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Incidence of endogenous viral genes in two strains of White Leghorn chickens selected for egg production and susceptibility or resistance to Marek's disease *

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Summary. Endogenous viral (*ev*) genes related to the avian leukosis virus were classified in two differentially selected strains of Leghorns in order to investigate whether such genes affect production traits. Strain K had been selected for resistance to Marek's disease (MD) and for high egg production and egg weight, whereas strain S had been selected only for MD susceptibility. Except that founders of strain K included a few commercial birds, both strains were derived from a common genetic base. DNA restriction fragment length analyses of 110 strain K and 94 strain S birds revealed the presence of 8 different *ev*-genes, 6 of which were identical to previously identified loci. This result was confirmed by assays for group specific antigen (gs-antigen), the product of the *gag* region of the *ev*-genes. The levels of gs-antigen in the birds closely followed what had been predicted from data obtained from previously described *ev*-genes. Both strains had a similar average number of *ev*-genes per bird (3.5 and 3.2 for strains S and K, respectively). However, strain K carried only five different *ev*-genes while strain S carried seven. Four of these loci were present in both strains. Among the *ev*-genes absent or occurring less frequently in strain K were those that code either for infectious endogenous virus (*ev*-10 and possibly *ev*-19) or for the internal viral *gag*-proteins (*ev*-3). Only those *ev*-genes which are transcriptionally silent or which code for the viral envelope gene were present in increased frequencies in strain K. The results indicate that selection for egg traits and/or Marek's disease resistance reduces the frequency of *ev*-genes which produce endogenous virus or the viral *gag*-proteins.

Key words: Egglayers – Marek's disease resistance – Egg production – Endogenous viral genes – Group specific antigen

Introduction

All commercial chickens thus far tested carry DNA sequences that are related to an endogenous avian leukosis virus (*ev*-genes). In White Leghorns, 21 different *ev*-genes have been identified, and many more appear to be present in other breeds of chickens. An individual White Leghorn might carry up to ten different *ev*-genes. Some of these are transcriptionally silent, whereas others produce protein components of the virus or even the complete infectious virus (Rovigatti and Astrin 1983; Smith 1986).

Crittenden et al. (1979) reported a reduced incidence of endogenous virus production in strains selected for egg yield and related traits. Further, chickens that possess *ev*-genes which produce endogenous virus or viral proteins display a reduced immune response upon infection with exogenous avian leukosis virus, indicating that they are partially immunotolerant (Crittenden et al. 1984). The presence of such genes might, therefore, lead to a higher incidence of viremia in chicken flocks (Crittenden et al. 1982), thus resulting in reduced productivity and increased mortality (Gavora 1986).

To obtain further evidence of the effects of *ev*-genes on productivity, we examined the influence of selection for production traits on the incidence of individual *ev*-genes in two strains of White Leghorn chickens derived from a common genetic base. Strain S was selected for susceptibility to Marek's disease (MD), whereas strain K was selected for resistance to MD and high egg production.

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Materials and methods

Strains of chickens

Strains S and K used in this study were developed at Cornell University (Hutt and Cole 1947; Cole and Hutt 1973; Gavora et al. 1979). In brief, the strains were derived from a common base population in 1935–1936, but founders of strain K included a few commercial birds ('Kimber') introduced in 1936 and 1940. Thereafter, the strains have reproduced as closed populations. Since 1936, strain S and strain K have been selected for susceptibility or resistance to the "avian leukosis complex", respectively. It was later recognized that Marek's disease was the most prevalent disease in the complex (Cole 1968). Strain K was also selected for high egg production and egg weight. In 1966, strains S and K were imported from Cornell University to the Animal Research Centre in Ottawa, where they have been maintained by random mating without selection. In a test conducted in Ottawa, MD incidence after challenge of 3-week-old birds with MD virus was 86% in strain S and 31% in strain K (Grunder et al. 1972).

