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Host Susceptibility to Endogenous Viruses: Defective, Glycoprotein-Expressing Proviruses Interfere with Infections

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Three defective endogenous avian leukosis viruses, *ev3*, *ev6* and *ev9*, interfered with subgroup E virus infections. *ev3*, *ev6*, and *ev9* expressed high levels of subgroup E envelope glycoproteins. These glycoproteins reduced the activity of cellular receptors for subgroup E viruses. *ev3* and *ev6* protected chickens and cultured cells from subgroup E virus infections.

In nature, retroviral information is transmitted by DNA proviruses and viral infections. Viruses that are transmitted as proviruses in the germ line are termed endogenous viruses to distinguish them from so-called exogenous viruses that are transmitted by infections. Endogenous avian leukosis viruses (ALVs) encode envelope glycoproteins which have a subgroup E host range and interference pattern. These envelope glycoproteins distinguish endogenous ALVs from exogenous ALVs which have subgroup A, B, C, and D glycoproteins (for a review, see reference 25).

Most hosts are resistant to infection by viruses expressed by their germ line proviruses. In chickens, the major form of this resistance is encoded by genes that affect the entry of virus into cells. Dominant genes at two unlinked loci have been shown to affect the entry of subgroup E viruses into cells. One of these, which we designate *Tv-E^s*, is thought to code for receptors for subgroup E virus (6, 8, 22, 23, 28). The second, *I-E*, codes for an inhibitor of subgroup E receptors (8, 22, 23, 28).

In this paper, we demonstrate that chickens are polymorphic for three dominant genes that inhibit the activity of subgroup E receptors. We further demonstrate that each of these genes is a defective endogenous virus that expresses high levels of envelope glycoproteins. These three defective viruses are designated *ev3*, *ev6*, and *ev9* (2-4).

Cells infected with endogenous and exogenous ALVs have reduced susceptibilities to viruses of the same subgroup (38). This phenomenon is called interference. Interference is characterized by slow penetration of superinfecting virus into cells (32). We demonstrate that *ev3*, *ev6*, and *ev9* reduce the susceptibility of cells to subgroup E, but not to other subgroups, of virus. We further demonstrate that *ev9* reduces the rate of penetration of subgroup E, but not of subgroup B, virus into cells. We conclude that glycoproteins expressed by defective endogenous viruses interfere with subgroup E virus infections.

MATERIALS AND METHODS

Chickens. K16, K18, K28, and K(-) chickens are random-bred lines of White Leghorns (28). K16 has a gs$^+$ chf$^+$V$^-$ phenotype for endogenous virus expression (gs represents the expression of antigenic determinants that are group specific to ALVs, chf represents the expression of glycoproteins that can serve as subgroup E envelope glycoproteins, and V represents the production of virus particles). At the time of these crosses K16 was homozygous for *ev1* and *ev3*, and segregating for *ev4*, *ev5*, and *ev6* (3). K18 has a gs$^-$ chf$^+$V$^-$ phenotype and at the time of these crosses was homozygous for *ev1*, and segregating for *ev4*, *ev6*, *ev8*, and *ev9* (4; H. Robinson and S. Astrin, manuscript in preparation). K(-) and K28 have gs$^-$ chf$^-$V$^-$ phenotypes. The K(-) and K28 chickens used in these crosses contained only *ev1*.

K16, K18, and K(-) chickens do not contain *Tv-E^s*, a dominant allele which codes for receptors for subgroup E virus (6, 8, 22, 23, 28). K28 is segregating for this allele (28). The *Tv-E^s* allele of K28 cosegregates with susceptibility to subgroup B virus (28) and is sometimes designated *Tv-B^{s2}*. It is not clear whether *Tv-E^s* and *Tv-B^{s2}* are identical or distinct genes. Genetic analyses place them within 1.7 map units or ~3 $\times$ 10^6 base pairs of each other. Functional studies indicate that subgroup B viruses interfere with subgroup B and E viruses, whereas most subgroup E viruses (including all of the endogenous origin) interfere with subgroup E, but not subgroup B, viruses (13, 24, 37). These interference patterns could result from subgroup B viruses having an affinity for receptors coded for by each of two closely linked genes or from B viruses having a greater affinity than E viruses for receptors coded for by a single gene.

