-1-1b (data not shown). Therefore, these interference tests indicated that these viruses are in subgroup F. They, therefore, are called RAV-F. (W8-1-1b is in subgroup F, although it has a low plating efficiency on duck cells. There is not always correspondence between subgroup and infection of duck cells. For example, B77 virus type I is in subgroup C, although it does not infect duck cells [25].)

A different test was used to determine the subgroup of some of the noncytopathic, nonduck-plating RAV (W8-1-4, W8-1-1, W8-1-2, and C2-4-A). Separate cultures of chicken cells were infected with these viruses, three RAV-F (W2-1-5a, C8-1-6a, and C2-4-F) and RAV-1, passaged three times, and inoculated with serial 10-fold dilutions of a cytopathic RAV-F (W2-1-2). The titers of the W2-1-2 are shown in Table 4. No cytopathic effect was seen on the RAV-F-infected cells. However, W2-1-2 had a high titer in the cells infected with the noncytopathic RAV from pheasant W8, C2-4-A, and RAV-1. Therefore, these RAV from pheasant W8 and C2-4-A do not appear to belong to subgroup F.

To determine with an interference test the

TABLE 2. *Assay of RAV in chicken, pheasant, and duck cells*[a]

Virus	Titer (units/0.2 ml) in cells of:		
	Chicken	Pheasant	Duck
RAV-61	10^6 PFU	10^5 DPFU	10^6 IU
W2-1-2	10^5 PFU	10^5 PFU	10^5 IU
W2-1-5a	10^5 PFU	10^5 PFU	10^4 IU
C8-2-6	10^5 PFU	10^5 PFU	10^5 PFU
C8-2-6a	10^4 PFU	10^3 PFU	10^4 PFU
C8-1-6	10^6 PFU	10^5 PFU	10^4 PFU[b]
C8-1-6a	10^5 PFU	10^5 PFU	10^4 PFU
C2-3	10^5 PFU	10^4 PFU	10^4 PFU
C2-4	10^6 DPFU[c]	10^7 DPFU	1 IU
W8-1-1b	10^6 PFU	10^5 DPFU	1 IU
W8-1-4	10^6 DPFU	10^5 DPFU	0 IU
W8-1-1	10^5 DPFU	10^4 DPFU	0 IU
W8-1-2	10^6 DPFU	10^4 DPFU	0 IU
W8-2-7a	10^2 DPFU[d]	10^3 DPFU[d]	0 IU

[a] Assay plates containing chicken, pheasant, or duck cells were inoculated with serial 10-fold dilutions of the viruses listed, and the end points for cytopathic effect (PFU) were determined. In addition, the supernatant media were harveted from all of the cultures approximately 6 and 7 days after infection, and the amount of sedimentable DNA polymerase activity in the pooled media was measured to determine the end point for DNA polymerase formation (DNA polymerase-forming units [DPFU]). In all but one case (C2-4) where there was a cytopathic effect, the end points for DPFU and PFU were similar. In these cases, only the PFU titer is given. In cases where there was no cytopathic effect, but sedimentable DNA polymerase activity was found, the titer is given in DPFU. In several cases, the supernatant media from duck cells inoculated with virus were assayed on chicken cells, and the measurements of sedimentable DNA polymerase activity were repeated to test for infectious units (IU).

[b] 10^6 IU.

[c] A cytopathic effect was seen at low dilutions of virus. Approximately 10^3 PFU were present.

[d] These titers were reproducibly lower than the others.

TABLE 3. *Interference by RAV-61*[a]

Virus	Cells infected with:	
	RAV-61	Control medium
RAV-61	0	+
W2-1-2[b]	0	+
C8-2-6[c]	0	+
C2-3	0	+
C2-4-F	0	+
W8-1-1b	0	+
RAV-2[d]	+	+
SR-RSV[e]	+	+
SNV[f]	+	+

[a] Cultures of chicken cells were mock-infected or infected with RAV-61 and were transferred three times until the cytopathic effects had disappeared. Assay plates were prepared and inoculated with approximately 10^4 PFU or focus-forming units of the viruses listed. The cultures were examined for cytopathic effects or focus formation. + indicates the expected amount of cytopathic effect or focus formation; 0 indicates no cytopathic effect.