The birds used in this study received no vaccines or medication and were free of exogenous lymphoid leukosis viruses, MD virus and *Mycoplasma gallisepticum*. They were housed in a positive-air-pressure building supplied with filtered air to minimize the chances of introducing pathogens (Grunder et al. 1975). The populations studied here were hatched in 1985. Strains S and K had, respectively, an average annual egg production of 163 and 232 eggs per hen housed and average egg weights at 240 days of age of 48.39 and 52.8 g. Blood samples for *ev*-analysis were collected between the ages of 8 and 12 months. The feather pulp samples for ELISA analyses were collected when the birds were 9½ months old. Mortality in both strains was low and hence unlikely to have affected the frequencies of the various *ev*-genotypes.

Detection of group specific antigen

Group specific antigen (gs-antigen), the product of the *gag*-gene, was measured in feather pulp by an ELISA assay (Smith et al. 1979). For details on this procedure, see Spencer (1986). Briefly, for each chicken four feather tips containing live pulp were added to 2 ml PBS and homogenized with a Polytron homogenizer fitted with a PT 10 probe generator (Brinkmann Instruments). After centrifugation, the supernatant was tested for gs-antigen using rabbit antiserum against the viral p27 protein (Life Sciences). The results were expressed as absorbance at a wavelength of 440 nm.

DNA isolation

Red blood cells were separated from serum by centrifugation at 1,000 *g*. Aliquots of 30 µl packed blood cells were resuspended in 4 ml buffer containing 100 m*M* NaCl, 20 m*M* TRIS-HCl (pH 8.0) and 10 m*M* EDTA; 100 µl 20% SDS and 35 µl proteinase K (10 mg/ml) were then added, and the samples were incubated overnight at room temperature. The samples were extracted twice with phenol-chloroform-isoamyl alcohol (25 : 24 : 1) and precipitated with ethanol. The precipitate was recovered with a glass-rod, washed with 100% ethanol, air-dried and dissolved in 0.5 ml 5 m*M* TRIS-HCl (pH 7.5) and 0.1 m*M* EDTA. DNA yield was approximately 250 µg.

Southern blotting

Two microlitres DNA were incubated for 4 h at 37 °C with 10 units *Bam*HI or *Sac*I restriction enzyme using the reaction buffers recommended by the supplier (Boehringer Mannheim). The samples were then subjected to gel electrophoresis on a 0.8% agarose gel in TPE buffer (Maniatis et al. 1982) at 1.2 V/cm for 16 h. Thirty well combs were normally used to form 2 sets of wells in a single 15 cm × 25 cm gel slab; this enabled us to run 60 samples on a single gel. After the run, the gels were stained with ethidium bromide to visualize the DNA and to check for proper digestion and migration of the DNA. The gels were subsequently treated for 15 min with 0.25 *M* HCl to depurinate the DNA. Blotting was carried out onto a charged nylon membrane (Zeta-Probe, Bio-Rad Labs.) in 0.4 *M* NaOH (Reed and Mann 1985) according to the suppliers' instructions. After transfer, the filters were washed with 2 × SSC (0.3 *M* NaCl, 0.03 *M* trisodium citrate) containing 0.1% SDS.

Hybridization

Prehybridization was carried out in plastic bags at 42 °C for at least 2 h in 4 × SSPE (0.72 *M* NaCl, 40 m*M* sodium phosphate (pH 7.4), 4 m*M* EDTA), 1% SDS, 50% formamide, 0.5 mg/ml herring sperm DNA and 5 mg/ml skim milk powder (Carnation). Hybridization was carried our overnight at 42 °C. The hybridization mixture (15 ml) was the same as that used for prehybridization, except that it contained 10% dextran sulfate, 100 ng denatured ^{32}P-labelled pRAV-2 and no herring sperm DNA. After hybridization, the filters were washed successively at room temperature with 2 × SSC, 0.5 × SSC and 0.1 × SSC (all buffers also contained 0.1% SDS). A final wash was carried out with 0.1 × SSC (+0.1% SDS) for 30 min at 52 °C. The filters were blotted dry and autoradiographed overnight at −70 °C using Kodak XAR-5 film and DuPont Cronex intensifying screens.