Cell culture and virus assays. Cells were cul-

tured from 10- to 12-day-old embryos in Dulbecco-modified Eagle medium supplemented with 10% tryptose phosphate broth, 4% calf serum, and 1% chicken serum. Virus infections were done in the presence of 10 μg of Polybrene per ml.

Endogenous virus loci. Endogenous virus loci were identified by testing for characteristic ALV-related restriction endonuclease *SstI* fragments and *Bam*HI fragments in DNA isolated from bloods of breeding birds or cultured cells or muscle of embryonic progeny (2, 4). DNAs were digested with restriction endonucleases, fractionated on agarose gels, transferred to nitrocellulose filters, and analyzed for ALV-related fragments by using Rous-associated virus-2 (RAV-2) [^{32}P]RNA (specific activity, ~10^7 cpm/μg of viral RNA) (2).

RESULTS

Segregation of *ev3* with an intermediate susceptibility to subgroup E virus, and *ev6* and *ev9* with low susceptibilities to subgroup E virus. In 1977 and 1979, genetic crosses were performed to define the inheritance of endogenous viral loci and endogenous virus expression in gs$^+$chf$^+$ K16 chickens and gs$^-$ chf$^+$ K18 chickens (3, 4). Because research from several laboratories indicated that the cells cultured from chf$^+$ *Tv-E^s* embryos had lower susceptibilities to subgroup E viruses than cells cultured from chf$^-$ *Tv-E^s* embryos (1, 8, 23, 28), crosses were designed so that we could follow susceptibility to endogenous virus as well as endogenous proviruses and endogenous virus expression. In 1977 and 1979, this meant backcrossing relevant hens [K16 × K(−) or K18 × K(−)] with K28 chickens that were homozygous for *Tv-E^s*.

All progeny of the 1977 and 1979 crosses had similar high susceptibilities to subgroup B and C virus. In striking contrast to their relatively uniform susceptibilities to subgroup B and C virus, progeny had widely different susceptibilities to subgroup E virus. The plating efficiency of subgroup E avian sarcoma viruses ranged from 1×10^1 to 2×10^5 focus-forming units per (FFU) ml on the progeny of the 1977 matings and from 1×10^1 to 10^6 FFU/ml on the progeny of the 1979 matings (Fig. 1).

Comparison of assays for endogenous virus expression with assays for susceptibility to subgroup E virus indicated that all chf$^-$ embryos had a high level of susceptibility to subgroup E virus (Fig. 1). In contrast to the chf$^-$ progeny which had high and relatively uniform susceptibilities to subgroup E virus, chf$^+$ progeny had lower and less uniform susceptibilities to subgroup E virus. In the 1977 crosses, BH-RSV(RAV-0) had an intermediate plating efficiency on chf$^+$ progeny of three K16 × K(−) hens (Fig. 1A, 1977 matings) and a low plating efficiency on chf$^+$ progeny of a fourth K16 × K(−) hen (Fig. 1B, 1977 matings). In the 1979 matings, BH-RSV(RAV-60) had a low plating efficiency on all chf$^+$ progeny.

Analysis of endogenous virus loci in the progeny of the 1977 and 1979 matings indicated that *ev1*, *ev4*, *ev5*, and *ev8* segregated with the gs$^-$ chf$^-$ phenotype; *ev3*, with the gs$^+$ chf$^+$ phenotype; and *ev6* and *ev9*, with the gs$^-$ chf$^+$ phenotype. Quite interestingly, embryos that contained only *ev3* exhibited an intermediate susceptibility to subgroup E virus (Fig. 1A, 1977 matings), whereas embryos containing *ev6* exhibited low susceptibilities to subgroup E virus (Fig. 1B, 1977 matings). Thus, in the 1977 matings, intermediate levels of susceptibility to subgroup E virus segregated with *ev3*, and low levels of susceptibility to subgroup E virus segregated with *ev6*.

ev6 and *ev9* segregated in the 1979 matings. These loci segregated with low susceptibilities to subgroup E virus. These levels were comparable to those observed for the *ev6* progeny in the 1977 matings.

