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Gs, an Allele of Chickens for Endogenous Avian Leukosis Viral Antigens, Segregates with *ev* 3, a Genetic Locus That Contains Structural Genes for Virus

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Gs is an allele of chickens for the expression of endogenous avian leukosis virus-related core (gs) and envelope (chf) antigens. Progeny of a genetic cross in which *Gs* was segregating were analyzed for endogenous viral DNA as well as for the expression of endogenous viral antigens. Viral genetic information was identified by cleavage of embryo DNA with restriction endonucleases, electrophoretic separation of the resulting fragments, and identification of bands containing viral sequences by hybridization of the DNA to ^{32}P-labeled viral RNA. Four different chromosomal sites of residence of endogenous viral sequences were identified by this method. These sites were the same as those previously assigned to the endogenous viral loci *ev* 1, *ev* 3, *ev* 4, and *ev* 5. *ev* 1 was present in all of the progeny of the cross. *ev* 3, *ev* 4, and *ev* 5 were present in various combinations with *ev* 1. *ev* 3 cosegregated with the gs^+chf^+ phenotype. Cells which did not contain *ev* 3 but contained *ev* 1, *ev* 4, and/or *ev* 5, did not express detectable levels of viral antigens. We suggest that *Gs* contains the structural genes for endogenous virus which reside at *ev* 3 and that these structural genes code for gs and chf in gs^+chf^+ cells.

Four different phenotypes for the expression of avian leukosis viruses (ALVs) have been described in uninfected cells from White Leghorn chickens (for review see 17). These phenotypes include the gs^-chf^+ phenotype for the expression of subgroup E ALV envelope antigens (chf), the gs^+chf^+ phenotype for the coordinate expression of ALV internal (gs) and subgroup E envelope antigens, the V-E^+ phenotype for the production of infectious subgroup E virus, and the V-15_B^+ phenotype for the spontaneous production of a noninfectious ALV. Dominant alleles which reside at distinct genetic loci appear to code for the expression of each of these phenotypes (3, 17). Alleles for endogenous virus expression have been postulated to be control rather than structural genes since chickens which both did and did not express endogenous viruses appeared to have similar copy numbers of endogenous viral DNA (estimates ranged from one to ten copies per haploid genome) (12, 14, 21, 23, 27).

Recently, ten independent genetic loci which contain ALV-related DNA have been identified in chickens (1; S. Astrin, L. Crittenden, and H. Robinson, unpublished observations). These loci have been designated endogenous viral loci (*ev*) and numbered 1 through 10. Cultured cells which contain *ev* 1, *ev* 4, *ev* 5, and *ev* 8 do not appear to express endogenous viruses or viral antigens, whereas cultured cells which contain *ev* 2, *ev* 3, *ev* 6, *ev* 7, *ev* 9, and *ev* 10 have characteristic phenotypes for the expression of endogenous viruses.

This is the first in a series of papers which will report that each endogenous viral locus segregates in genetic crosses with one and only one phenotype for virus expression. In this paper we characterize endogenous virus expression and endogenous viral loci in progeny of crosses of three random-bred lines of chickens: K16, K(−), and K28. K16 is homozygous for *Gs*, the allele which codes for the expression of the gs^+chf^+ phenotype (18, 19). K(−) and K28 do not express endogenous viruses. The question we have asked is: does *ev* 3, a locus which has been observed in gs^+chf^+ cells, segregate with *Gs*, the allele which codes for this phenotype (4, 8, 9, 16, 31)? The results we present indicate that *ev* 3 cosegregates with *Gs* and suggest that *Gs* contains structural genes which code for the gs^+chf^+ phenotype.

MATERIALS AND METHODS

Chickens and embryos. K16 is a random-bred line of White Leghorns which is homozygous for an allele, *Gs*, which codes for the gs^+chf^+ phenotype (18, 19). K(−) and K28 are random-bred lines of White

Leghorns which do not express endogenous viruses or endogenous viral antigens (18, 19). The phenotype for no virus expression is designated gs^-chf^-, and the allele for no expression of the gs^+chf^+ phenotype is designated *gs*. K16, K(−), and K28 chickens are maintained at the Worcester Foundation for Experimental Biology.