Probes and reference DNA

The plasmid pRAV-2 is a pBR322 plasmid which contains RAV-2 sequences ligated into the *Sal*I site. The RAV-2 sequences originated from the Charon 21A bacteriophage clone RAV-2-1 (Ju et al. 1980) which contains the DNA complementary to the RNA of Rous-associated virus-2. The plasmid was a generous gift from Dr. L. B. Crittenden, Regional Poultry Laboratories, East Lansing, Michigan. ^{32}P-labelled plasmid was prepared with the nick translation kit from Boehringer Mannheim and purified using the spun column method (Maniatis et al. 1982). Specific activities were $1-2 \times 10^8$ dpm per µg DNA.

Reference genomic chicken DNAs containing either *ev*-3, *ev*-6, *ev*-1/*ev*21, or *ev*-1/*ev*-8 were a generous gift of Dr. E. J. Smith (Regional Poultry Laboratories, East Lansing, Michigan). Two additional genomic DNAs carrying the *ev*-loci 1, 3, 7, 19 and 20 and the *ev*-loci 1, 7 and 10 were kindly provided by Dr. E. H. Humphries (Department of Microbiology, University of Texas, Dallas).

Results

Analysis of ev-loci

Ev-loci were classified as summarized by Rovigatti and Astrin (1983) and Smith (1986). This classification is based on the lengths of the junction fragments obtained when genomic DNA is digested with either *Sac*I or *Bam*HI restriction enzyme. Junction fragments are those fragments which contain viral as well as cellular DNA. The length of such fragments represents the distance between the restriction sites on the viral and cellular DNA which are nearest to the junction of the viral and cellular DNA.

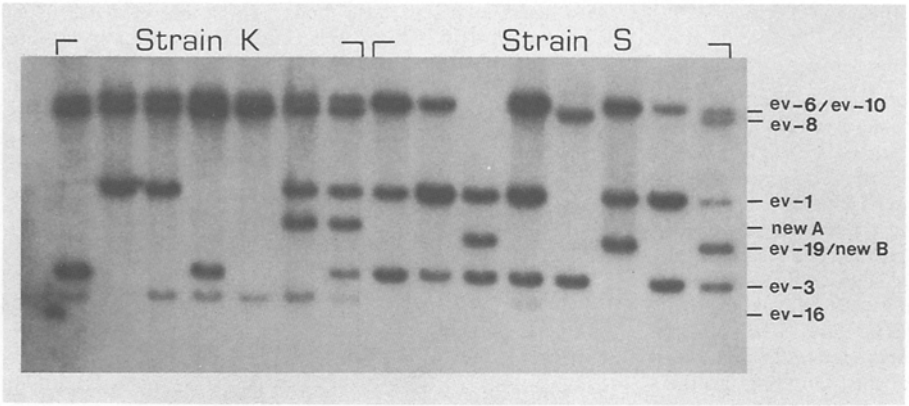

Fig. 1. Southern blot of *Sac*I-digested DNA from strain K and strain S chickens hybridized with pRAV-2. *Ev*-loci were identified by comparing them with DNAs known to contain the particular locus under study, with the exception of *ev*-16. The identification of *ev*-16 is based solely on a comparison of the molecular weights of the digestion fragments with those published

