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Endogenous viral genes of the White Leghorn chicken: Common site of residence and sites associated with specific phenotypes of viral gene expression

(restriction endonuclease/blotting technique/Rous associated virus type 0)

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ABSTRACT The DNAs from >150 individual White Leghorn chickens were digested with restriction endonucleases *Bam*HI, *Eco*RI, *Hin*dIII, and *Sst* I, fractionated by gel electrophoresis, denatured, and transferred to nitrocellulose filters. Fragments containing the endogenous viral genes were detected by hybridization with 70S Rous associated virus type 2 [^{32}P]RNA. Embryos of several different phenotypes with respect to production of the endogenous virus and expression of viral group-specific antigen and viral envelope protein were analyzed. DNA from birds of each phenotype produced a distinctive pattern of fragments containing viral genetic information. Individual fragments were seen to segregate as genetic loci in mating experiments. From the fragment patterns and the segregation data, the following conclusions were drawn with respect to the sites of residence in the chicken chromosome of the endogenous viral genes: (*i*) the DNA of all chickens contains viral genetic information in at least one site, and this site of residence appears to be the same in all chickens analyzed; (*ii*) four other sites have been identified, and the presence of viral information at each of these sites is always accompanied by a specific phenotype of endogenous viral gene expression; (*iii*) in addition to the above-mentioned five sites, a small number of other sites have been identified which are not associated with a known phenotype.

Initiation of infection by the type C RNA viruses of chickens involves the production, in the cytoplasm of the infected cell, of a DNA copy of the infecting RNA genome (1, 2). This DNA copy is then integrated into the cell chromosome and serves as a template for the synthesis of viral RNA (3). However, there also exists type C viral genetic information in the chromosome of uninfected chicken cells (4–8), and cells occasionally produce a type C virus, Rous associated virus 0 (RAV-0) (9), the genome of which is completely homologous to the information carried in the uninfected cell (6).

We initiated the present studies in an effort to determine whether all chickens contain identical endogenous viral genetic information and whether this information always resides at the same site in the chicken chromosome. It is of particular interest to determine whether any differences in these parameters exist among chickens that differ in the expression of the endogenous virus. Embryos have been described that spontaneously produce the endogenous virus (designated V^+) (9–11) or can be induced to produce virus with BrdUrd treatment (11). Other embryos, although producing no virus, make viral group-specific antigen (a phenotype designated as gs^+) (12) and viral envelope protein (designated chf^+) (13, 14); some make envelope but little or no gs antigen (gs^-chf^+); and a third group (gs^-chf^-) make no viral antigens although they appear to contain complete RAV-0 genetic information (6, 7). In order to determine the arrangement of the endogenous viral genes in each of these phenotypes of chicken, we extracted DNA from embryos or erythrocytes of individuals of each phenotype, cleaved the DNA with restriction endonucleases *Eco*RI, *Bam*HI, *Hin*dIII, or *Sst* I and fractionated the fragments by agarose gel electrophoresis. The DNA was then denatured *in situ* in the gel, transferred to nitrocellulose filters, and hybridized with 70S [^{32}P]RNA extracted from virions of Rous associated virus 2 (RAV-2), a virus that has greater than 80% sequence homology with RAV-0 (15). DNA from embryos of each phenotype produced a band or bands common to all embryos as well as a distinctive set of bands. Individual bands were observed to segregate as genetic loci in mating experiments. From the patterns and the segregation data we conclude that all the chickens tested contain viral genetic information at one common site in the genome and that each phenotype in which the viral genes are actively expressed is associated with viral genetic information at one or more additional sites.