Genetic crosses were performed by artificial insemination and are indicated by ×, with the rooster preceding the × and the hen following the ×. Genotypes are designated as *Gs/gs*, with the slash indicating a genetic locus and the symbols preceding and following the slash indicating the alleles present at the locus.

gs^+chf^+ chicken embryos were also obtained from SPAFAS, Norwich, Conn. (non-inbred embryos), Heisdorf and Nelson, Redmond, Wash. (non-inbred embryos), and the U.S. Department of Agriculture, Regional Poultry Research Laboratory, East Lansing, Mich. (inbred embryos of lines 6_3, 7_1, and 15_1).

Cells and cell DNAs. Chicken embryo cultures were prepared from 11-day-old White Leghorn embryos as described by Rubin (22). Cells were cultured in Dulbecco-modified Eagle medium supplemented with 10% tryptose phosphate broth, 4% calf serum, and 1% chicken serum.

Cells were typed for the expression of antigens which are group specific (gs) to ALVs (10) by assaying for complement fixation in reactions of hamster antiserum to gs (Microbiological Associates) with homogenates of cultured cells (10^8 cells per ml) or embryo viscera (20%, wt/vol). Homogenates were scored as gs^+ if complement fixation was observed with homogenate dilutions of 1:4 or greater. Materials scored as gs^- showed no complement fixation.

Cells were typed for chick helper factor (chf), a glycoprotein (7) which can serve as a subgroup E envelope antigen for the envelope-defective Bryan Rous sarcoma virus (28, 30) as described in method 3 of Robinson and Lamoreux (18). Cells were scored as chf^+ if, after infection with Bryan Rous sarcoma virus (Rous-associated virus type 7 [RAV-7]), culture supernatants contained more than 10^4 focus-forming units of Bryan Rous sarcoma virus per ml with a subgroup E host range. Supernatants of chf^- cultures contained less than 5×10^2 focus-forming units per ml of Bryan Rous sarcoma virus with a subgroup E host range.

DNA was prepared from cultured cells as described by Varmus et al. (26).

Enzymes, gels, and transfer of DNA to nitrocellulose filters. Restriction endonuclease *Sst*I was purchased from Bethesda Research Labs. DNAs at a concentration of 250 μg/ml were digested using 250 U of endonuclease per ml at 37°C for 4 h. Digestion was monitored by the addition of simian virus 40 or λ DNA to an aliquot of the digestion mixture, and visualization of the products, after digestion and electrophoresis, by staining with ethidium bromide. Electrophoresis of digested DNAs was carried out in 1% agarose (Sea Kem) slab gels as described by Ketner and Kelly (11). A 25-μg amount of DNA was run in each well. DNA was transferred to nitrocellulose filters (type HAWP00010, Millipore Corp.) by the method of Southern (24) as modified by Ketner and Kelly (11).

Hybridization and autoradiography. ^{32}P-labeled RAV-2 RNA was used as a probe for endogenous viral sequences. RAV-2 RNA has greater than 80% sequence homology with the RNAs of other members of the ALV group of avian retroviruses (6, 13). RAV-2 RNA was chosen for these studies since RAV-2 grows to high titer, which facilitates the preparation of the hybridization probe. RAV-2-infected cell cultures were labeled with ^{32}P as described by Parsons et al. (15), using 1.5 mCi of ^{32}P (carrier-free; New England Nuclear Corp.) per ml of culture fluid. Virus was collected and pelleted at 24-h intervals, and 70S RNA was purified by pronase-sodium dodecyl sulfate treatment followed by sucrose gradient sedimentation as described by Robinson et al. (20). Purified 70S RNA was hybridized at 70°C to DNA immobilized on nitrocellulose filters in a mixture containing 4× SSC (1× SSC is 0.015 M sodium citrate plus 0.15 M NaCl, pH 7.4), 500 μg of carrier wheat embryo RNA per ml, 0.1% sodium dodecyl sulfate, and 0.3 μg of ^{32}P-labeled 70S RAV-2 RNA (specific activity, 1×10^7 to 2×10^7 cpm/μg) per ml. The salt and temperature conditions chosen for hybridization are those described by Baluda et al. (2). The filters were incubated in hybridization mixtures for 10 to 20 h, washed with 2× SSC, treated with pancreatic RNase (20 μg/ml in 2× SSC for 0.5 h at 37°C), washed with 4× SSC–0.1% sodium dodecyl sulfate at 70°C, and dried. Hybrid bands were detected by autoradiography at −70°C, using Kodak XR-5 film and a Dupont Quanta II intensifying screen.