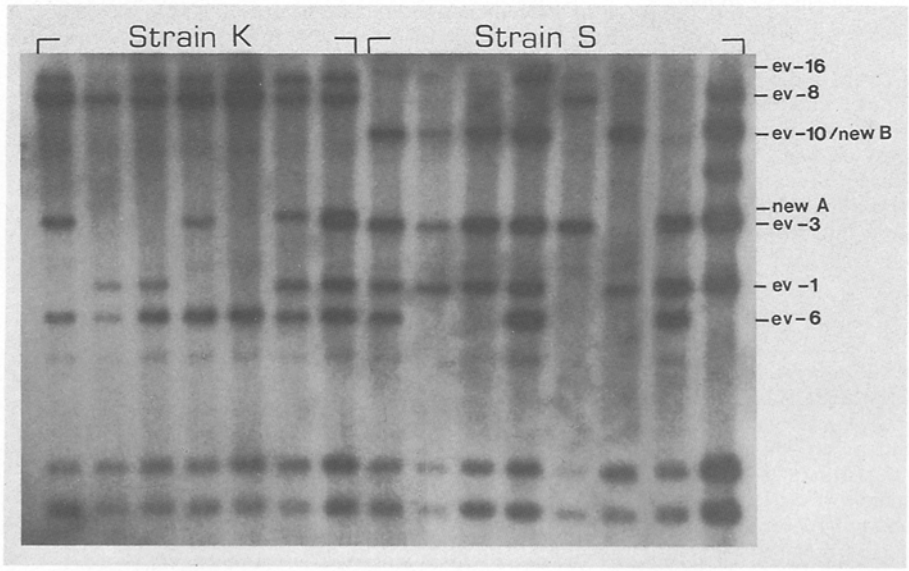

Fig. 2. Southern blot of *Bam*HI-digested DNA from strain K and strain S chickens hybridized with pRAV-2. DNA samples are the same and follow the same order as in Fig. 1

This distance generally depends on the cellular location of the proviral integration site and is, therefore, characteristic for a particular *ev*-locus.

Endogenous viral genomes contain *Sac*I and *Bam*HI sites close to the 5′-junction (Shank et al. 1981). Consequently, only the 3′-junction fragment will hybridyze to the pRAV-2 probe used to detect retroviral sequences. Southern blots of *Bam*HI digests also reveal two internal fragments whose molecular weight is the same for all *ev*-loci unless some of the internal sequences of the *ev*-gene have been deleted (Rovigatti and Astrin 1983).

Figures 1 and 2 show Southern blots of *Sac*I and *Bam*HI digests of a series of strain S and strain K chickens. Seven distinct bands were observed upon *Sac*I digestion and eight bands upon *Bam*HI digestion. The *Sac*I and *Bam*HI fragments belonging to the same *ev*-locus were determined by matching their blots to those of birds which had a single *Sac*I band and a single characteristic *Bam*HI band, and consequently must have a single *ev*-locus. This approach made it possible to match bands in birds with increasingly complex banding patterns. The pairing of *Sac*I bands and *Bam*HI bands proved to be

Table 1. Frequencies and phenotypes of *ev*-genes in females of strains S and K

Size of 3'-specific DNA fragment (kb)		Locus	Phenotype[a]	Frequency[d]	
				Strain S ($N=94$)	Strain K ($N=110$)
*Sac*I	*Bam*HI				
9.5	5.2	*ev*-1	gs^-chf^-	0.78	0.36
6.3	7.3	*ev*-3	gs^+chf^+	0.96	0.63
21	4.4	*ev*-6	gs^-chf^+	0.28	1.00
18.0	23	*ev*-8	gs^-chf^-	0.28	0.95
21	14.0	*ev*-10	$V\text{-}E^+$	0.65	0.00
7.6	9.8	*ev*-19	$V\text{-}E^+$ (?)[b]	0.24	0.00
8.2	7.4	new A	gs^- (?)[c]	0.00	0.24
7.6	14.0	new B	NI	0.35	0.00

[a] Summarized by Smith (1986). Phenotypes are abbreviated as follows: $V\text{-}E^+$, production of infectious endogenous virus; gs^+, expression of internal viral *gag* proteins; chf^+, expression of the viral envelope protein

[b] The phenotype of *ev*-19 is uncertain. Chickens carrying this locus together with *ev*-20 are $V\text{-}E^+$ (Humphries et al. 1984). Our analysis allowed us to assign a specific *Bam*HI fragment to *ev*-19. This was not possible in previous analyses due to the lack of segregation from other *ev*-loci (Humphries et al. 1984)

[c] Two of three chickens from strain K which carry this locus but not *ev*-3 were gs^-

[d] At each locus, the frequencies were significantly different between strains S and K ($P<0.0001$)

unambigous with the exception of a few birds which contained *ev*-loci with overlapping *Sac*I or *Bam*HI bands and where signal strength had to be taken into account.