MATERIALS AND METHODS

Cells and Cell DNA. Chicken embryo cultures were prepared from 11-day White Leghorn embryos as described by Rubin (16). Cells were typed for chick helper factor (chf) as described by Weiss *et al.* (17) and for group-specific antigen (gs) as described by Payne and Chubb (12). Chicken embryos were obtained from the following sources: SPAFAS (Norwich, CT; gs^+chf^+, gs^-chf^-, and gs^-chf^+ embryos), Heisdorf and Nelson (Redmond, WA; gs^-chf^- and gs^+chf^+ embryos), Regional Poultry Research (East Lansing, MI; gs^+chf^+ embryos of line 6_3, V^+ embryos of lines 100 and 7_2, and BrdUrd-inducible embryos of line 15) and Harriet Robinson (Worcester, MA; gs^-chf^- embryos of lines K28 and K^-, gs^+chf^+ embryos of line K16, and gs^-chf^+ embryos of line K18 (18). Howard Temin kindly provided C/O and C/E cells of line 100 and Harriet Robinson kindly provided V^+ cells of line C. DNA was prepared from cultured cells or from erythrocytes of adult birds as described by Varmus *et al.* (19).

Enzymes, Gels, and Transfer of DNA to Nitrocellulose Filters. Restriction endonucleases *Eco*RI, *Hin*dIII, and *Bam*H1 were purchased from New England BioLabs; *Sst* I was purchased from Bethesda Research Laboratories. DNAs at a concentration of 250 μg/ml were digested by using 250 units of endonuclease per ml at 37°C for 4 hr. 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 (20). Each well contained 25 μg of DNA. DNA was transferred to nitro-

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Abbreviations: RAV-0, Rous associated virus type 0; RAV-2, Rous associated virus type 2; chf, chicken helper factor; gs, group-specific antigen; *ev* 1, *ev* 2, etc., endogenous viral locus 1, locus 2, etc.

cellulose filters (Millipore HAWP00010) by the method of Southern (21) as modified by Ketner and Kelly (20).

Hybridization and Autoradiography. For preparation of hybridization probe, RAV-2-infected cell cultures or RAV-0-producing cells (line 100) were labeled with ^{32}P as described by Parsons *et al.* (22) with 1.5 mCi of [^{32}P]phosphate (carrier-free, New England Nuclear) per ml of culture fluid. Virus was collected and pelleted at 24-hr intervals, and 70S RNA was purified by Pronase/sodium dodecyl sulfate treatment followed by sucrose gradient sedimentation as described by Robinson *et al.* (23). Purified 70S RNA was hybridized at 70°C to DNA immobilized on nitrocellulose filters in a mixture containing 0.60 M NaCl, 0.06 M Na_3 citrate (pH7) 500 μg of carrier wheat embryo RNA per ml, 0.1% sodium dodecyl sulfate, and 0.3 μg of 70S RAV-0 or RAV-2 [^{32}P]RNA (specific activity, 1–2 $\times 10^7$ cpm/μg) per ml. The filters were incubated in this mixture for 10–20 hr, washed with 0.30 M NaCl/0.03 M Na_3 citrate, treated with pancreatic RNase (20 μg/ml in 0.30 M NaCl/0.03 M Na_3 citrate for 0.5 hr at 37°C), washed with 0.60 M NaCl/0.06 M Na_3 citrate/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

Endogenous Viral Sequences in gs$^+$chf$^+$, gs$^-$chf$^-$, and V$^+$ Chickens. DNA was prepared from embryos or erythrocytes of birds of the gs$^+$chf$^+$, gs$^-$chf$^-$, and V$^+$ phenotypes and digested to completion with restriction endonuclease *Eco*RI, *Hin*dIII, or *Sst* I. The fragments were separated on agarose gels, denatured *in situ*, and transferred to nitrocellulose filters (21). Endogenous viral genes were detected by annealing to RAV-2 [^{32}P]RNA followed by autoradiography. Initial experiments using RAV-0 [^{32}P]RNA as probe gave results identical to those with the RAV-2 probe; however, because the RAV-2 probe was much easier to produce in quantity, it was used in subsequent experiments. Fig. 1 shows patterns typical of virus-specific fragments seen in the three types of chickens as well as results obtained with *Eco*RI-cleaved mouse embryo and *Hin*dIII-cleaved quail embryo DNA (wells M and Q). No annealing was seen with either the mouse or quail DNA (identical results were obtained with duck embryo DNA), demonstrating that the probe was specific for the endogenous viral genes of the chicken.