Inverse correlation between level of chf expression and susceptibility to subgroup E virus in *ev3*, *ev6*, and *ev9* cells. Several workers have reported higher levels of expression of chf in gs$^-$ chf$^+$ cells (extremely high helper cells) than in gs$^+$ chf$^+$ cells (1, 14, 28). To determine the relative levels of expression of chf in cells that contained *ev1*, *ev1* and *ev3*, *ev1* and *ev6*, and *ev1* and *ev9*, relevant cells were assayed for chf. Three experiments were performed in which one or two embryos of each of the desired genotypes were assayed for chf. In these assays, cells were infected at a high multiplicity of infection with BH-RSV(RAV-7), a subgroup C ALV. Two days after infection, the culture medium was harvested and assayed for BH-RSV(RAV-7) and for BH-RSV(chf). The titer of BH-RSV(RAV-7) was used as an indicator of the extent of infection of cells, and the titer of BH-RSV(chf) was used as a measure of the level of chf expressed by cells.

Two days after infection, all infected cultures produced high titers of BH-RSV(RAV-7) (10^5 to 10^6 FFU/ml). In contrast to the relatively uniform production of BH-RSV(RAV-7), cultures produced different levels of BH-RSV(chf). *ev1* cells produced 10 to 60 FFU/ml of BH-RSV(chf), *ev1* plus *ev3* cells produced 10^4 to 10^5 FFU/ml, *ev1* plus *ev6* cells produced 2×10^4 to 4×10^5 FFU/ml, and *ev1* plus *ev9* cells produced 1×10^5 to 7×10^5 FFU/ml (Fig. 2). Repeat assays on selected embryos indicated that independent assays on the same cultures gave titers of BH-RSV(chf) that varied as much as fivefold.