The analysis of 94 strain S hens and 110 strain K hens revealed the presence of eight different *ev*-loci (Table 1). The initial identification of these loci was based on the molecular weights of the 3'-junction fragments: those from the present study were compared with those described in the literature. A second, more definite assignment of the loci was made using DNA from chickens with known *ev*-genotypes as a standard: six of the eight *ev*-loci could be identified with previously described *ev*-loci. Two loci, designated 'new A' and 'new B', did not appear to correspond to any of those previously identified (Smith 1986). An additional locus, characterized by a 5.6 kb *Sac*I and a larger than 23 kb *Bam*HI fragment, was also present in both strains. Based on the molecular weight of the *Sac*I fragment and the low strength of the hybridization signal, the locus was determined to contain only a small segment of retroviral sequences. It might be identical to *ev*-16 (reported MW of the *Sac*I band 5.4 kb) which consists of a solitary long terminal repeat (Smith 1986; element D of Hughes et al. 1981). The weakness of the hybridization signal did not allow consistent scoring of this locus, and therefore it was not included in the analysis. However, based on screening of some selected Southern blots, it occurred more often in strain K than in strain S.

Table 1 also lists the phenotypes of the identified *ev*-loci as summarized by Rovigatti and Astrin (1983) and Smith (1986). The phenotype of *ev*-19 is uncertain. Primary fibroblast cultures from the chicken line containing *ev*-19 produced endogenous infectious virus, indicating that this locus is $V\text{-}E^+$ (Humphries et al. 1984). However, the line also contained *ev*-20, *ev*-7 and *ev*-3. The *ev*-gene 'new A' does not express gs-antigen – inferred from the absence of gs-antigens in the feather pulp of two strain K birds which carried this locus but not *ev*-3. The other new locus ('new B') which was only detected in strain S did not segregate from *ev*-3.

The average number of *ev*-loci per genome was 3.5 in strain S and 3.2 in strain K. Of the eight loci observed, three occurred exclusively in strain S, one exclusively in strain K and four were common to both strains (Table 1). The frequency of each locus differed significantly between the two strains (Chi-square test, $P<0.0001$). Among the *ev*-loci absent or occurring less frequently in strain K were those which either produce endogenous virus (*ev*-10 and possibly *ev*-19) or the gs-antigen (*ev*-3). The frequency of *ev*-1, which codes for complete virus, but whose expression is normally suppressed by DNA methylation, was also reduced in strain K (Groudine et al. 1981; Rovigatti and Astrin 1983). The *ev*-genes occurring at a higher frequency in strain K than in strain S are either silent or produce the viral envelope, but not the *gag* proteins.

Comparison of expected and observed gs-antigen levels

To confirm the validity of the classification of the *ev*-genes, gs-antigens were measured in the feather pulp of individual birds (Table 2). Based on the phenotypes expected to be associated with the various *ev*-loci (Table 1), the birds were grouped into four genotypic classes: Type I, birds without *ev*-genes coding for gs-antigen or infectious virus ($gs^-/V\text{-}E^-$); Type II, birds with the gs-antigen producing gene *ev*-3, but without genes coding for infectious virus ($gs^+/V\text{-}E^-$); Type III, birds containing both, the gs-antigen producing gene *ev*-3 and the virus producing genes *ev*-10 and *ev*-19 ($gs^+/V\text{-}E^+$); and Type IV, birds with genes *ev*-10 and *ev*-19 coding for infectious virus but lacking *ev*-3 ($gs^-/V\text{-}E^+$).

All birds of strain K were of Types I and II. As expected, the absorbance readings from the ELISA test for gs-antigens were low for Type I birds and differed significantly from the high readings of strain K Type II birds (analysis of variance, $P<0.01$). Birds from strain S were predominantly of Types II and III, and ELISA readings were similar to those obtained on the Type II birds of strain K. There was no significant difference between Type II and Type III birds in strain S, indicating that in birds carrying the gs-antigen producing gene *ev*-3, the

Table 2. Comparison of mean levels of gs-antigen (gsa) in feather pulp among hens classified on the basis of the *ev*-genes present

Class[a]	Expected phenotypes			Strain S ($N=90$)		Strain K ($N=109$)	
	chf	gs	V–E	Frequency	Average gsa[b]	Frequency	Average gsa[b]
I	+	–	–	0		0.38	0.38[c]
II	+	+	–	0.27	1.92[d]	0.62	1.64
III	+	+	+	0.69	1.85	0	
IV	+	–	+	0.04	2.79[e]	0	