Sst digestion of DNA from a bird of the gs$^-$chf$^-$ phenotype (Fig. 1, lane A) yielded a single band at 5.8 $\times 10^6$ daltons. The majority of viral information in these cells is thus contained within a continuous segment of DNA smaller than 5.8 $\times 10^6$ daltons. The *Eco*RI pattern of this DNA contained three bands of 11.0, 5.1, and 2.4 $\times 10^6$ daltons, whereas the *Hin*dIII pattern contained bands of 3.2, 1.9, and 1.0 $\times 10^6$ daltons. Fifteen other DNAs, all from birds of the gs$^-$chf$^-$ phenotype, yielded the patterns shown in the A lanes of Fig. 1. In addition, the bands in these lanes are included in the respective fragment patterns of all other chicken DNAs (>150) that have been analyzed. One probable interpretation of these patterns is that they represent viral information located at a single site in these cells. The viral genes at this site would contain two *Eco*RI-cleavage sites and two *Hin*dIII sites. The fact that, in more than 150 DNAs analyzed, the three *Eco*RI bands are never found individually but always appear together, as do the three *Hin*dIII bands, is consistent with this interpretation as are the fragment maps of the closely related Rous sarcoma virus (25). Further work will be required to verify this interpretation. With *Sst* I, *Eco*RI, and *Hin*dIII, the total molecular weight of the virus-specific fragments produced from a single DNA sample is different (5.8, 18.5, and 6.1 $\times 10^6$, respectively); therefore, nonviral sequences must be present in one or more of the fragments detected. Indeed, the *Sst* I fragment of 5.8 $\times 10^6$ daltons and the *Eco*RI fragments of 11 and 5.1 $\times 10^6$ daltons are larger than RAV-0 DNA [4.5 $\times 10^6$ daltons or 20% less than that of Rous sarcoma virus, the DNA transcript of which is 5.5 $\times 10^6$ daltons (25)] and most likely contain both viral and cellular sequences. The fact that these putative viral cell junction fragments are included in the fragment patterns of all the chicken DNAs analyzed leads to the interpretation that there is a set of viral genetic information located at the same site in the genome of all chickens. The viral sequences located at this site and characterized by the *Sst* I band at 5.8 $\times 10^6$ daltons will be referred to as endogenous viral locus 1 (*ev* 1).

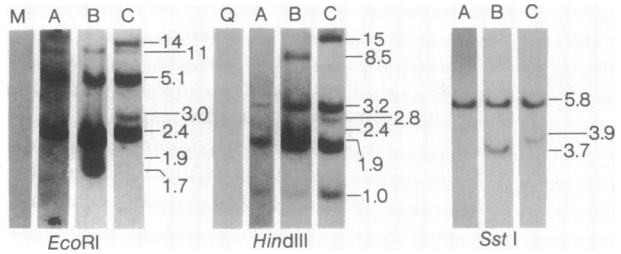

FIG. 1. *Eco*RI, *Hin*dIII, and *Sst* I digestion of DNAs and identification of endogenous viral sequences. A marker of *Eco*RI-digested λ [^{32}P]DNA was used to determine molecular weights which are shown $\times 10^{-6}$. Lane M contains mouse embryo DNA. Lane Q contains quail embryo DNA. The three A lanes contain DNA from a gs$^-$chf$^-$ chicken embryo. The three B lanes contain DNA from a V$^+$ chicken embryo of line 7_2. The three C lanes contain DNA from a gs$^+$chf$^+$ chicken embryo.