[b] W2-1-5a was similar.

[c] C8-1-6 and C8-1-6a were similar. C8-2-6a interfered with the cytopathic effects of RAV-61 and of W8-1-1b and, therefore, is also of subgroup F.

[d] RAV-2, a cytopathic avian leukosis virus of subgroup B.

[e] Schmidt-Ruppin RSV-D, a transforming avian leukosis virus of subgroup D.

[f] Spleen necrosis virus (SNV), a cytopathic reticuloendotheliosis virus.

subgroup of the RAV that did not interfere with RAV-F (W8-1-4, W8-1-1, W8-1-2, and C2-4-A), chicken cells were infected with these viruses, passaged three times, and inoculated with avian sarcoma viruses of subgroups A, B, C, and D. The chronically infected cells were sensitive to the viruses of subgroups B, C, and D and were resistant to the viruses of subgroup A (data not shown). This resistance was confirmed by titration of Schmidt-Ruppin-RSV-A and RSV-RAV-1 on these chronically infected cells (Table 5). Reductions in titer of $>10^3$ to $>10^4$ were found on these cells and not on the uninfected or RAV-F-infected cells. Therefore, these noncytopathic RAV appear to be of subgroup A, and they are, therefore, called RAV-A.

Type-specific differences among newly isolated RAV. Some type-specific differences among the RAV-F have already been presented. For example, W8-1-1b differs from the other RAV-F (W2-1-2, W2-1-5a, C8-2-6, C8-2-6a, C8-1-6, C8-1-6a, C2-3, and C2-4-F) in having a much lower titer in duck cells.

A criterion in addition to host range and interference that is used for subgroup classification and that often recognizes type-specific differences is neutralization by antibody to the virion envelope. Therefore, some of the newly isolated RAV were incubated with antibody to RAV-61 and assayed (Table 6). RAV-61 and four of the nine newly isolated RAV-F (C8-2-6a, C8-1-6, C8-1-6a, and C2-4-F) were neutralized; two of the nine newly isolated RAV-F (W2-1-2 and W2-1-5a) were somewhat neutralized; and the other three newly isolated RAV-F (C8-2-6, C2-3, and W8-1-1b) and all of the RAV-A were not neutralized by the antibody to RAV-61. These results are consistent with the previous subgroup classification of RAV and also confirm that there are type-specific differences among RAV-F.

TABLE 4. *Interference with RAV-F*[a]

Cells infected with:	Virus titer (PFU/ml)
Nothing	5×10^6
SNV	5×10^5
RAV-F	
W2-1-5a	<5
C8-1-6a	<5
C2-4-F	<5
W8-1-4	5×10^4
W8-1-1	5×10^4
W8-1-2	5×10^4
C2-4-A	ca. 5×10^4[b]
RAV-1[c]	5×10^5[b]

[a] Chicken cells were inoculated with the viruses listed and were transferred three times. Assay plates were prepared and were inoculated with serial 10-fold dilutions of W2-1-2, a cytopathic RAV-F, and the number of plaques was determined. In a parallel experiment, no significant reduction in titer of C8-1-6a was found on RAV-A-infected cells (see text). SNV, Spleen necrosis virus.

[b] Separate experiments.

[c] Subgroup A.

TABLE 5. *Interference with viruses of subgroup A*[a]

Cells infected with:	Virus titer (FFU/ml)	
	SR-RSV-A	RSV-RAV-1
Nothing	10^4	10^5
RAV-F: W2-1-5a	10^4[b]	10^4[b]
W8-1-4	<5	<5
W8-1-1	<5	<5
W8-1-2	<5	<5
C2-4-A	<5[c]	<5[c]

[a] Chicken cells were inoculated with the viruses listed and were transferred three times. Assay plates were prepared and inoculated with serial 10-fold dilutions of Schmidt-Ruppin RSV-A (SR-RSV-A) or RSV-RAV-1, and the number of foci were determined. FFU, Focus-forming units.

[b] Approximate titer only because of some residual cytopathic effect in these cells.

[c] Separate experiment.