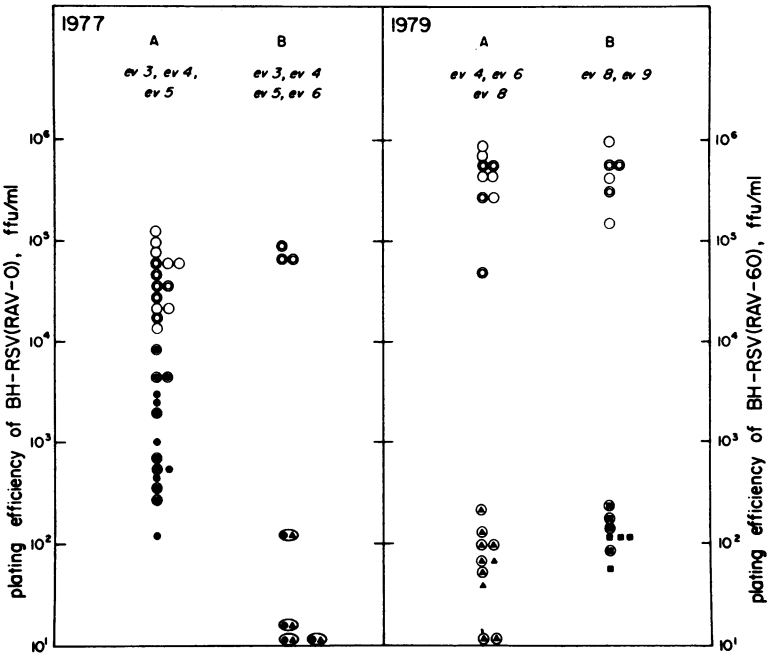

FIG. 1. *Plating efficiency of subgroup E avian sarcoma viruses on Tv-E^s embryos which were segregating for ev3, ev4, ev5, ev6, ev8, and ev9. 1977 Matings, [K16 × K(−)] × K28; 1979 matings, [K18 × K(−)] × K28. Primary cultures from the 1977 matings were tested for susceptibility to BH-RSV(RAV-0) and from the 1979 matings to BH-RSV(RAV-60). BH-RSV(RAV-0) and BH-RSV(RAV-60) are subgroup E avian sarcoma viruses. Differences in titer of subgroup E viruses on chf$^-$ embryos in the 1977 and 1979 matings reflect differences in the titer of BH-RSV(RAV-0) and BH-RSV(RAV-60). Stocks of BH-RSV(RAV-60) have fivefold-higher titers than do stocks of BH-RSV(RAV-0). Data for 1977 (A) came from three matings, 1977 (B) came from one mating, 1979 (A) came from two matings, and 1979 (B) came from two matings. All embryos exhibited comparable susceptibilities to BH-RSV(RAV-2). Symbols: ○, ev1 Embryos; ●, ev1 ev3 embryos; ▲, ev1 ev6 embryos, and ■, ev1 ev9 embryos. Embryos that contained ev4, ev5, and/or ev8 in addition to the loci designated by ○, ●, ▲, and ■ are circled.*

Data from repeat assays, however, fell within the range of values previously observed for each genotype. From these assays we conclude that *ev6* and *ev9* express similar amounts of chf that are approximately fourfold higher than the amount of chf expressed by *ev3*.

Table 1 correlates the expression of chf (data taken from Fig. 2) with susceptibility to subgroup E virus (data taken from Fig. 1). These data indicate that the susceptibility *Tv-E^s* cells to subgroup E virus is inversely proportional to the level of expression of chf in the cells.

Reduced rate of penetration of subgroup E virus into chf$^+$ cells. ALV-infected chicken cells exhibit superinfection resistance to viruses that belong to the subgroup of infecting virus (38). This phenomenon is called viral interference. Studies on the mechanism of interference indicate that the few viruses that establish infections in cells that exhibit interference undergo slow rates of penetration into cells (32). To determine whether chf affected the rate of penetration of subgroup E virus into cells, *Tv-E^s* chf$^-$, *Tv-E^s* chf$^+$, and subgroup E virus-infected *Tv-E^s* chf$^-$ cells were analyzed for adsorption and penetration of subgroup B and E viruses.

Studies on the adsorption of subgroup E virus to chf$^-$, chf$^+$, and subgroup E virus-infected chf$^-$ cells revealed that under standard assay conditions, adsorption of subgroup E virus to cells is nonspecific. Both susceptible and resistant cells adsorbed 30% of the virus that was added to assay plates. The presence of Polybrene increased the amount of virus adsorbed but did not affect the specificity of adsorption (data not shown).

To follow penetration of virus into cells, cells were infected and then at various times after infection briefly treated with mild acid (Fig. 3). Brief acid treatments inactivate ALVs that have not undergone penetration (32).

The rate of penetration of subgroup E virus into chf$^+$ and subgroup E virus-infected cells was four times slower than the rate of penetration of

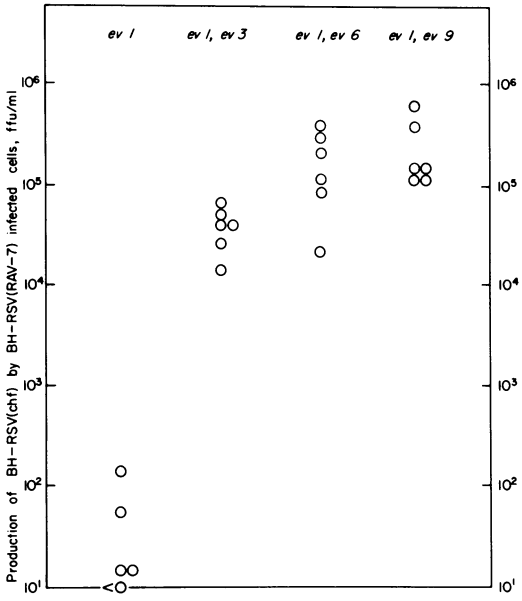

FIG. 2. *Expression of chf by ev3, ev6, or ev9. Cells with the indicated combinations of endogenous proviruses were infected at a multiplicity of infection of 5 with BH-RSV(RAV-7), 2 days after the infection medium was harvested and assayed for BH-RSV(RAV-7) on subgroup E virus-resistant K(−) cells and BH-RSV(chf) on subgroup C virus-resistant 15_B cells.*