The *Sst* fragment pattern of DNA obtained from a V$^+$ embryo of line 7_2 (Fig. 1, lane B) contained two major bands, one at 5.8 $\times 10^6$ daltons and one at 3.7 $\times 10^6$ daltons. In addition, a minor band at 0.9 $\times 10^6$ daltons could be detected after a long autoradiographic exposure. As described above, the band at 5.8 $\times 10^6$ daltons was found in the *Sst* I patterns of all chicken DNAs tested. The *Eco*RI pattern contained, in addition to the three bands found in all chickens, new bands at 1.9 and 1.7 $\times 10^6$ daltons. The *Hin*dIII pattern contained the three *Hin*dIII bands common to all DNAs as well as bands at 8.5 and 2.4 $\times 10^6$ daltons. The two new *Sst* I bands (3.7 and 0.9 $\times 10^6$ daltons) as well as the two new *Hin*dIII and two new *Eco*RI bands never occurred individually; in DNAs from more than 40 chickens yielding these bands, the bands always appeared in pairs. In addition, the two new *Sst* I bands segregated together in mating experiments as did the new *Eco*RI and *Hin*dIII bands (unpublished data). The new bands are thus probably part of a single genetic locus. The *Hin*dIII band at 8.5 $\times 10^6$ daltons is larger than RAV-0 DNA (4.5 $\times 10^6$ daltons) and most likely contains both viral and cellular sequences. The appearance of this new viral cell junction band as well as the appearance of the other new bands indicates that the line 7_2 embryos contain a new site of residence of viral information; the viral sequences at this site will be designated endogenous viral locus 2 (*ev* 2). The bands characteristic of *ev* 2 have been found in the pattern of all 20 birds of line 7_2 that have been analyzed. In addition, these bands have never been observed in the patterns of more than 125 birds of the V$^-$ phenotype that have been analyzed. *ev* 2 is thus characteristic of line 7_2 and may contain the genetic information for the virus produced by these cells.

In addition to the major band of *ev* 1, the *Sst* I fragment patterns of DNA from a bird of the gs$^+$chf$^+$ phenotype (Fig. 1, lane C) contained a band migrating at 3.9 $\times 10^6$ daltons. This band could be seen to segregate in mating experiments as an individual genetic locus (data presented below) and has been

designated *ev* 3. In the *Eco*RI and *Hin*dIII fragment patterns of this DNA, in addition to the three bands common to all chickens, each pattern contained two new bands interpreted to represent the viral–cell junctions of *ev* 3. The bands of *ev* 3 are all included in the respective fragment patterns of more than 60 gs^+chf^+ birds from four independent flocks that have been analyzed. In addition, no bird of the gs^-chf^-, gs^-chf^+, or V^+ phenotype has been shown to contain these bands. *ev* 3 is thus characteristic of the gs^+chf^+ phenotype and may be responsible for the production of gs and chf in these birds.

Endogenous Viral Sequences in Additional gs^-chf^- Chickens and in gs^-chf^+ Chickens. The patterns shown for DNA from gs^-chf^- chickens in Fig. 1 represent the simplest pattern present in birds of this phenotype. The DNAs of 20 other gs^-chf^- birds that were analyzed yielded more complicated patterns. The *Bam*HI and *Sst* I digests of two of these DNAs are shown in lanes A and B of Fig. 2. In addition to the major band of *ev* 1, the *Sst* I digest in lane A contained a band migrating at 5.4×10^6 daltons. This band could be seen to segregate in mating experiments as an individual genetic locus (data presented below) and has been designated *ev* 4. The *Bam*HI digest of this DNA contained four bands; the three smallest fragments were common to all chicken DNA patterns (data not shown) and the fourth fragment (4.5×10^6 daltons) was characteristic of *ev* 4. In the lanes labeled B in Fig. 2 are the *Bam*HI and *Sst* I fragment patterns for DNA from another gs^-chf^- bird. In this case, the *Sst* pattern contained a new band at 12×10^6 daltons in addition to the *ev* 1 and *ev* 4 bands. This band has also been found to segregate in mating experiments as an individual genetic locus (data presented below) and has been designated *ev* 5. The *Bam*HI digest contained, in addition to the bands of *ev* 1 and *ev* 4, a band at 7.8×10^6 daltons which is characteristic of *ev* 5. *ev* 4 and 5 have been found, occurring either individually or together, in 20 gs^-chf^- birds and in about 20 gs^+chf^+ birds. These loci have not been correlated with any known phenotype as yet.