TABLE 6. *Neutralization of RAV by antibody to RAV-61*[a]

Virus	Antibody	DNA polymerase activity (cpm of [^{3}H]TMP incorporated/15 min)
RAV-F		
RAV-61	0	1,170
	+	60
W2-1-2[b]	0	2,100
	+	370
W2-1-5a[c]	0	1,610
	+	290
C8-2-6[c]	0	1,550
	+	780
C8-2-6a[b]	0	1,430
	+	60
C8-1-6	0	325
	+	0
C8-1-6a	0	1,810
	+	30
C2-3[b]	0	150
	+	110
C2-4-F[d]	0	4,700
	+	60
W8-1-1b[b]	0	6,100
	+	8,700
RAV-A		
W8-1-4[f]	0	810
	+	700
C2-4-A	0	800
	+	920

[a] Portions of virus diluted to contain roughly 10^2 to 10^3 DNA polymerase-forming units in 0.1 ml were incubated for 30 min at 37°C with equal volumes of a 20-fold dilution of immune or control chicken sera. The mixtures were diluted 5- and 50-fold with complete medium and were inoculated on assay plates containing chicken cells. The cells were overlaid with complete medium; 6 and 7 days after infection the supernatant media were harvested, and the amounts of sedimentable DNA polymerase activity were determined. The rates of incorporation were linear for 30 min. Only the values for the cultures inoculated with the fivefold dilutions of the neutralization mixtures are given, except for C8-2-6. The relative amounts of neutralization seen with the cultures inoculated with the 50-fold dilutions were similar. The relative amounts of neutralization were reproduced in usually two other experiments. Since non-neutralized virus may proliferate, the relative amounts of neutralization should be considered minimal estimates.

[b,c,d] Separate experiments.

[e] Supernatant medium from cultures inoculated with 50-fold dilution of neutralization mixture.

[f] W8-1-1, W8-1-2, and W8-2-7a were similar in not being neutralized by this antibody.

The type-specific differences among the RAV-F were also confirmed with another antibody to RAV-61, number 822. C8-1-6a, C8-2-6a, W2-1-2, and W2-1-5a were neutralized, whereas C8-2-6 and W8-1-1b were not (data not shown).

In addition, some of the newly isolated RAV were incubated with antibody to RAV-1 and assayed (Table 7). The RAV-F were not neutralized. Four of the RAV-A (W8-1-1, W8-1-2, W8-2-7a, and C2-4-A) were neutralized by the antibody to RAV-1, whereas one RAV-A (W8-1-4) was not significantly neutralized, indicating type-specific differences among the RAV-A.

A further type-specific difference among the newly isolated RAV-F was in the extent of cell killing. (The newly isolated RAV-A caused no detectable cell killing.) Most of the RAV-F, for example, W2-1-2 and C8-1-6, caused a cytopathic effect with only a small decrease in cell number, whereas two of the RAV-F (C8-1-6a and W8-1-1b) caused a cytopathic effect with a larger decrease in cell number in relation to the control cultures (Table 8).

DISCUSSION

Bryan high-titer strain RSV-RAV-0 is a mixture of two viruses, Bryan high-titer RSV and

TABLE 7. *Neutralization by antibody to RAV-1*[a]

Virus	Antibody	DNA polymerase activity (cpm of [^{3}H]TMP incorporated/15 min)
RSV-RAV-1	0	2,095
	+	550[b]
RAV-F		
RAV-61	0	3,340
	+	3,300
C8-1-6	0	1,350
	+	1,570
W8-1-1b	0	2,350
	+	4,775
RAV-A		
W8-1-4	0	360
	+	240[c]
W8-1-1	0	400
	+	0
W8-1-2	0	1,005[d]
	+	270
W8-2-7a	0	850[e]
	+	180
C2-4-A	0	1,515
	+	0

[a] Experimental procedures were as described in footnote *a*, Table 6, except the antibody was only diluted fivefold.

[b] The number of foci per plate was also reduced five- to tenfold.

[c] Not a significant reduction in DNA polymerase activity.

[d] A 75% decrease in amount of DNA polymerase activity was also found in two other experiments.