TABLE 1. *Expression of chf and susceptibility to subgroup E viruses in different classes of chf⁺ embryos*

Endogenous proviruses[a]	chf[b]	Susceptibility to subgroup E virus[c]
ev1 (*ev4*, *ev5*, *ev8*)	15	1.0
ev1, *ev3* (*ev4*, *ev5*)	4.0×10^4	0.03[d]
ev1, *ev6* (*ev4*, *ev5*, *ev8*)	1.5×10^5	0.0002[d] 0.0001
ev1, *ev9* (*ev8*)	1.5×10^5	0.0002[e]

[a] Proviruses in parentheses were segregating in embryos.

[b] Median level of chf expression; data were taken from Fig. 2.

[c] Median titer of BH-RSV(RAV-0) or BH-RSV(RAV-60) on embryos which contained *ev3*, *ev6*, or *ev9* divided by the median titer of these viruses on embryos which did not contain these loci. Data were taken from Fig. 1.

[d] 1977 Matings.

[e] 1979 Matings.

subgroup E virus into chf⁻ *Tv-E*^{s} cells. As expected, subgroup B virus had a similar rate of penetration into all three cell types. Since viral interference is characterized by low, subgroup-specific rates of penetration of virus into cells, we suggest that the expression of chf establishes interference for subgroup E viruses.

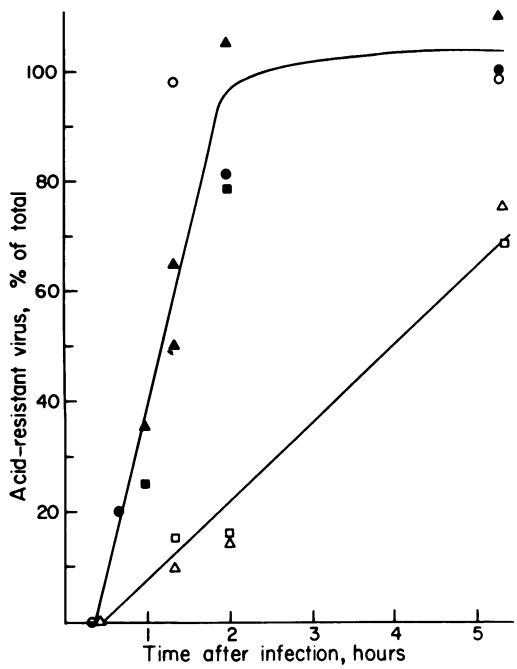

FIG. 3. *Penetration of subgroup B and E RSV into chf⁻ cells, subgroup E virus-infected chf⁻ cells, and chf⁺ cells. BH-RSV(RAV-2) (a subgroup B avian sarcoma virus) or BH-RSV(RAV-60) (a subgroup E avian sarcoma virus) was adsorbed to relevant cells for 10 min at 37°C. After adsorption, cells were washed with growth medium, incubated at 37°C in growth medium, and acid treated (0.05 M glycine·HCl; pH 2.2) at the indicated times. Acid-resistant virus, a percentage of the total, is FFU of virus on acid-treated plates per FFU of virus on untreated plates times 100. Symbols: ● and ○, C/O chf⁻ cells, K28 × K(−) embryo 1706; ■ and □, NTRE-7 (subgroup E ALV)-infected 1706 cells; ▲ and △, C/O chf⁺ cells, K28 × [K18 × K(−)] ev1 ev9 embryo 1865; ●, ■, ▲, BH-RSV(RAV-2) for which titers were determined at 2.7×10^6, 2.4×10^6, and 2.5×10^6; ○, □, and △, BH-RSV(RAV-60) for which titers were determined at 2.5×10^4, 3.2×10^2, and 2.9×10^2 FFU/ml on the respective cell types.*