[a] All birds tested belonged to one of the four classes. *Ev*-19 was scored as $V-E^+$ and 'new A' as gs^- (see Table 1)
[b] Based on absorbance reading of an ELISA
[c] The average gsa values of type I and type II birds in strain K differed significantly (analysis of variance, $P<0.01$)
[d] Not significantly different from type II strain K birds or type III strain S birds (analysis of variance)
[e] Not amenable to analysis of variance, since only four birds were of type IV

Table 3. Chi-square analyses of expected and observed genotype frequencies

Strain	Genotype								Frequency[b]	
	ev-1	*ev*-3	*ev*-6	*ev*-8	*ev*-10	*ev*-19	new A	new B	Observed	Expected
S ($N=94$)	+	+	–	–	+	–		–	0.16	0.12
	+	+	–	–	–	–		–	0.07	0.07
	+	+	–	–	+	–		+	0.04	0.07
Other genotypes[a]									0.72	0.74
K ($N=110$)	–	+	+	+			–		0.32	0.29
	–	–	+	+			–		0.17	0.17
	+	+	+	+			–		0.10	0.17
	+	–	+	+			–		0.13	0.10
	–	+	+	+			+		0.12	0.09
	–	–	+	+			+		0.03	0.05
	+	+	+	+			+		0.05	0.05
Other genotypes									0.08	0.08

[a] This group contains genotypes whose expected number of occurrence was <5
[b] The expected and observed frequencies did not differ significantly for strain S (Chi-square $=1.9$, $df=3$, $P>0.2$) or for strain K (Chi-square $=6.5$, $df=7$, $P>0.2$)

additional presence of endogenous virus producing genes did not further increase gs-antigen levels.

Four of the birds of strain S carried the virus-producing genes *ev*-10 and/or *ev*-19, but not the gs-antigen producing gene *ev*-3 (Type IV). The ELISA readings for gs-antigen of these four birds were the highest among all birds screened. The chance probability for such a ranking within strain S is less than 4×10^{-7}. The high levels of gs-antigen in these birds could be the result of viral proliferation in the absence of *ev*-3 (see Discussion).

Linkage disequilibrium and frequencies of genotypes

Linkage disequilibrium was observed for three pairs of *ev*-loci (Hill 1974; likelihood ratio statistic). The pairs were *ev*-3/*ev*-8, *ev*-3/*newB* and *ev*-1/*ev*-8. The first two pairs occurred only in strain S, while the third pair occurred in both strains and showed linkage disequilibrium in both. In all three cases, the disequilibrium constant was negative, indicating an excess of repulsion gametes. *Ev*-1 and *ev*-8 have been mapped to the long and short arm of chromosome 1, respectively, while *ev*-3 is located on a minichromosome (Tereba and Astrin 1982; Tereba 1981). The different chromosomal locations indicate that these linkage disequilibria among *ev*-loci reflect epistatic interactions.

Despite the linkage disequilibria between some of the *ev*-genes, the frequencies of the more common genotypes did not differ significantly from the expected frequencies calculated under the assumption of independent segregation of *ev*-loci (Table 3).

Discussion

The analysis of strains S and K revealed the presence of eight different *ev*-loci. Of these, six could be identified as being identical to previously described *ev*-loci, whereas two appeared to be new loci and were subsequently designated 'new A' and 'new B'. 'New A' had characteristic *Bam*HI and *Sac*I fragments which differed from previously characterized *ev*-loci (Smith 1986). In our analy-

sis, three birds which were phenotypically gs^- were determined to be carrying this locus together with *ev*-6 and *ev*-8. As feather pulp analysis was negative for gs-antigen in two of the three birds, the definitive phenotypic identification of this locus will require the analysis of additional birds. The other new locus, 'new B', had a *Bam*HI band indistinguishable from the virus-producing locus *ev*-10 and a *Sac*I band indistinguishable from *ev*-19. Such an overlapping banding pattern would be compatible with 'new B' originating through recombination within the 3'-flanking regions of *ev*-10 and *ev*-19. The assignment of a gs-antigen phenotype was not possible since the locus did not segregate from other, gs-positive *ev*-loci.