[e] Complete neutralization was found with antibody diluted twofold.

RAV-0, which replicate in pheasant cells with transformation and no cytopathic effect. Bryan high-titer RSV-RAV-0 does not infect C/E chicken cells since Bryan high-titer RSV is defective in an envelope glycoprotein and C/E chicken cells have no receptor for the glycoprotein of RAV-0. When Bryan high-titer RSV-RAV-0 was serially passed in pheasant cells and supernatant media were inoculated on C/E chicken cells, variant avian leukosis viruses capable of replicating in C/E chicken cells were found.

Eight variant viruses were isolated from the 12 different passage series described in Fig. 1 and 2, whereas in the other four passage series no variant viruses were detected. There were approximately 10^6 pheasant cells/culture, 11 days/passage, and 3 to 7 passages/series, with an average of about 5 passages/series. Therefore, a variant virus capable of replicating in C/E chicken cells appeared approximately once in 5×10^7 RSV-RAV-0-infected pheasant cell-days (10^6 cells $\times$ 11 days/passage $\times$ 5 passages gave a probability of about 1 [8/12] of appearance of a variant virus capable of replicating in C/E chicken cells).

TABLE 8. *Cytopathic effects of RAV-F*[a]

Virus	Cytopathic effect	No. of cells
None	0	4.7×10^6
RAV-61	+	4.1
W2-1-2	+	4.2
C8-1-6	+	4.1
C8-1-6a	+	3.1
W8-1-1b	+	2.9

[a] Duplicate cultures containing 6×10^5 chicken cells were inoculated with approximately 10^5 DNA polymerase-forming units of the viruses listed and were overlaid with complete medium. Medium and distilled water were added at 4 days after infection, and, at 7 days after infection, the cultures were examined for the presence of a cytopathic effect, and the number of cells per culture was determined. The values listed are the average of the values for duplicate cultures. Similar differences in extent of cell killing were found after inoculation of lower concentrations of virus.

Two major classes of viruses capable of replicating in C/E chicken cells were found (Table 9). One class, RAV-F, includes viruses similar to the previously described RAV-61 and ring-necked pheasant virus. The RAV-F included both of the viruses isolated from pheasant cells W2 (W2-1-2 and W2-1-5a), the four viruses isolated from pheasant cells C8 (C8-2-6, C8-2-6a, C8-1-6, and C8-1-6a), two of the three viruses isolated from ILV (C2-3 and C2-4-F), and one of the five viruses isolated from pheasant cells W8 (W8-1-1b). The RAV-F had a subgroup F envelope and were cytopathic, and eight of nine replicated well in duck cells.

Viruses of the second major class, RAV-A, had a subgroup A envelope, were not cytopathic, and did not replicate in duck cells. The RAV-A consisted of four of the five viruses isolated from pheasant cells W8 (W8-1-4, W8-1-1, W8-1-2, and W8-2-7a) and one of the viruses isolated from I-ILV (C2-4-A). C2-4 was originally a mixture of RAV-A with some RAV-F.

No RSV-A or RSV-F were found. This observation is consistent with the previous failures to secure nondefective Bryan high-titer RSV (24). The early focus formation seen in Fig. 1 must have been the result of phenotypic mixing.

However, there also were type-specific differences among the RAV. C8-1-6a caused more cell killing than most of the other RAV-F; C8-2-6, C2-3, and W8-1-1b were not neutralized by antibody to RAV-61; and W8-1-1b had a low titer in duck cells and caused more cell killing than most of the other RAV-F. W8-1-4 was not neu-

TABLE 9. *Comparison of newly isolated viruses, RAV-61, and RAV-0*

Virus	ALV group-specific antigens	ALV DNA polymerase	Relative titer		Interference with:		Proportion of neutralization by:		% Cell killing
			Ph/Ch[a]	Du/Ch[b]	RAV-61	RSV-A	Ab-RAV-61[c]	Ab-RAV-1[d]	
RAV-61	+	+	0.1	1	$>10^{5}$[e]	0[e]	0.95	0	10[f]
RAV-F	+	+	0.1-1	0.1-1[g]	$>10^5$	0	0-0.95	0	10-35
RAV-A	+	+	0.1-1	$<10^{-5}$	0-10	$>10^4$	0	0.35-1.0	0
RAV-0	+	+	$>10^6$		0	0	0	0	0