RESULTS

Observation of four fragments which contain endogenous viral sequences in *Sst*I-cleaved DNAs of progeny of *gs/gs* × *Gs/gs* chickens. A K(−) *gs/gs* rooster was crossed with a K16 *Gs/Gs* hen. Progeny of this cross which were heterozygous for *Gs* were mated with a K28 *gs/gs* rooster. Twenty-five of 47 progeny of this second cross were gs^+chf^+. This result indicates that the gs^+chf^+ phenotype of K16 is coded for by a single dominant allele, *Gs*.

To determine whether *ev* 3 segregated with *Gs*, DNA was extracted from progeny of the second cross and analyzed for endogenous viral sequences. DNA to be analyzed for endogenous viral loci was cleaved with restriction endonuclease *Sst*I. *Sst*I was chosen for these digestions since cleavage of chicken DNA with this enzyme produces a single major fragment for each endogenous viral locus (1). The digested DNA was fractionated by gel electrophoresis, denatured in situ, and transferred to nitrocellulose filters (24). Endogenous viral genes were detected by annealing the DNA on the nitrocellulose filters to ^{32}P-labeled 70S RNA extracted from virions of RAV-2. Bands containing viral sequences were visualized by autoradiography.

DNA from 31 progeny from three *gs/gs* × *Gs/gs* matings (A026-95 × V369-84, A026-95 × V369-85, and A026-95 × V369-87) were analyzed

for endogenous virus DNA. Various combinations of four different *SstI* fragments which contained endogenous viral sequences were observed (Fig. 1). These fragments had apparent molecular weights of 12×10^6, 5.8×10^6, 5.4×10^6, and 3.9×10^6.

Four patterns of fragments were observed in the DNA from progeny of A026-95 × V369-84. These are shown in lanes A through D. DNA from progeny of A026-95 × V369-85 yielded six different patterns (lanes E through J). DNA from progeny of A026-95 × V369-97 displayed four different patterns of fragments (lanes K through N). The number of progeny from each mating which had a specific pattern is indicated below the lane which exhibits the pattern. No ALV-related DNA was detected in mouse, duck or quail DNA (lanes O, P, and Q). This result demonstrates that the RAV-2 probe is specific for endogenous viral genes of chickens.

Evidence that the four *SstI* fragments represent distinct genetic loci for endogenous viruses. In the patterns shown in Fig. 1, four *SstI* bands are present. Three lines of evidence suggest that each of these bands represents a distinct genetic locus which contains endogenous viral sequences.

First, the bands occurred independently of each other. For example, all of the patterns in Fig. 1 contain the band at 5.8×10^6 daltons, but in patterns of eight of the progeny this is the only band observed (lanes A, E, and K). Various combinations of the 5.8×10^6-dalton common band and three others can be seen in the remaining patterns. Each of the three additional bands occurred independently of the other two. For example, lanes C and F contain only the common band and the 5.4×10^6-dalton band, lanes G and M contain the common band and the 12×10^6-dalton band, and lanes B and L contain the common band and the 3.9×10^6-dalton band.

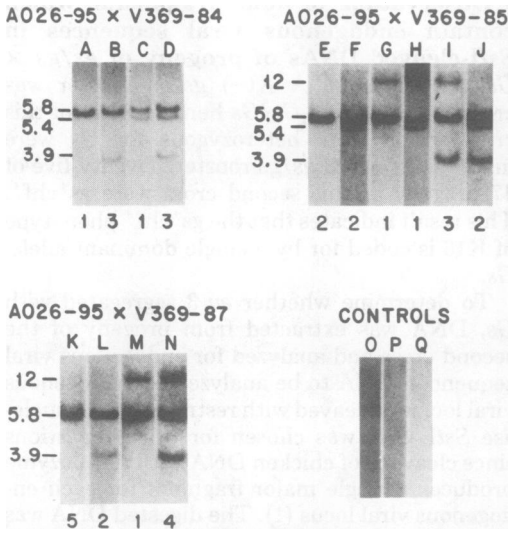

FIG. 1. *SstI restriction endonuclease patterns of endogenous viral sequences in the DNAs of progeny of gs/gs × Gs/gs chickens. The rooster and hen for each mating are indicated in the figure. The number of progeny of each mating exhibiting each pattern is shown below each lane. Lanes O, P, and Q contain digested mouse, duck, and quail DNA, respectively. Molecular weights (×10⁻⁶) were calculated using a marker of EcoRI-digested ^{32}P-labeled λ DNA (not shown).*