Protection of chickens against endogenous virus infections by chf. 15_B Roosters, which are homozygous for *Tv-E*^{s} and *ev7* (19), were crossed with *ev1*, *ev1 ev3*, and *ev1 ev6* hens to obtain *Tv-E*^{s} progeny that had *ev1* and *ev7* in addition to *ev3* or *ev6* (26). *ev7* is a defective endogenous virus that codes for low levels of expression of noninfectious particles (15_B-ILV). Cells that contain *ev7* in addition to *ev1*, *ev3*, or *ev9* occasionally produce infectious subgroup E viruses. These infectious viruses are recombi-

nants of the product of *ev7* with the products of *ev1*, *ev3* or *ev9* (27). Recombinants occur at a frequency of about one in 4×10^8 cell days in bromodeoxyuridine-induced *ev1 ev7* cells and about one in 5×10^6 cell days in induced *ev1 ev3 ev7* or *ev1 ev7 ev9* cells. All *Tv-E^s ev1 ev7* chickens are viremic within a few months of birth.

Tv-E^s ev1 ev7, *ev1 ev3 ev7*, and *ev1 ev6 ev7* chickens were hatched and reared. By 1 year of age, 14 of 14 *Tv-E^s ev1 ev7* chickens were either viremic or had neutralizing sera for subgroup E virus. In contrast, none of seven *ev1 ev3 ev7* or *ev1 ev6 ev7* chickens was viremic. Four *ev1 ev3 ev7* and *ev1 ev6 ev7* birds were maintained to 2 years of age. At 2 years of age, none of these birds exhibited evidence for infection by subgroup E virus (Table 2).

We suggest that the expression of chf by *ev3* or *ev6* prevented the horizontal spread of spontaneously expressed subgroup E viruses in *ev7*-containing chickens. This result is particularly impressive because cultured *ev1 ev3 ev7* cells express infectious subgroup E virus at about 100 times the rate of *ev1 ev7* cells.

DISCUSSION

Interference with endogenous virus infections by defective glycoprotein-expressing proviruses. One salient feature of the relationship of endogenous viruses to their hosts is the resistance of hosts to the horizontal spread of their virus. In this paper, we demonstrate that one form of host resistance to endogenous virus infections is coded for by endogenous proviruses that express high levels of envelope glycoproteins. These glycoproteins establish viral interference presumably by binding to receptors for viruses.

Our studies indicate that interference by proviruses is analogous to interference by endogenous or exogenous virus infections. First, for both horizontally and vertically transmitted viruses, the establishment of interference depends on the presence of a provirus that codes for envelope glycoproteins (Fig. 1) (30, 39). Expression of complete virus or internal proteins is not necessary. Second, the extent of interference depends on the amount of glycoprotein that is present in a cell (Fig. 2; Table 1) (30). High levels of glycoprotein correlate with high levels of interference. Third, the specificity of interference is determined by the subgroup or type of the envelope glycoprotein. Envelope glycoproteins interfere only with the activity of receptors for which they have an affinity (Fig. 3) (38). Finally, interference results in reduced rates of penetration of viruses with envelope glycoproteins of the same subgroup as the interfering virus (Fig. 3) (32).

TABLE 2. *Interference by ev3 and ev6 with the spread of endogenous viruses in chickens*

Endogenous proviruses[a]	Endogenous virus infections[b]	
	1 yr old	2 yr old
ev1, ev7	14/14[c]	NT[d]
ev1, ev3, ev7	0/3	0/2
ev1 (ev4), ev6, ev7	0/4	0/2

[a] Viruses that were present in some, but not all, chickens are indicated in parentheses.

[b] The presence in sera of particulate RNA-directed DNA polymerase (for assay conditions, see reference 24) or neutralizing activity for subgroup E virus was used as evidence for the horizontal spread of virus.

[c] Number of chickens which exhibited evidence for the spread of endogenous viruses/number tested.

[d] NT, Not tested.

The experiments we performed to elucidate the mechanism of interference by chf indicate that chf slows the rate of penetration of subgroup E viruses into cells. Although these experiments demonstrate that interference by chf occurs at the same point in infection as interference by exogenous virus infections, they unfortunately do not add to our current knowledge of viral interference. When we undertook these studies, we had hoped that by working with subgroup E viruses instead of subgroup A viruses (32) we would be able to study receptor-specific adsorption of virus. This hope was based on two observations which suggested that subgroup E viruses might undergo less nonspecific adsorption to chicken cells than subgroup A viruses: subgroup E viruses are shed more efficiently by infected cells than subgroup A viruses; adsorption of subgroup E virus but not subgroup A virus is enhanced by cations.