[a] Ph/Ch, Titer in pheasant cells/titer in chicken cells.
[b] Du/Ch, Titer in duck cells/titer in chicken cells.
[c] Ab-RAV-61, Antibody to RAV-61.
[d] Ab-RAV-1, Antibody to RAV-1.
[e] Amount of reduction of titer of RAV-61 or RSV-A when assayed on infected chicken cells.
[f] Reduction in number of cells 7 days after infection with viruses.
[g] W8-1-1b had a relative titer of 10^{-5}.

tralized by antibody to RAV-1 as were the other RAV-A, and W8-2-7a had a lower titer than the other RAV-A.

The results indicate that the pheasant embryo was the major determinant of the kind of RAV that appeared. The two RAV that appeared after RSV-RAV-0 passage in pheasant cells W2 were both of subgroup F and had similar type-specific characteristics. The four RAV from pheasant cells C8 were all of subgroup F and, with the exception of more cell killing by C8-1-6a and no neutralization of C8-2-6 by antibody to RAV-61, had similar type-specific characteristics. Four of the five RAV from pheasant cells W8 were RAV-A with similar type-specific characteristics, except for less neutralization of W8-1-4 by antibody to RAV-1 and a lower titer for W8-2-7a. The relative uniformity of the RAV from cells of a single embryo and the differences between the RAV isolated from cells of different embryos strongly supports recombination between virus and cellular nucleotide sequences as the mechanism for the origin of the RAV-A and RAV-F. Such a mechanism has been established by Hayward and Hanafusa (6) for the origin of RAV-60 in chicken cells and by Shoyab and Baluda (16, 17) for the origin of RAV-61 in pheasant cells.

The rate of appearance of RAV-A and RAV-F, one per 5×10^7 infected cell-days, is also consistent with such a mechanism. The rates of spontaneous mutation and of deletion are much greater (11, 21, 25). The rate of appearance of RAV-A and RAV-F is similar to those for the appearance of RAV-60 after passage in chick helper factor-negative cells and of RAV-61 and ring-necked pheasant viruses after passage in pheasant cells (2, 4, 5).

If the RAV-F and RAV-A arose by recombination with cellular nucleotide sequences, these results indicate that the endogenous avian leukosis virus-related nucleotide sequences in cells from different pheasant embryos are different; that is, pheasant cells W2 and C8 had subgroup F specificity, whereas pheasant cells W8 had subgroup A specificity. The properties of W8-1-1b indicate that W8 also had subgroup F specificity. One could speculate that the subgroup A specificity arose by duplication and mutation of the gene for subgroup F specificity or the reverse. C2-4 was originally a mixture of RAV-A and RAV-F. This mixture is consistent with such a relationship between subgroups A and F.

The type-specific differences among the RAV-F from C8 and the RAV-A from W8 and the origin of both RAV-A and RAV-F from cells of pheasant W8 could be the result of recombination at different sites in the endogenous avian leukosis virus-related nucleotide sequences.

The origin of the genes for the cytopathic effect is unclear. To confirm that the induction of a cytopathic effect was a new property of the RAV-F, RSV-RAV-0 and three RAV-F (W2-1-2, C8-1-6a, and W8-1-1b) were assayed on turkey cells. All of the RAV-F caused a definite cytopathic effect, with plaques appearing at higher dilutions of virus. However, the RSV-RAV-0 caused no cytopathic effect, although there was virus replication as shown by the production of sedimentable DNA polymerase activity (data not shown). (A similar problem occurs in understanding the origin of the genes for transformation in Kirsten murine sarcoma virus [15].) Since almost all of the RAV-F were cytopathic, and the RAV-A were not, the cytopathic effect seems linked to the subgroup F character.

Finally, these experiments may indicate that the envelope gene of subgroup A avian leukosis viruses originated from endogenous avian leukosis virus-related nucleotide sequences in pheasant cells, and, therefore, that subgroup A viruses may not have originated exclusively from the chicken cell genome.

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