A second line of evidence that each of the *SstI* fragments represents a distinct chromosomal locus is that, with the exception of the 3.9×10^6-dalton band, all the bands contain DNA which has a molecular weight greater than that of the double-stranded DNA copy of an endogenous virus, RAV-0. The double-stranded DNA copy of RAV-0 is estimated to be 4.5×10^6 daltons, or 20% less than that of Rous sarcoma virus (25). Therefore, the bands of molecular weights 12×10^6, 5.8×10^6, and 5.4×10^6 most likely contain both viral and cellular sequences, with each set of viral sequences adjacent to a different set of cellular sequences. Experiments reported elsewhere (1) have shown that the viral sequences present in the 3.9×10^6-dalton band are also flanked by a unique set of cellular sequences. The fact that the 3.9×10^6-dalton *SstI* band is too small to contain complete RAV-0 genetic information may indicate that the viral sequences in this band have a deletion for endogenous viral information or that *SstI* cleaves within this proviral genome and that the 3.9×10^6-dalton band contains most, but not all, of the viral sequences present at this cellular locus.

The third line of evidence that these fragments represent distinct genetic loci is given in Table 1, where the frequency of occurrence of the 12×10^6-, 5.4×10^6-, and 3.9×10^6-dalton bands in the three matings is tabulated. In each case the frequency for the occurrence of these bands is close to 50%. This is the frequency which would be expected for the occurrence of individual alleles in the progeny of matings where one parent is heterozygous for an allele and the other parent does not contain an allele. The DNA of one of our parents, A026-95, contained only the 5.8×10^6-dalton fragment. We know this from having analyzed the DNA of 13 of the A026-95 progeny which are part of the current K28 line of chickens (data not shown). Thus, the occurrence of the 12×10^6, 5.4×10^6, and 3.9×10^6 fragments in close to 50% of the progeny of matings in which they were segregat-

TABLE 1. *Occurrence of specific SstI fragments in the DNA of progeny of matings which were segregating for these fragments*

Sst I fragment (daltons)	Mating[a]	No. with fragment/ no. tested	% with fragment
12×10^6	V369-85	5/11	46
	V369-87	5/12	
5.4×10^6	V369-84	4/8	47
	V369-85	5/11	
3.9×10^6	V369-84	6/8	55
	V369-85	5/11	
	V369-87	6/12	

[a] Matings were between rooster A026-95 and the indicated hens. The DNA of A026-95 did not contain the 12×10^6-, 5.4×10^6-, or 3.9×10^6-dalton *SstI* fragments.

ing is consistent with these fragments representing genetic loci which were heterozygous in V369-84, V369-85, and/or V369-87.

Assignment of the *SstI* fragments to previously identified endogenous viral loci. Each of the *SstI* fragments had a molecular weight similar to that of *SstI* fragments which have been used to assign specific genetic loci to ALV-related endogenous viral sequences (1). An *SstI* fragment of 5.8×10^6 daltons has been used to define the locus *ev* 1 which appears to be present in all chickens. *ev* 1 is not expressed in cultured cells. An *Sst* fragment of 3.9×10^6 daltons has been used to define the locus *ev* 3. *ev* 3 has been observed in chickens with a gs^+chf^+ phenotype. *Sst* fragments of 5.4×10^6 and 12×10^6 daltons have been used to define *ev* 4 and *ev* 5. *ev* 4 and *ev* 5 appear to contain viral information which is not expressed in cultured cells. To determine whether the *SstI* fragments observed in Fig. 1 revealed the same genetic loci as previously defined as sites of residence for endogenous viral genes, selected DNAs were digested with *Bam*HI or *Hin*dIII.

When DNAs which contained the 5.8×10^6- and 3.9×10^6-dalton *SstI* bands were digested with *Hin*dIII, patterns containing fragments with molecular weights of 15×10^6, 3.2×10^6, 2.8×10^6, 1.9×10^6, and 1.0×10^6 were observed (Fig. 2, lanes A and B). The 3.2×10^6-, 1.9×10^6-, and 1.0×10^6-dalton fragments have been previously defined as characteristic of *Hin*dIII-cleaved *ev* 1 (1). The 15×10^6- and 2.8×10^6-dalton *Hin*dIII fragments have been defined as representing *ev* 3. The presence of these five bands in the *Hin*dIII patterns confirms the identity of the 5.8×10^6- and 3.9×10^6-dalton *SstI* fragments as *ev* 1 and *ev* 3, respectively.