Lanes C and D of Fig. 2 show the *Bam*HI and *Sst* I fragment patterns for DNA from two embryos of the gs^-chf^+ phenotype. The *Sst* I digest in lane C shows the presence of the *ev* 1 and *ev* 4 bands as well as a new band at 13×10^6 daltons and a faint band at 1.8×10^6 daltons. The new band at 13×10^6 daltons segregated as an individual genetic locus in mating experiments (unpublished data) and has been designated *ev* 6. The band at 1.8×10^6 daltons has been observed in the patterns of many other DNAs but usually can be visualized only after a long autoradiographic exposure. The relationship of this band to the other endogenous viral loci is not known. The *Bam*HI pattern shown in lane C contains, in addition to the common bands and the *ev* 4 band, a new fragment migrating at 2.7×10^6 daltons which is characteristic of *ev* 6. *ev* 1, 4, and 6 were present in 14 of 15 DNAs from gs^-chf^+ birds that have been analyzed. In addition, this pattern of loci has not been seen in birds of any other phenotype. *ev* 6 appears to be unique to birds of the gs^-chf^+ phenotype and may be responsible for the presence of viral envelope protein in these birds. This pattern of loci, however, was not the only one observed with DNA from birds of this phenotype. The two lanes labeled D in Fig. 2 show the *Bam*HI and *Sst* I patterns for DNA from a gs^-chf^+ bird that did not contain *ev* 6. This DNA contained two new *Sst* I bands at 11 and 14×10^6 daltons and new *Bam*HI bands at 14 and 7×10^6 daltons. The relationship of these bands to the gs^-chf^+ phenotype is not known.

Segregation of *ev* 3, 4, and 5 in Genetic Experiments. As described above, cleavage with *Sst* I appears to produce a single major band for each site in the chicken genome containing viral genetic information. If this postulate is correct, the individual *Sst* I bands should segregate as distinct genetic loci in mating experiments. Fig. 3 shows the results of the mating of a rooster whose DNA, upon cleavage with *Sst* I, yielded major fragments at 12, 5.8, 5.4, and 3.9×10^6 daltons with a hen whose DNA yielded a single major *Sst* I fragment at 5.8×10^6 daltons. When DNAs from 12 of the progeny were analyzed, eight different *Sst* I patterns were observed (lanes C through J). The DNA patterns of all the progeny contained the band at 5.8×10^6 daltons; the pattern of one of the progeny contained only this band. Each of the three other *Sst* I bands segregated as a separate genetic locus; each was found in roughly 50% of the progeny and all possible combinations of the three bands were produced. The segregation data verify the existence of multiple loci for endogenous viral genes in the chicken and validate the hypothesis that the major *Sst* I bands represent individual genetic loci.

Endogenous Viral Sequences in Line 100, Line 15, and Line C. Fig. 4 shows the patterns obtained from *Sst* I digestion of DNA from V^+ embryos of lines 100 and C and from two embryos of line 15_B, a line that releases viral particles after treatment with BrdUrd. All the patterns contained *ev* 1 as well as one or more additional loci. The patterns for DNAs from line 100 C/E (lane A) and line 100 C/O (lane B) embryos were identical, containing only *ev* 1 and *ev* 2 (the locus found in line 7_2 birds). The presence of these loci has been confirmed by digestion of the DNA with *Eco*RI, *Hin*dIII, and *Bam*HI (data not shown). These loci were also the only ones seen in a second