Our experiments on interference by chf do, however, indicate that reduced rates of penetration are not the primary cause of interference (Fig. 3). Reductions in the rate of penetration by 3- to 4-fold do not account for 10^3- to 10^4-fold-lower susceptibilities of cells to viruses. Presumably, the primary cause of interference is competition of envelope glycoproteins with virus for receptors. Endogenously synthesized glycoproteins are shed from cells (31). Consequently, interference by chf could result from the binding of intracellular or extracellular glycoprotein to receptors.

Survival value of defective glycoprotein-expressing endogenous viruses. Several lines of evidence are consistent with defective proviruses having survival value. Recent work suggests that most endogenous viruses result

from reinfection of the germ line by endogenous viruses (D. Steffen, personal communication). The vast majority of retroviral infections do not result in proviruses that express envelope antigens in the absence of normal internal proteins (16, 17, 30). Twenty-five percent (3 of 12) of endogenous avian proviruses have this phenotype. The relatively frequent occurrence of defective envelope-expressing endogenous viruses suggests that nature selects germ line proviruses for this phenotype. Second, *ev3*, *ev6*, and *ev9* are the only endogenous viruses that are expressed at high levels (15). All others are unexpressed or expressed at extremely low lines. The fact that these and only these proviruses are expressed at high levels strongly suggests that expression of envelope glycoproteins has survival value. A third line of evidence, which is consistent with the expression of chf having survival value, is the broad distribution of the chf$^+$ phenotype in nature. The vast majority of commercial chickens (12, 28) have this phenotype (7, 36).

Recent evidence indicates that *ev3* has survival value for chicks infected with exogenous ALVs (5; L. Crittenden et al., manuscript in preparation). Chickens that do not express chf frequently display lethal inflammatory responses to exogenous virus infections. Such chickens have unusually high titers of neutralizing sera to viruses. chf is known to establish tolerance to antigenic determinants that are group specific to ALV glycoproteins (11). Thus, *ev3*, *ev6*, and *ev9*, by establishing partial tolerance to ALV envelope antigens, may protect the host from debilitating immune responses to exogenous virus infections.

Endogenous viruses that express envelope antigens may also have survival value in that they protect their hosts from endogenous virus infections (Table 2). Endogenous ALVs do not cause disease in chickens (20; H. Robinson et al., manuscript in preparation). Replication competent endogenous viruses do, however, reinfect the germ line of hosts (29). Analyses of recombinant inbred strains of mice suggest that germ line infections occur at remarkably high frequencies in mice that have a replication competent endogenous virus and are susceptible to infection by that virus. Of 15 such lines, 3 acquired new endogenous viruses during a period of 10 years (D. Steffen, personal communication). Each integration of a virus into the chromosome of a host is a mutational event. Each such event has the potential for altering the phenotype of a cell. Thus, the presence of endogenous viruses that interfere with endogenous virus infections would protect the germ line from the accumulation of proviruses and provirus-associated mutations.

Occurrence of defective glycoprotein-expressing proviruses in other species. The expression of envelope glycoproteins by germ line proviruses does not appear to be limited to endogenous avian viruses. Several workers have reported the expression of murine retroviral envelope antigens in cells and sera of mice which do not appear to have replicating viruses (9, 19, 21, 33, 34). Although these glycoproteins have not been shown to play a role in determining the susceptibility of mice to virus infections, our bias is that these glycoproteins will interfere with endogenous virus infections. Recently two genes, both of which are dominant, have been reported to reduce the susceptibility of mice to infection by ecotropic, but not amphotropic, viruses (10, 35, 40). We suggest that these genes, *Fv-4^r* and *Akvr-1*, are defective endogenous viruses that express high levels of ecotropic envelope glycoproteins. We further speculate that *Fv-4^r* and *Akvr-1* are but the first in a series of defective envelope-expressing proviruses that will be found to reduce the susceptibility of mice to ecotropic, xenotropic, and amphotropic virus infections.

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