When DNAs which yielded patterns containing the 5.8×10^6-, 5.4×10^6-, and 12×10^6-dalton *SstI* fragments were digested with *Bam*HI, patterns containing bands with molecular weights of 7.8×10^6, 4.5×10^6, 3.2×10^6, 1.0×10^6, and 0.7×10^6 were observed (Fig. 2, lanes B and C). *Bam*HI fragments with molecular weights of 3.2×10^6, 1.0×10^6, and 0.7×10^6 define *ev* 1. The 4.5×10^6- and 7.8×10^6-dalton *Bam*HI fragments are characteristic of *ev* 4 and *ev* 5, respectively (1). The presence of these bands confirms the identity of the 5.4×10^6- and 12×10^6-dalton *SstI* bands as *ev* 4 and *ev* 5, respectively.

Does *ev* 3 segregate with the gs^+chf^+ phenotype? Table 2 lists the phenotype of each of the 31 progeny along with the pattern of endogenous viral loci observed for each. The data in the table show that the gs^+chf^+ phenotype is always accompanied by the presence of *ev* 3. All embryos whose DNA contained *ev* 3 were of the gs^+chf^+ phenotype. Thus, *ev* 3 cosegregates in every instance with the gs^+chf^+ phenotype.

As expected from previous work, cultured cells

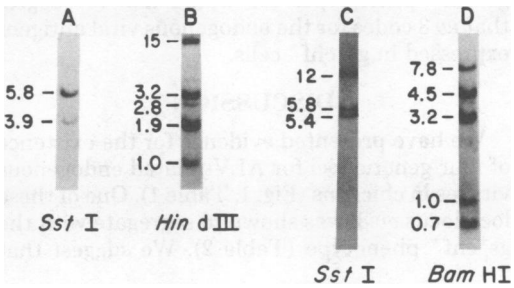

FIG. 2. *SstI, HindIII, and EcoRI cleavage patterns of endogenous viral sequences in DNA of a gs^+ chf^+ chicken (lanes A and B) and a gs^-chf^- chicken (lanes C and D). Molecular weights ($\times 10^{-6}$) were calculated using a marker of EcoRI-digested ^{32}P-labeled λ DNA (not shown).*

TABLE 2. *Endogenous viral loci and endogenous virus expression in the progeny of matings which were segregating for endogenous viral loci and the expression on the gs^+chf^+ phenotype*

No. of embryos[a]	Genotype[b]				Phenotype[c]
	ev 1	*ev* 4	*ev* 5	*ev* 3	
8	+				gs^-chf^-
3	+	+			gs^-chf^-
1	+	+	+		gs^-chf^-
2	+		+		gs^-chf^-
5	+			+	gs^+chf^+
5	+	+		+	gs^+chf^+
7	+		+	+	gs^+chf^+

[a] All of the embryos with a given genotype exhibited the same phenotype.

[b] +, Presence of a particular locus.

[c] gs and chf tests were performed and scored as described in the text.

which contained *ev* 1 and *ev* 4, and/or *ev* 5, did not express gs or chf (1). Assays for chf typically reveal the expression of low levels of chf in chf$^-$ cells (18). Low levels of expression of chf did not correlate with the presence of *ev* 4 or *ev* 5 in cells.

Occurrence of *ev* 3 in other gs$^+$chf$^+$ chickens. More than 60 gs$^+$chf$^+$ birds from six different sources have been analyzed for endogenous viral loci. In each case, the *Sst*I-cleaved DNA contained the 3.9 × 10^6-dalton fragment which is characteristic of *ev* 3. Figure 3 shows these patterns. In each case the bands characteristic of loci *ev* 1 and *ev* 3 are present. In most of the DNA patterns other bands are present. These bands most likely represent other genetic loci for endogenous viral genes. DNAs from over 100 birds of phenotypes other than gs$^+$chf$^+$ have been analyzed; in no case have any of these patterns shown the presence of *ev* 3. *ev* 3 thus appears to be characteristic of birds of the gs$^+$chf$^+$ phenotype. We would like to suggest that *ev* 3 codes for the endogenous viral antigens expressed in gs$^+$chf$^+$ cells.