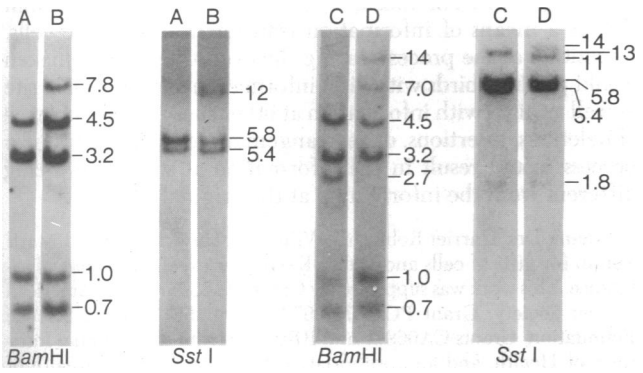

FIG. 2. *Bam*HI and *Sst* I digestion of chicken embryo DNAs and identification of endogenous viral sequences. A marker of *Eco*RI-digested λ [^{32}P]DNA was used to determine molecular weights which are shown $\times 10^{-6}$. The A lanes contain DNA from a gs^-chf^- embryo; the B lanes contain DNA from a second gs^-chf^- embryo; the C lanes contain DNA from a gs^-chf^+ embryo; the D lanes contain DNA from a second gs^-chf^+ embryo.

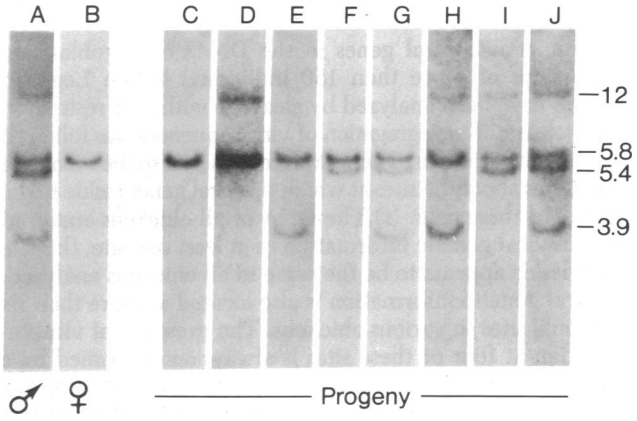

FIG. 3. *Sst* I digestion of chicken DNAs and identification of endogenous viral sequences. Molecular weights are shown $\times 10^{-6}$. Lanes A and B contain DNAs from erythrocytes of a rooster and of a hen from the SPAFAS flock. Lanes C through J contain DNAs from eight of their progeny.

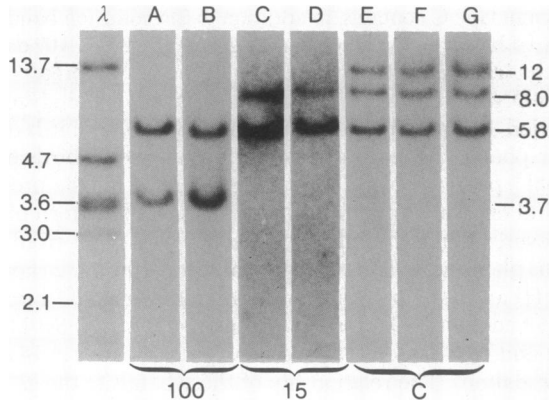

FIG. 4. *Sst* I digestion of chicken embryo DNAs and identification of endogenous viral sequences. A marker of *Eco*RI-digested λ [^{32}P]DNA is shown at the left. Molecular weights are shown × 10^{-6}. Lanes A and B contain DNA from lines 100 C/E and C/O, respectively. Lanes C and D contain DNA from line 15 and lanes E, F, and G contain DNA from line C.

pair of line 100 C/O and C/E embryos that have been analyzed (data not shown). It is noteworthy that the patterns for these two types of line 100 embryos are identical because the C/O cells undergo infection by the virus that is produced whereas the C/E cells do not (24). One possible interpretation of this result is that the infecting virus can integrate at many different sites and therefore is not detected as a specific band in DNA from a population of cells.

Lanes C and D of Fig. 4 show the *Sst* I patterns for two birds of line 15_B, a line that produces viral particles after treatment with BrdUrd (11). The patterns contained *ev* 1 as well as a new band at 8.0 × 10^6 daltons which has been designated *ev* 7. Thirteen other birds of line 15_B have been analyzed and found to contain only *ev* 1 and 7. *ev* 7 may thus contain the genetic information for the viral particles produced by line 15 after BrdUrd treatment (11).