Results from the analysis of feather pulp for gs-antigen were generally in good agreement with our assignment of loci. In strain K, the only gs-antigen expressing *ev*-gene was *ev*-3, and 90% of the birds carrying this gene were positive for gs-antigen (ELISA reading >0.5). Of the birds free of *ev*-3, 88% were negative for gs-antigen. It may be important that the exceptions in this latter class were almost exclusively birds containing *ev*-1. As mentioned earlier, the expression of *ev*-1 is normally suppressed by methylation, but embryos containing *ev*-1 can be induced to produce non-infectious viral particles (Rovigatti and Astrin 1983; Groudine et al. 1981). One such embryo was identified which spontaneously produced elevated levels of viral particles containing the *gag* proteins.

In strain S, 96% of all birds carried *ev*-3, which is phenotypically chf^+gs^+. All of these birds showed high readings of gs-antigen (ELISA reading >1.3) at levels which appeared to be independent of the presence of other *ev*-genes. The highest gs-antigen values were found in four birds containing the virus-producing genes *ev*-19 and/or *ev*-10, but not *ev*-3. It is possible that the absence of *ev*-3 led to a high production of endogenous virus in feather pulp and hence to a high level of gs-antigen. Cells which harbor *ev*-3 are less susceptible to endogenous virus infection (Robinson et al. 1981; Smith 1986). This protective effect is probably mediated by the viral envelope proteins expressed by *ev*-3 rather than by the viral *gag* proteins since it is also observed with the proviruses *ev*-6 and *ev*-9 which are phenotypically gs^--chf^+. Viral envelope proteins could provide resistance to infection by endogenous virus by blocking viral receptor sites.

The frequencies of the various *ev*-loci in strains S and K differed significantly. Most strikingly, all *ev*-genes producing the complete virus were absent in strain K, indicating that selection for egg production and/or Marek's disease resistance reduced the incidence of such genes. This result is in agreement with the finding that the incidence of endogenous virus production in strains selected for egg production traits was lower than in corresponding control strains (Crittenden et al. 1979). Further, most of the *ev*-genes which produce endogenous virus have been identified in experimental inbred lines, but are not present in non-inbred commercial White Leghorns (SPAFAS lines, Heisdorf and Nelson lines and Kimber lines; Tereba and Astrin 1980).

The two other identified *ev*-genes that occurred at a low frequency in strain K were *ev*-3, which expresses high levels of the viral *gag* and envelope proteins, and *ev*-1, which occasionally produces noninfectious viral particles. These two genes may therefore also be detrimental to egg production and/or Marek's disease resistance (except in birds which carry *ev*-genes coding for infectious endogenous virus, see above). In the nine commercial non-inbred White Leghorn strains surveyed by Tereba and Astrin (1980), *ev*-1 was found to be present in all birds in all strains. *Ev*-3 was only present in three of the nine strains surveyed, but occurred in 100% of the birds of these three strains. It is thus possible that the founder birds of these commercial strains were homozygous for *ev*-3 and/or *ev*-1, thus precluding elimination of these genes by the selection exercised by breeders.

Genes *ev*-6 (gs^-chf^+) and *ev*-8 (gs^-chf^-) occurred at levels close to 100% in strain K, as compared to 28% for both in strain S. *Ev*-6 has been located on the long arm of chromosome 1 and *ev*-8 on its short arm (Tereba 1981; Tereba and Astrin 1982). As expected from the distance between these two loci, we did not observe significant linkage in our segregation analysis. It is thus likely that the simultaneous increase in the frequencies of these two loci in strain K was not due to linkage. In commercial strains, the two loci are present at variable frequencies.

Although we have interpreted the differences in the frequencies of endogenous viral genes in strains S and K to be the result of genetic selection, we can not exclude the possibility that these differences are due to genetic drift or that they reflect the particular breeding history of the two strains. However, other well-defined Leghorn strains which had been kept non-selected or which were selected for egg production contained several *ev*-genes whose frequency was unaltered despite of 30 years of segregation. Other *ev*-genes shared with strains S and K showed the same response to selection as did those of the two strains analysed here (unpublished results).

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