DISCUSSION

We have presented evidence for the existence of four genetic loci for ALV-related endogenous viruses in chickens (Fig. 1, Table 1). One of these loci, locus *ev* 3, was shown to segregate with the gs$^+$chf$^+$ phenotype (Table 2). We suggest that the presence of gs antigens and viral envelope proteins in gs$^+$chf$^+$ cells reflects the transcription and translation of *ev* 3 endogenous viral sequences (Table 2, Fig. 3).

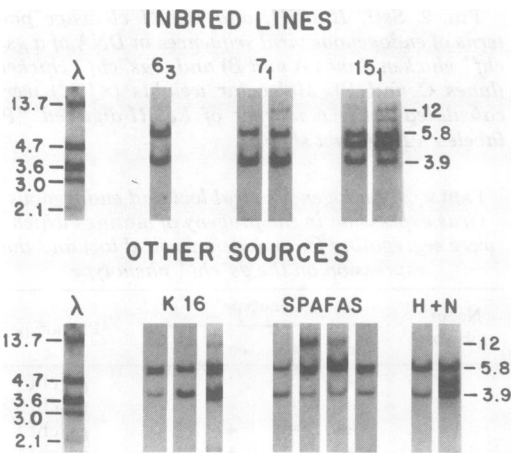

FIG. 3. *SstI restriction endonuclease patterns of endogenous viral sequences in the DNA of gs$^+$chf$^+$ chickens from different sources. Sources are indicated above the lanes. Lanes display representative patterns observed in DNA of embryos from each source. Sources are grouped according to whether or not they are inbred. A marker of EcoRI-digested ^{32}P-labeled λ DNA is shown at the left. Molecular weights (×10^{-6}) are given.*

ev 3 was not, however, the only locus present in cells of the gs$^+$chf$^+$ phenotype (Fig. 1 and 3, Table 2). DNAs from all chickens, regardless of their phenotype, also contained locus *ev* 1. Therefore, it is possible that *ev* 1 codes for the proteins seen in the cells and that this locus is activated by the presence of *ev* 3 or an element that cosegregates with *ev* 3. Resolution of this problem awaits the production of a bird that contains only *ev* 3.

Several lines of evidence suggest that the genes that code for viral proteins in gs$^+$chf$^+$ cells lack a small segment of genetic information for gs antigens and a large segment of information for RNA-directed DNA polymerase. Virus-specific RNA produced in gs$^+$chf$^+$ cells seems to lack about 1,900 nucleotides in portions of the ALV *gag* and *pol* genes (29). *gag* codes for the proteins p27, p19, p15, and p12, which are the core proteins of ALVs. These four proteins react with hamster antiserum to gs. *pol* codes for RNA-directed DNA polymerase. gs$^+$chf$^+$ cells produce a 120,000-dalton protein which contains tryptic peptides found in p27, p19, and p12, but lacks tryptic peptides found in p15. The 120,000-dalton protein contains two or possibly three out of the 16 tryptic peptides found in the β subunit of the RNA-directed DNA polymerase of ALVs (5). Thus, p120 could reflect the transcription and translation of a defective endogenous virus which has partial deletions in its *gag* and *pol* genes. Experiments to determine whether *ev* 3 or *ev* 1, or both, contains such a deletion are in progress.

The V-E$^+$ phenotype of chickens is expressed by alleles which reside at at least two distinct genetic loci (3). Each of these alleles segregates with characteristic ALV-related *Sst*I fragments (S. Astrin and L. Crittenden, unpublished observations). The observation that gs$^+$chf$^+$ chickens from different sources all contain a common ALV-related *Sst* fragment, *ev* 3, suggests that this phenotype is coded for by an allele or closely related alleles which reside at a single genetic locus (Fig. 3).

When this work was undertaken, we did not anticipate finding four genetic loci which contained endogenous viral information in K(−), K28, and K16 chickens. Since chickens with defined endogenous viral loci are essential to the rational study of these viruses, we are currently breeding chickens for their endogenous viral loci. We do this by extracting DNA from the erythrocytes of birds, identifying which loci are pres-

ent, and then selectively breeding. The current K28 line of chickens has been bred to contain only *ev* 1. In the 1979 reproduction of K16 we plan to breed for *ev* 1 and *ev* 3. Similarly, in the 1979 reproduction of K(−), we will breed for *ev* 1, *ev* 1 and *ev* 4, and *ev* 1 and *ev* 5.

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