Lanes E, F, and G of Fig. 4 show the *Sst* I patterns for DNA from three embryos of line C. These embryos contained *ev* 1 and 7 as well as a third locus. The band at 12 × 10^6 daltons, which represents the third locus, is similar in mobility to the band representing *ev* 5, a locus present in gs$^-$chf$^-$ birds (see Fig. 2, lane B of the *Sst* I digestions). Additional experiments will be necessary to determine whether these loci are indeed the same and to determine which locus codes for the virus produced by line C.

DISCUSSION

The endogenous viral genes in the DNAs of fibroblasts or erythrocytes of more than 150 individual White Leghorn chickens have been analyzed by cleavage with four restriction endonucleases. The segregation of viral sequences was followed in mating experiments. These experiments lead to the following conclusions about the sites at which the viral genes reside in the genome of these cells. (*i*) The DNA of all chickens analyzed contains viral genetic information in at least one site; this site of residence appears to be the same in all chickens analyzed. (*ii*) Viral genetic information is also located at more than six additional sites in various chickens. The presence of viral information at four of these sites is always accompanied by a particular phenotype such as gs$^+$chf$^+$, gs$^-$chf$^+$, V$^+$ (line 7_2), or V$^+$ (line 15). Table 1 summarizes the loci and respective phenotypes that have been observed.

Three of the viral loci, including the one common to all chickens, can be found in the fibroblasts of gs$^-$chf$^-$ chickens and must be repressed in these birds because these cells make extremely low amounts of either viral RNA or viral protein products (26). Among other possibilities, these loci may (*i*) lack an appropriate promotor for RNA polymerase, (*ii*) contain premature termination signals, (*iii*) be located in a repressed or heterochromatic region of the chromosome, or (*iv*) be part of a set of genes that is not expressed in fibroblasts but may be expressed in other tissues of the chicken or developing embryo.

Table 1. Endogenous viral loci of the White Leghorn chicken

Locus	Molecular weight of major *Sst* I fragment × 10^{-6}	Associated phenotype*
ev 1	5.8	None
ev 2	3.7	V$^+$ (line 7_2)
ev 3	3.9	gs$^+$chf$^+$
ev 4	5.4	None
ev 5	12	None
ev 6	13	gs$^-$chf$^{+\dagger}$
ev 7	8.0	V$^+$ (line 15_B)

* All birds containing a given locus are of the phenotype indicated.
† The birds of this phenotype that I have tested also contain *ev* 4.

The finding of viral genes at a particular site in chickens of a given phenotype suggests that the genes at that site serve as a template for the mRNA that specifies the viral gene products characteristic of the phenotype. The phenotype would thus be determined by the nature of the genes at that locus. For example, it is known that the RNA and protein produced in gs$^+$chf$^+$ cells is defective (26, 27), lacking regions coded for by part of the gs antigen gene and a large segment of the polymerase gene. This situation could be the consequence of a rather large deletion in the endogenous viral genes at the locus correlated with this phenotype (*ev* 3). The presence of such a deletion could be taken as evidence that *ev* 3 serves as a template for the RNA specifying the viral products in those cells.

Two models can explain the presence of multiple viral loci in the chicken. First, each locus may have arisen independently as a consequence of infection of the germ line or evolution of cellular genes (28). The frequency of occurrence of each locus would then be a consequence of how recently in evolution each event took place. *ev* 1 would be the oldest because it is found in all chickens analyzed. A second model is that all the loci are derived from *ev* 1. Viral genes may originally have existed at only this locus. This information may then have been transcribed into RNA, the RNA copied into DNA by reverse transcriptase, and the DNA copy integrated into a second site in the chicken genome. This mechanism has been suggested by Temin (28) as a means of information transfer in eukaryotic cells. Repetition of the process during the evolution of the chicken would produce birds with viral information at the original site as well as birds with information at other sites. The occurrence of deletions, insertions, or rearrangements during the transfer process would result in the information at some sites being different from the information at the original site.

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